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A MORPHOLOGICAL STUDY OF THE CLOSURE OF THE NEURAL TUBE IN RAT EMBRYOS

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A thesis submitted in partial fulfilment

of the requirements for the degree of

MASTER OF DENTAL SCIENCE (PAEDIATRIC DENTISTRY)

Discipline of Paediatric Dentistry

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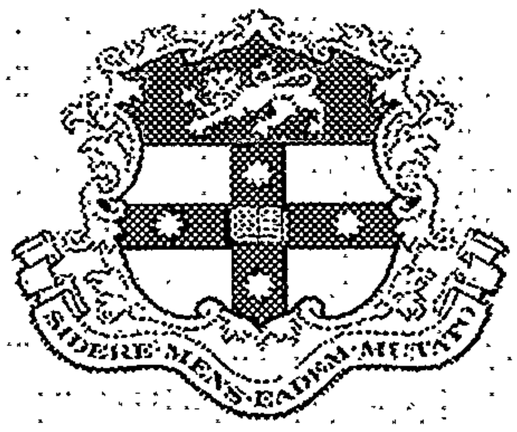

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ABSTRACT

As the formation of a patent neural tube constitutes the first fusion process in embryology, a knowledge of the cellular events relating to this process may provide a better understanding of subsequent fusion events. Both this event and palatogenesis demonstrate fusion processes that are frequently perturbed in human embryology resulting in neural tube defects and cleft palate malformations respectively. While recent work has conclusively demonstrated morphological and histochemical evidence of apoptosis in the medial edge epithelium during palatal fusion, to date, no such evidence has been forthcoming for the closure of the neural tube. Although there has been a great deal of research into the process of neurulation, most work focuses on the bending and shaping of the neural folds and little deals with the mechanisms of fusion.

It was the aim of this study to define the role of apoptotic death within the fusing neural folds of rat embryos by employing an *in-situ* end-labelling technique for the detection of the endonuclease-induced DNA fragmentation characteristic of apoptosis. The use of immunohistochemical techniques for phenotypic marking and the identification of replicating cells were trialed in order to provide further information about the cells taking part in the fusion process.

In this study it has been possible to make observations of the fusion process at a cellular level and arrive at a more comprehensive description of the process. During the stages of initial fusion, migratory neural crest cells were found within the cell-free zone. Other cells appeared to be losing adhesion to the surrounding neurectoderm, suggestive of pre-migratory neural crest cells. This population of cells exhibits an intimate relationship with the neurectodermal cells during the fusion process. However, at present it is not understood if these cells play a direct role in the fusion of the neural folds or if they merely coexist in close proximity.

A frequent observation found in recently fused neural folds, was the presence of one or two cells hanging from the fusion line into the lumen of the neural tube. Although, it was expected that these cells would be undergoing apoptosis, the use of the *in-situ* end-labelling technique did not give consistent labelling of these cells. Without the use of phenotypic markers it was impossible to know if these cells were of neural crest or neurectodermal origin.

The level of cell death, as detected by the *in-situ* end-labelling technique, in the neural folds during fusion was very low. In fact, there was almost complete absence of cell death in any other areas of the embryo apart from the tips of the fusing neural folds. In contrast, there was a high level of cell proliferation within the neurectoderm as seen from the high number of mitotic figures and confirmed by the use of PC10 labelling for the detection of Proliferating Cell Nuclear Antigen (PCNA).

The results of the study therefore show a low level of death associated with the fusion of the neural folds. It is possible that these labelled cells were presumptive neural crest cells. The cell death observed in the study of the caudal neural tube may, therefore, represent a regulation process whereby neural crest cells are eliminated if they become trapped within the fusing neural folds, are surplus or embark on an inappropriate migratory pathway.

Many details of the closure of the neural tube remain unexplained at this time. It is hoped that future work employing new techniques and equipment such as vital dye analysis, organ culture and confocal microscopy will lead to further advances in the understanding of this very important fusion process.

STATEMENT OF AUTHORSHIP

The experiments described in this thesis were performed at Westmead Hospital Dental Clinical School, the Institute for Clinal Pathology and Medical Research (ICPMR) and the Institute of Dental Research, Sydney, between January 1993 and December 1995. Some work was carried out by Dr Neil Hunter and support staff in the Institute of Dental Research, Sydney. The work described is that of the author unless otherwise stated and has not been submitted in whole or in part for any other degree.

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ABBREVIATIONS

AM	adrenomedullary cells
bp	base pairs
BrDU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CNS	central nervous system
CSPG	chondroitin sulphate proteoglycan
°C	degree Celcius
DiI	1,1-dioctadecyl-3,3,3',3',-tetramethylindocarbocyanine perchlorate
DLHP's	dorso-lateral hinge points
DLP	dorso-lateral pathway
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
ER	endoplasmic reticulum
GAG	glycosaminogycan
g	globular cell
HSPG	heparin sulphate proteoglycan
ICE	Interleukin-1β converting enzyme
IDR	Institute of Dental Research
IL	interleukin
IL-1β	interleukin 1β
IL3	interleukin 3
iw	inverted wedge-shaped
L	lateral neuroepithelial cells
M	molar
mM	millimolar
MEE	medial edge epithelium

mg^{2+}	magnesium ion
MHP	median hinge point
µg	microgram
µL	microlitre
µm	micrometre (micron)
mg	milligram
mL	millilitre
M phase	mitotic phase
mRNA	messenger ribonucleic acid
2n	diploid amount of DNA
4n	tetraploid amount of DNA
NC	neural crest
NCC	neural crest cell
NCC's	neural crest cells
nm	nanometre
Non-S phase	Non-DNA synthetic phase
PBS	phosphate buffered saline
%	percent
RA	retinoic acid
r	rhombomere
s	spindle-shaped cell
S phase	DNA synthetic phase
tg	total generation time of cells
tg1	duration of G1 phase of cell cycle
tg2	duration of G2 phase of cell cycle
tm	duration of M phase of cell cycle
ts	duration of S phase of cell cycle
TUNEL	TdT-mediated dUTP-biotin nick end-labelling
w	wedge-shaped cell
VP	ventral pathway

1

APOPTOSIS IN EMBRYOGENESIS

The field of developmental biology was founded over a century ago by Wilhelm Roux and his colleagues. Since that time, some understanding of how a single fertilised egg undergoes the complex series of changes to become an entire organism has slowly been gained. However, many questions of the intricate details involved in embryogenesis still remain unanswered today. In a recent survey of over 100 leading developmental biologists around the world (Barinaga, 1994), the consensus on the most important unanswered question in the field was clearly that of how the body's specialised organs and tissues are formed. This topic, known as morphogenesis was also voted as an area where rapid progress is expected over the next five years .

Morphogenesis encompasses the formation of all tissues and organs, from the first embryonic tissue layers to the intricate structure of the specific organ systems. The development of a body plan that directs cells to migrate and differentiate appropriately must occur very early in development. The dorso-ventral axis of the embryo is specified following gastrulation and from the appearance of the primitive streak the antero-posterior axis is also determined. Despite the complexities involved in morphogenesis on a whole-body level, there are a restricted number of cellular activities which take part during this process. This link between morphogenesis and cell fate is gene regulation. While it is understood that the cell's genome contains specific instructions that influence the pattern of development, cells also respond to environmental signals.

Molecular biologists have identified genes which are primary candidates for establishing regional identity for different parts of the body. These discoveries began with the finding that single gene mutations in *Drosophila melanogaster* could result in major transformations of the body plan. For example, the second thoracic segment which normally bears a pair of balancing organs called 'halteres' is transformed into an extra first thoracic wing-bearing segment, thus producing a mutant phenotype (*bithorax*) with two pairs of wings rather than one. It became clear that the mutant phenotype was produced by a mistake at a critical stage in the sequence of genetically determined cell differentiations.

By analysing these and other mutants a whole series of genes has been identified which are normally involved in specifying regional identity within the *Drosophila* embryo (De Robertis *et al.*, 1990). All of these genes contain a region of 180 nucleotide base pairs called the 'homeobox' which encodes for a DNA binding domain within the specified protein. Such homeobox-containing genes have a very widespread distribution (from yeast to human) and have been highly conserved throughout evolution. All vertebrates have four homeobox complexes, each located on separate chromosomes. These complexes may have arisen during evolution through duplications of the single cluster of homebox genes in invertebrates.

The homeobox genes divide the embryo along its head-to-tail axis into bands with different developmental potentials. The location of a homeobox gene on a chromosome corresponds to where it is expressed in the body. Proceeding from left to right, the genes control body areas closer to the anterior end of the animal. Homeobox genes are therefore arranged in the chromosomal DNA in the same order in which they are expressed along the antero-posterior body axis (De Robertis *et al.*, 1990).

The polypeptide chain in the homeodomain consists of four helices, one of which is responsible for recognising a specific DNA sequence. This helix is nearly the same in all homeodomain proteins and so the proteins all bind to similar DNA sequences. When they bind to genes in a cell, homeodomain proteins activate or repress the expression of subordinate genes.

Although it is not known exactly how genes cooperate to organise cells from the stage of a single fertilised egg to a complex vertebrate body plan, it seems that gradients of proteins and other molecules (such as growth factors and retinoic acid) act as signposts for specifying the positions of cells and efficiently directing cell fate.

Induction is a process where one tissue directs the patterning of another and thereby has a role to play in the coordination of morphogenesis. The relationship between the ectoderm and its underlying mesenchyme is of utmost importance during organogenesis. Virtually every vertebrate tissue and organ is formed by some type of induction.

The formation of the mesoderm between the ectoderm and the endoderm is the first major inductive event in embryogenesis. Mesoderm, which ultimately develops bone, muscle and blood, drives the shape change from a round embryo to an elongated adult. Dorsal mesoderm then induces areas of neighbouring ectoderm to become neural, diverting it from an epidermal fate. Although this was first demonstrated in the 1920's the chemical signals that mediate the process have only just begun to be identified. Two substances, noggin (Lamb *et al.*, 1993) and follistatin (Hemmati-Brivanlou *et al.*, 1994) have been identified in *Xenopus* that can induce vertebrate embryonic tissue to become neural and are expressed, *in vivo*, in an appropriate spatial and temporal manner.

Polypeptide growth factors, such as Transforming Growth Factor-β (TGF-β), provide the signals that drive important biological processes such as differentiation, proliferation, migration and cell death. Such signals must be secreted by the signalling tissue at the correct stage of development and in an adequate quantity to have an inductive effect. The activity of these proteins produces an effect on target tissues that is dependent on a concentration gradient. In mammals, the family of TGF-β proteins and other distantly related molecules, are expressed widely during embryogenesis. One of these proteins, noggin, has been implicated in conferring dorso-ventral patterning during gastrulation in *Xenopus* (Slack and Tannahill, 1993).

During gastrulation, the ectoderm differentiates into neural and epidermal cell lines. The discovery that neurectodermal specification was produced under the influence of signals from the Spemann organizer was made in the 1920's. Ectodermal cells not receiving these signals became epidermal. However, a recent study by Wilson and Hemmati-Brivanlou (1995) demonstrated that members of the TGF-β family are potent epidermal inducers and neural inhibitors. These secreted growth factors, including Activin and bone morphogenetic protein-4 (BMP-4), inhibit cells from differentiating into neurectoderm thus driving the production of epidermal cells. This work provides the first evidence for the inhibitory control of neural specification in vertebrates.

Recently, serotonin has been implicated in the regulation of cranial neural crest migration in the mouse (Moiseiwitsch and Lauder, 1995) thus making it an important morphogen in craniofacial development. The influences of serotonin on craniofacial development appear to be through the regulation of neural crest cell migration in a temporally specific, dose-dependent, and receptor-mediated manner. As neural crest cells emerge from the neurectoderm, they appear to be stimulated by low to medium concentrations of serotonin. Higher concentrations appear to be inhibitory or have no effect. Furthermore, once neural crest cells have been formed, serotonin uptake and receptor activation may be involved in down-regulating the inherent migratory activity of these cells as they differentiate into craniofacial mesenchyme.

The extracellular matrix is another important factor in morphogenesis, but it is not known whether its influence on cell behaviour acts directly or indirectly. While it has always been thought that its action is indirect, serving as a substratum which alters cell shape (Watt, 1986), examples of direct signalling by the extracellular matrix are being discovered. Thorogood (1988) implicated collagen II in directing the sites of cartilage formation and Menko and Boettiger (1987) found evidence that the extracellular matrix directly controls muscle differentiation.

In relation to epithelial-mesenchymal interactions the mode of action of the matrix has yet to be defined. While there does not seem to be any other mode of communication between the two layers, the matrix alone does not seem to be the inducer. Wolpert (1988) suggested that the extracellular matrix may function as a channel for an undetermined diffusible signal.

Craniofacial development and palatogenesis, in particular, is an area of specific interest in Paediatric Dentistry. Congenital craniofacial malformations are amongst the most prevalent birth defects in man, with 1 in 1000 infants born with a cleft lip and palate (Vanderas, 1987). The rehabilitation of these children is complex and extends over many years. In order to understand how these perturbations occur, it is necessary, first, to have a thorough understanding of the processes involved in normal palatogenesis.

The exact nature of the mechanisms that lead to fusion of the palatal processes remains the subject of much research today. It is known, however, that the process of fusion necessitates a breakdown of the medial edge epithelium, allowing mesenchymal confluence. One of the current hypotheses of this remodelling procedure is that the cells of the medial edge epithelium undergo apoptosis.

Parallel studies in progress at the Institute of Dental Research, Sydney are investigating the role of apoptosis within the medial edge epithelium of the palate of rat embryos. Figures 1.1A - 1.1D show low and high power views of the fusing palate in two 16.5 day rat embryos. Figures 1.1A and 1.1B are Haematoxylin and Eosin stained, paraffin sections in the coronal plane showing the anterior palate. The opposing palatal shelves have recently fused with each other and the nasal septum. The medial edge epithelium remains intact at this stage, but the cells of the epithelial triangles that have formed on the nasal and oral surfaces display abundant apoptosis.

Figures 1.1C and 1.1D are Toluidine Blue stained, semi-thin resin-embedded sections of a more posterior section of palate. The stage of fusion is slightly more advanced than in Figures 1.1A and 1.1B as the medial edge epithelium has begun to disintegrate, forming epithelial islands within the mesenchyme. In the high power view (Figure 1.1D) the arrows indicate some of the cells that show the morphological characteristics of apoptosis. The majority of these apoptotic cells have been phagocytosed, but incompletely digested, by neighbouring cells within the medial edge epithelial seam. Surh and Sprent (1994) have addressed the issue of the apparent rate of apoptotic cell death found in a study of the thymus. The detection of apoptotic cells *in-situ* was dependent on the efficiency of the thymic macrophages in the removal and digestion of the apoptotic bodies. Therefore, the tissue environment, particularly in regard to the availability of efficient phagocytic cells, will have a key bearing on the interpretation of the apparent rate of apoptosis. Further work employing electron microscopy and an *in-situ* end-labelling technique for the detection of the DNA fragmentation characteristic of apoptosis is expected to provide direct evidence for the role of apoptosis in normal palatogenesis.

FIGURES 1.1A - 1.1D: Parallel studies at the Institute of Dental Research, Sydney are examining the process of palatal fusion. The following photomicrographs show sections through recently fused palatal shelves of 16.5 day rat embryos. Figures 1.1B - 1.1D are shown on subsequent pages.

FIGURE 1.1A: Coronal section through the anterior palate and nasal septum of a paraffin-embedded rat embryo showing an uninterrupted medial edge epithelium (arrow) [110X].

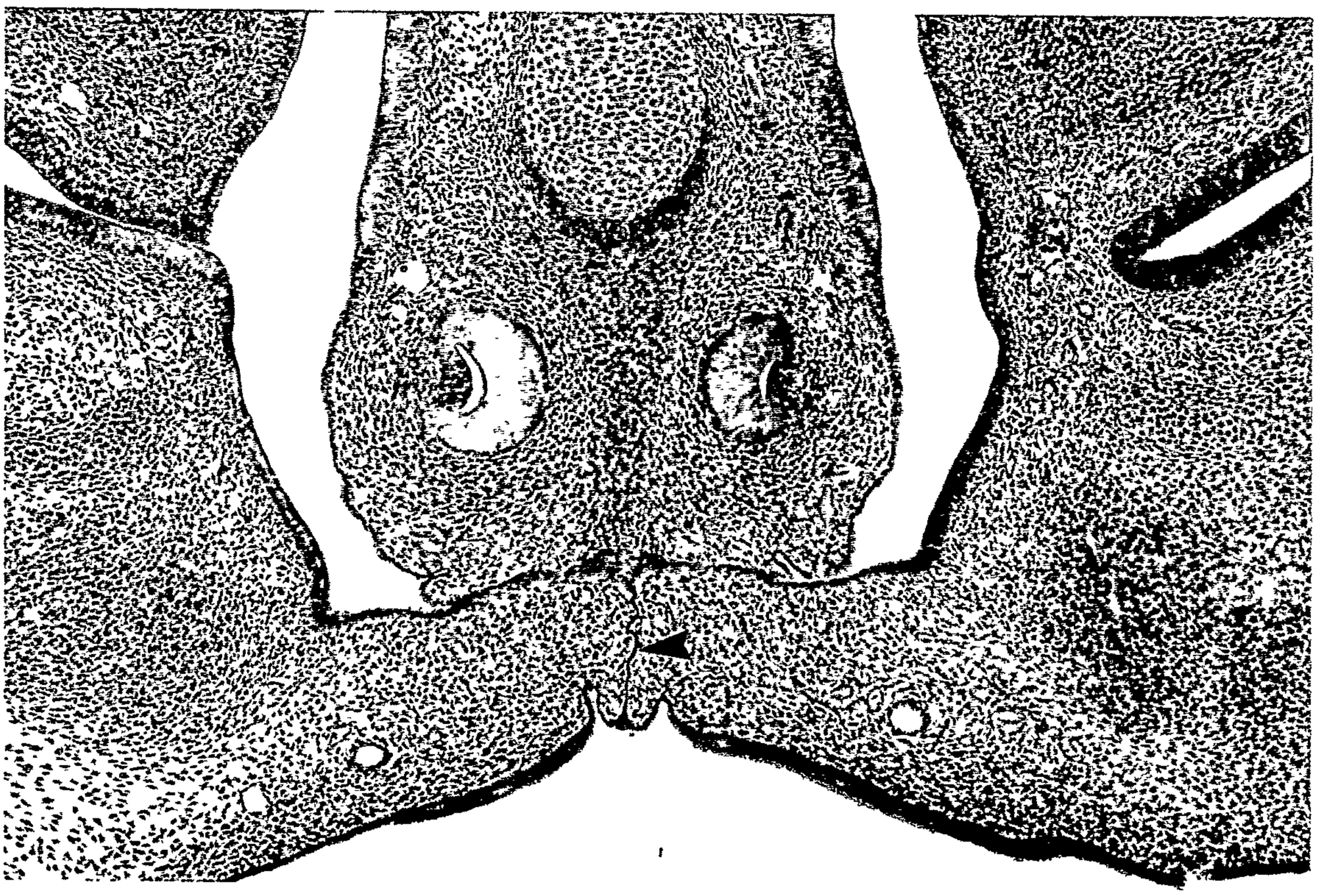


FIGURE 1.1B: High power view of the area of fusion shown in Figure 1.1A. The medial edge epithelium (MEE) is intact and separates the mesenchymal cores (M) of the two palatal shelves. The nasal (N) and oral (O) epithelium are continuous on the nasal and oral surfaces of the palate. There is evidence of abundant apoptosis within the epithelial triangle at the nasal surface (ET) [430X].

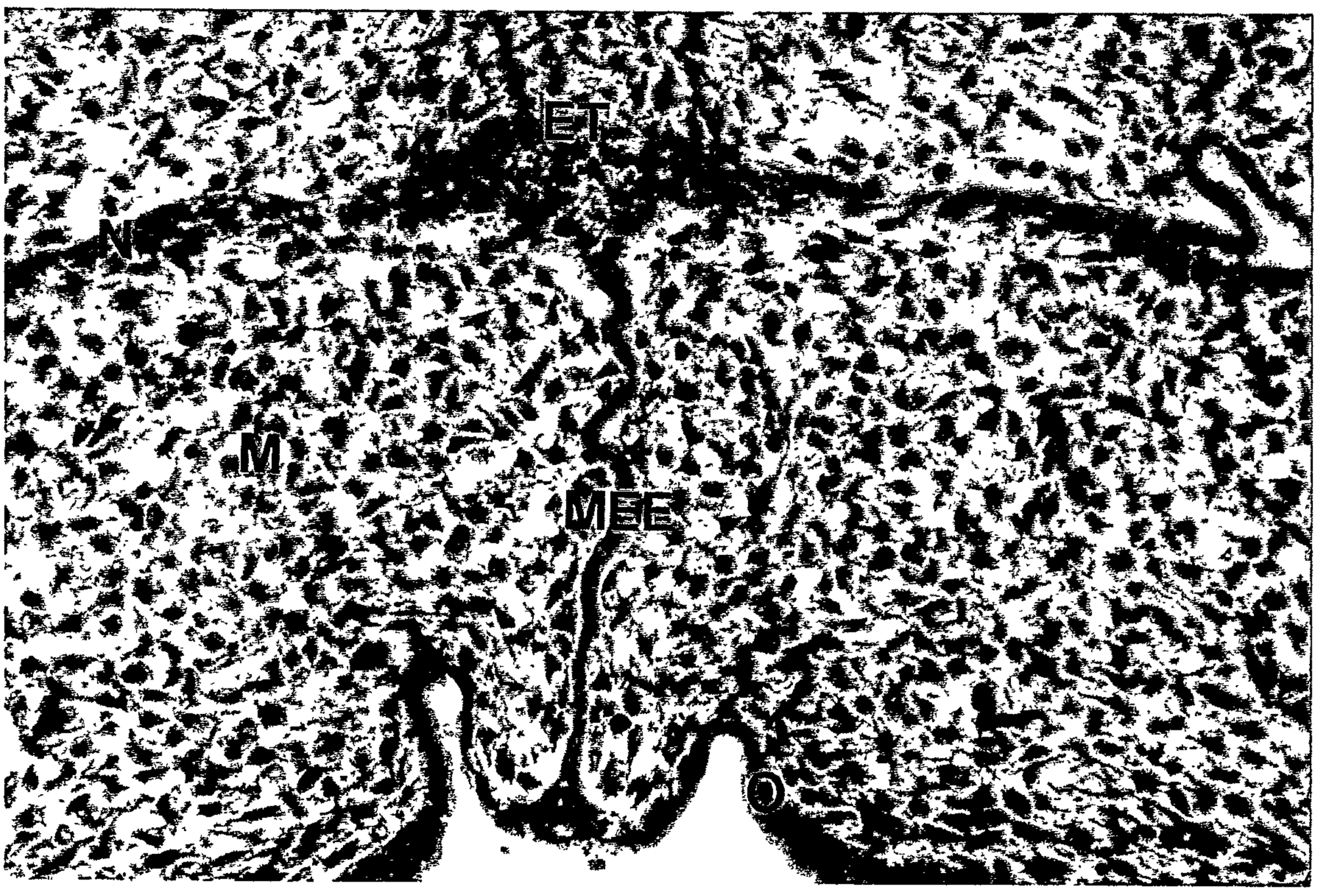


FIGURE 1.1C: Semi-thin coronal section of a resin-embedded rat embryo showing the recently fused palatal shelves. This region of palate is more posterior to the one shown in Figures 1.1A and 1.1B. The nasal (N) and oral (O) cavities are separated by the palate. The medial edge epithelium has begun to disintegrate forming epithelial islands (arrows) within the mesenchyme [110X].

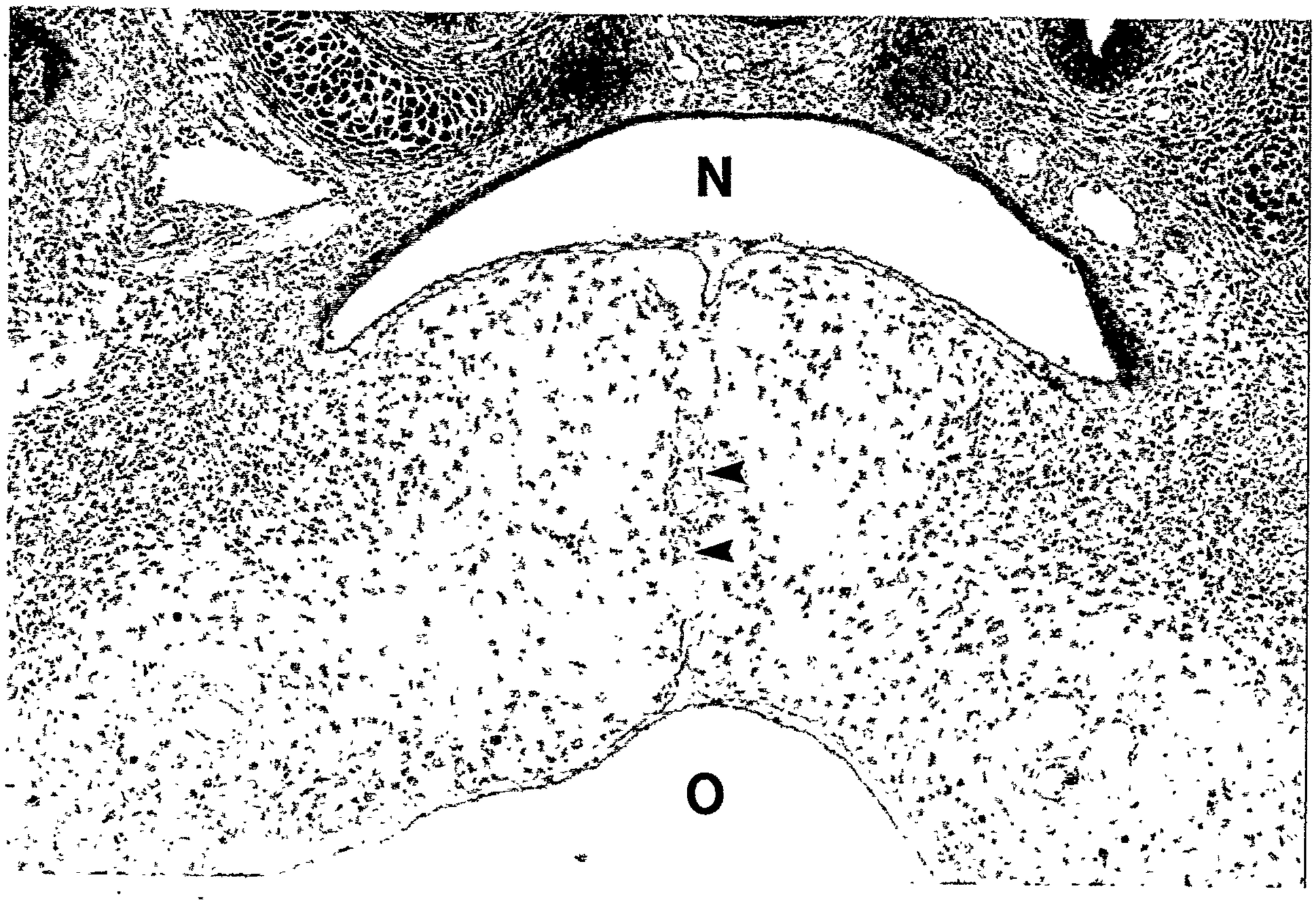
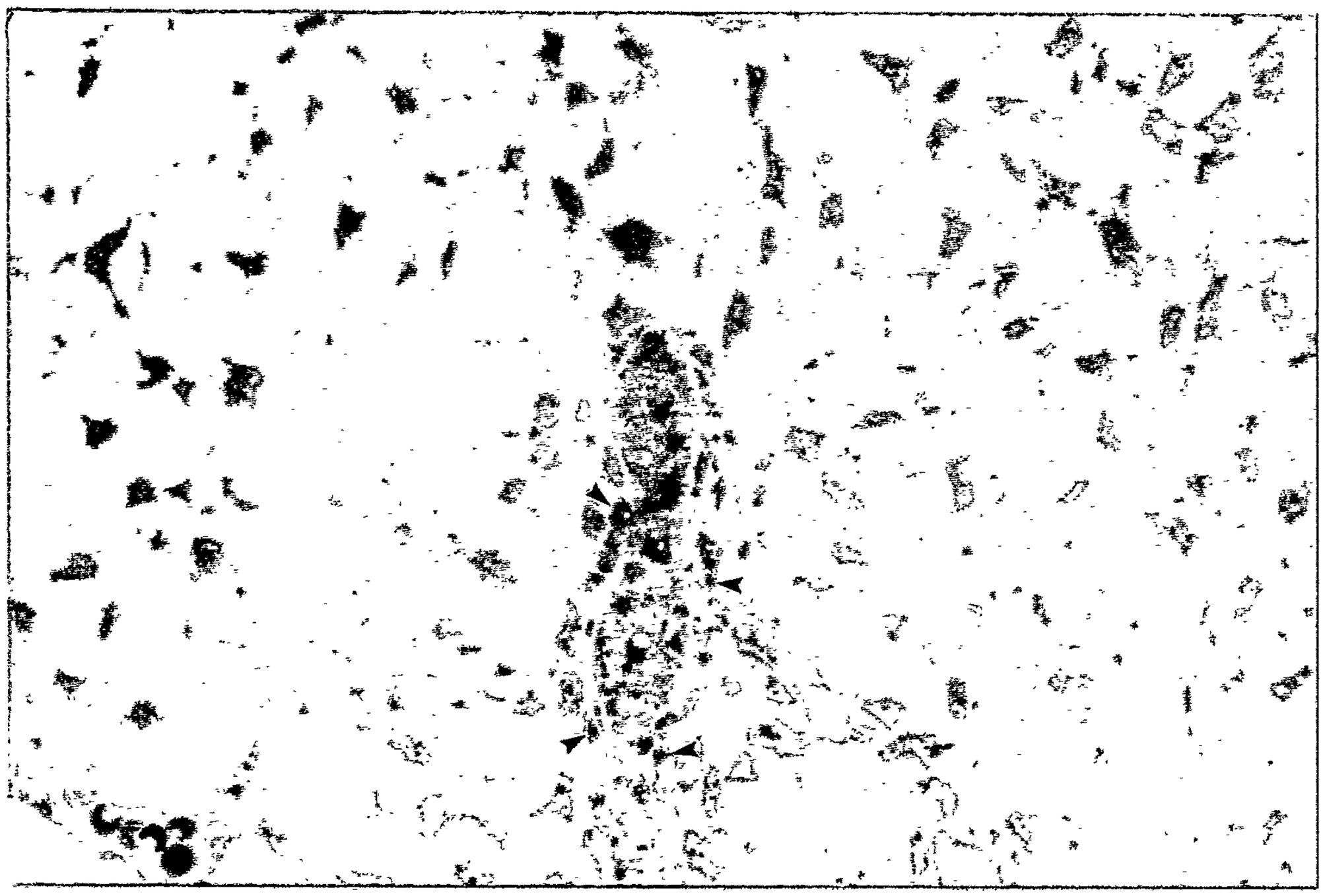


FIGURE 1.1D: High power view of the large epithelial island seen in Figure 1.1C. Several cells (arrows) show the characteristic morphological features of apoptosis. These cells have undergone shrinkage and condensation of the nuclear chromatin [430X].



In 1972, Kerr *et al.*, reported a physiological form of cell death called "apoptosis". In this innovative paper, apoptosis was described as "an important basic biological phenomenon that plays a complementary but opposite role to mitosis in the regulation of animal cell populations". This form of cell death was recognised in scattered single cells and showed characteristic morphological changes without evoking an inflammatory response. The role of cell death in embryogenesis and the homeostasis of normal adult tissues has long been recognised (Glücksmann, 1951, Saunders, 1966). However, the wide-ranging implications of this process during early morphogenesis are only now beginning to be realised.

Apoptosis appears to require the activation of specific genes within dying cells, suggesting that it is a programmed event much like any form of differentiation. The possibility that cell death is a highly programmed event under genetic control has been extensively studied in *Caenorhabditis elegans* by Horvitz and colleagues since 1977 (Sulston and Horvitz, 1977; Horvitz *et al.*; 1982; Sulston *et al.*, 1983). In *C. elegans* certain cells are programmed to die at a specific time in development. The genetic regulation of these precisely controlled deaths has been linked to three important genes; *ced-3*, *ced-4* and *ced-9*, which are responsible for either turning on or turning off the death command. The study of genetic regulation of cell death in *C. elegans* has lead to the discovery of homologs that control cell death in mammalian cells.

Apoptosis has long been known to play a major role in morphogenesis. Examples include the development of the nervous system where about a half of all developing neurones undergo cell death (Raff *et al.*, 1993); and digit formation where many cells in the interdigital spaces die as a normal part of development (Ballard and Holt, 1968). However, these processes are not programmed in the same manner as *C. elegans*. Instead death occurs as a result of deprivation of specific growth factors, in the case of the development of the nervous system, or in response to other morphogenic substances that signal cells in areas undergoing remodelling.

To date there has been limited research into the role of cell death in the early embryogenesis of mammals. Amongst the array of current research into the origin and final fate of neural crest cells is evidence for apoptotic death in this population of cells. In the area of the hindbrain where there is a characteristic segmentation of neural crest cell migration along the rostro-caudal axis, two specific areas are devoid of neural crest emigration and it has been shown that many presumptive neural crest cells in these areas undergo apoptosis (Jeffs *et al.*, 1992 and Graham *et al.*, 1993).

The work described in this thesis relates to an investigation of the morphology of the closure of the neural tube as a possible model for subsequent fusion events such as palatogenesis. The specific area of interest was the events leading to the fusion of the apposed neural folds. Particular emphasis was placed on whether or not apoptosis played a role during this event, as it has been shown to do in palatogenesis (Mori *et al.*, 1994).

The question of whether apoptosis has a major role to play in early embryogenesis is of particular relevance to this study. For this reason, a review of the current status of apoptosis in the literature, is presented in the succeeding chapter. An overview of mammalian embryogenesis with particular emphasis on palatogenesis follows in Chapter 3 and the historical and contemporary perspectives of the process of neurulation and a review of the neural crest are found in Chapter 4. The final two chapters contain a description of the present study and a discussion of the findings, with particular reference to the role of apoptosis in neurulation.

2

APOPTOSIS

2.1 INTRODUCTION

There are two distinct types of cell death which differ in their applications, morphological appearances and biochemical processes. Necrosis can be thought of as a pathological form of cell death that is most often the result of severe and sudden injury. Due to the aetiological nature of the process, it is common to find many necrotic cells within a specific area.

Cells undergoing necrosis show early changes in mitochondrial shape and function associated with the loss of plasma membrane integrity and cellular homeostasis. The final fate of a necrotic cell is to swell, burst and either undergo autolysis or be removed by phagocytes. The process of phagocytosis can involve the release of proteolytic enzymes or the generation of reactive oxygen metabolites with the leakage of cellular contents into the surrounding tissue spaces causing damage to adjacent cells and evoking an inflammatory response (Kerr *et al.*, 1972; Wyllie, 1993).

Degradation of DNA in necrotic cells occurs as a result of chromatin digestion by lysosomal proteases and endonucleases and results in a smear pattern on gel electrophoresis. The proteases destroy the histones and expose the entire length of DNA to nucleases (Wyllie, 1993).

In contrast, the process of apoptosis is quite distinct. The differences in the characteristics between apoptosis and necrosis are summarised in Table 2.1 and Figure 2.1. The ultrastructural changes of apoptotic cell death were described in detail by Kerr *et al.* (1972). While the mitochondria continue to function normally, early changes are seen within the nucleus. Affected cells shrink in volume and lose microvilli and cell-cell junctions. The chromatin rapidly forms dense crescent-shaped aggregates and complex invaginations develop in the nuclear membrane, resulting in a segmented nucleus. The plasma membrane also becomes convoluted so that the cell becomes separated into a cluster of membrane bound "apoptotic bodies" which often contain morphologically normal mitochondria and other cellular organelles.

The altered surface characteristics of the apoptotic bodies act as signals to phagocytes and adjacent viable cells to phagocytose the dying cell. The lysosomal enzymes of the apoptotic cells do not participate in the cell death process, although lysosomal activation occurs within the phagocytic cells (Morris *et al.*, 1984; Wyllie, 1993). As there is little leakage of cellular contents into the surrounding tissue space during removal of apoptotic cells an inflammatory response is not evoked.

2.2 THE PHYSIOLOGICAL AND PATHOLOGICAL ROLES OF APOPTOSIS

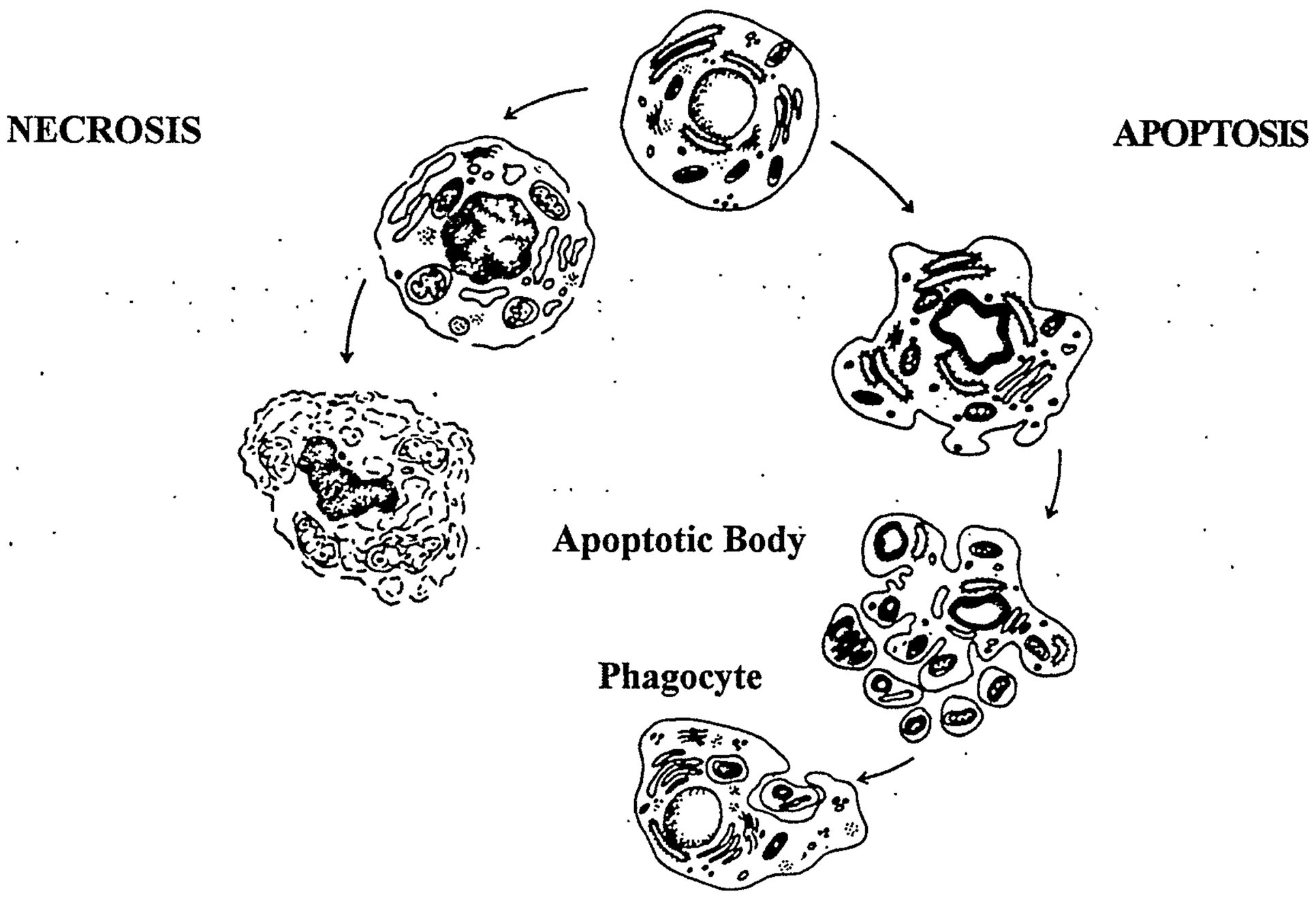
Apoptosis occurs in a wide range of physiological and pathological states. It has been widely researched in the area of clonal selection of lymphocytes in the thymus. During thymic development, pre-T cells enter the thymus and recombine the genes for the antigen receptor. After completion of this process, cells can differentiate into mature T cells possessing different receptor characteristics and are then selected for further maturation (positive selection). Sentman *et al.* (1991) showed that when the coupling between antigen receptor and antigen is incomplete, the T cells undergo apoptosis and are depleted (negative selection).

TABLE 2.1: The different characteristics of Necrosis and Apoptosis

NECROSIS	APOPTOSIS
Affects contiguous cell masses	Affects scattered individual cells
Chromatin marginates into small aggregates	Chromatin marginates as large crescent
Increase in cytoplasm and cell volume	Decrease in cytoplasm and cell volume
Organelles swell (Mitochondria and ER)	Organelles retain integrity
Cell ruptures	Cell fragments into "apoptotic bodies"
Cell contents are released	Phagocytosis of apoptotic bodies
Extensive inflammatory response	No inflammatory response
Smear pattern of DNA	Ladder of DNA fragmentation (~200bp)

(Adapted from Corcoran and Ray, 1992)

FIGURE 2.1: Morphological characteristics of Necrosis and Apoptosis



(From Ueda and Shah, 1994)

Apoptosis has also been implicated in cell-mediated immunity, the response to viral infection, development of autoimmunity and regression of neoplasms. In cell-mediated immunity, cells bearing foreign antigen can be killed through apoptosis by effector T cells. Cytolytic T lymphocytes can eliminate target cells without sustaining damage (Cohen and Duke, 1992). In contrast, T cells infected with a virus can limit viral replication by undergoing an altruistic death mediated by apoptosis.

Under normal conditions immature lymphocytes that bind autoantigens undergo apoptosis. Failure to deplete self-reactive T lymphocytes by apoptosis has been suggested by Cohen and Duke (1992) to be responsible for autoimmune disease.

In tumour development, it is possible that apoptosis may eliminate the cells carrying the cancerous genes and repress the development of neoplasms, while failure of apoptosis can result in progression of neoplasms. Alternatively apoptosis may trigger progression of neoplasms by affecting cell cycle progression through other signals (Ueda and Shah, 1994).

2.3 METABOLIC CHANGES DURING APOPTOSIS

The processes involved in the DNA fragmentation of apoptosis have not yet been identified. The mechanisms that have been proposed include a rise in cytosolic calcium, altered expression of mRNA and synthesis of a series of proteins.

Some stimuli have been shown to cause an uncontrolled rise in cytosolic calcium prior to DNA cleavage and cell death (Ueda and Shah, 1992). Cohen and Duke (1992) proposed that the rise in intracellular calcium concentration may activate calcium-dependent endonucleases. In some cells depletion of extracellular calcium or chelation of intracellular calcium has been shown to prevent apoptosis but in other cell types it does not (Batistatou and Green, 1993).

Apoptosis appears to be dependent on synthesis of mRNA and protein in some cell types because it can be arrested by application of inhibitors shortly after the stimulus has been applied (Cohen and Duke, 1984). However, the same inhibitors of mRNA and protein synthesis do not affect or stimulate apoptosis in other cell types.

It can be seen that there is no common sequence of metabolic events for cells during apoptosis. Instead different cell types have distinct regulatory systems in response to a variety of stimuli that can determine whether or not the cell undergoes apoptotic death.

2.4 DNA FRAGMENTATION IN APOPTOSIS

During apoptotic cell death the nuclear chromatin is cleaved by the action of endogenous Ca^{2+}/Mg^{2+}-dependent endonucleases that are inhibited by zinc (Cohen and Duke, 1984). Characteristically, the DNA is cleaved into oligonucleosome-length fragments of 180 - 200 base pairs, which can serve as a biochemical marker for apoptosis (Wylie, 1980). The fragmentation results from double stranded breaks in the linker regions between the nucleosomal cores of the DNA (Wylie, 1993). Because the nucleosomes are regularly spaced along the double helix, the DNA fragmentation can be detected as a ladder pattern on agarose gel electrophoresis. More recently end-labelling techniques have been developed to mark apoptotic cells *in-situ* (Gavrieli *et al.*, 1992; Wijsman *et al.*, 1993 and Gold *et al.*, 1993).

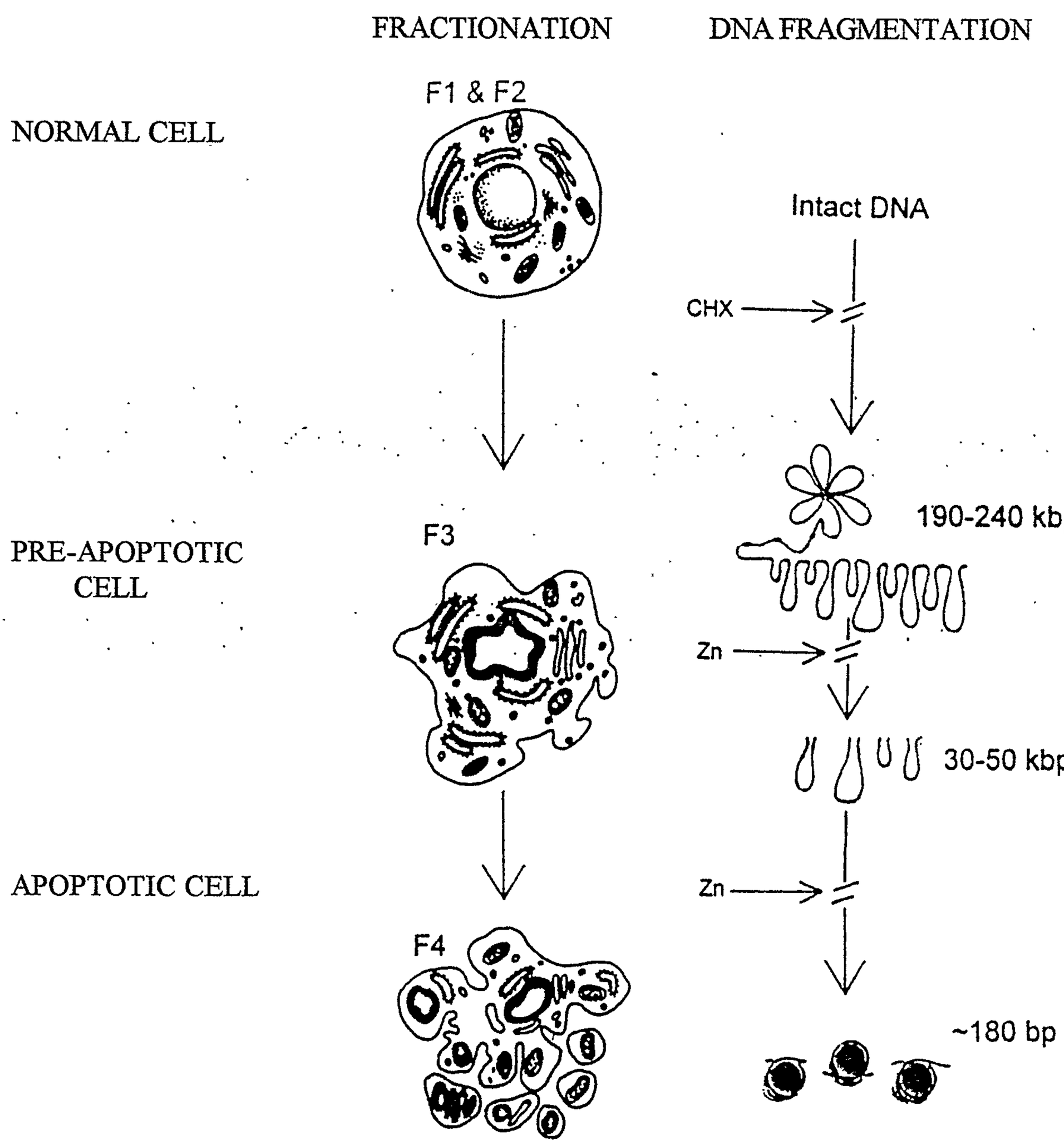
A study by Cohen *et al.* in 1994 showed an intermediate stage of DNA fragmentation in thymocytes. Following incubation with dexamethasone, a population of thymocytes displaying large molecular weight fragments of DNA (30-50 kilobase pairs) was identified. Using discontinuous Percoll density gradient fractionation, four fractions of decreasing size and increasing density (F1-F4) were found.

On the basis of ultrastructural and biochemical criteria, cells in F1 and F2 were normal thymocytes, cells in F3 were preapoptotic and those in F4 were apoptotic (See Figure 2.2). The results suggested that the formation of large fragments of DNA precedes endonuclease cleavage of DNA into the oligonucleosomal fragments that give rise to the characteristic DNA ladders.

Finally, Cohen *et al.* (1994) propose that normal thymocytes (F1 and F2) are converted to preapoptotic cells (F3) by a step that is dependent on protein synthesis and gives rise to large fragments of DNA. This stage appears to be mediated by an endogenous Mg^{2+}-dependent endonuclease (Sun and Cohen, 1994) that is less strongly inhibited by zinc than that of the final stage of DNA fragmentation into oligonucleosome fragments (F4). This altered inhibition by zinc may be the result of the action of two separate enzymes, the presence of two active sites on the same enzyme or an alteration of the conformation of the DNA allowing enhanced accessibility to the enzyme that mediates internucleosomal cleavage. Once the cells have reached the F3 stage they are committed to apoptosis and the DNA is rapidly and characteristically cleaved by a zinc-inhibitable endogenous Ca^{2+}/Mg^{2+} endonuclease giving rise to typical apoptotic cells (F4).

Although the initial steps in the induction of apoptosis may differ in different cell types it appears that there is convergence to a small number of common pathways in the later stages of apoptosis. The findings of Cohen *et al.* (1994) are of major importance in the recognition and identification of apoptotic cells at an early stage of commitment.

FIGURE 2.2: A proposed model correlating DNA fractionation and fragmentation of apoptosis in thymocytes. Intact DNA of normal thymocytes (F1 and F2) is cleaved initially into large fragments present in the preapoptotic population (F3) of cells containing condensed chromatin. These cells are then rapidly converted to cells with the classical ultrastructure and biochemistry of apoptotic thymocytes (F4).



(Adapted from Cohen *et al.*, 1994

2.5 PROGRAMMED CELL DEATH

The term apoptosis is often used interchangeably with programmed cell death. However, the two terms should be kept apart and used in the correct context. Apoptosis is a morphologic definition that describes a form of cell death characterised by specific cellular changes including shrinkage, nuclear condensation and cell fragmentation resulting in the formation of apoptotic bodies. The term "programmed cell death" is a functional definition, describing cell death that occurs at a precisely predicted time and place and is truly genetically determined. So it follows that not all apoptosis is programmed.

The best studied example of programmed cell death is that of the invertebrate *Caenorhabditis elegans* (*C. elegans*). Two features of *C. elegans* make it an excellent organism in which to study programmed cell death. Firstly, the divisions and deaths of individual cells can be observed in the live organism because it is transparent. Secondly, genetic and molecular analysis allows the identification of the genes that function during cell death. The complete pattern of cell divisions and cell deaths occurring during the development of *C. elegans* has been determined (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979 and Sulston *et al.*, 1983). Of the 1090 somatic cells formed during development of an adult hermaphrodite, 131 undergo programmed cell death. These cell deaths are precisely programmed; in every animal the same cells die, each at their own characteristic time in development. Considerable progress has been made in recent times in understanding the genetic regulation of cell death in *C. elegans*.

Two genes, *ced-3* and *ced-4*, have been identified in *C. elegans* that must function for cells to undergo programmed deaths. Mutations of either of these genes results in the survival of almost all cells that normally die (Ellis and Horvitz, 1986). *Ced-3* encodes a cysteine protease and *ced-4* encodes a calcium-binding protein.

The work of Hengartner *et al.*(1992) showed that the function of *ced-3* and *ced-4* can be antagonised by the expression of another gene, *ced-9*. They found that a dominant *ced-9* mutation prevents all cells from dying and that mutations that inactivate *ced-9* kill the animal. These observations suggested that the dominant *ced-9* mutation causes either ectopic or increased activity of the *ced-9* gene, which prevents *ced-3* and *ced-4* from killing cells that would otherwise die. Conversely, mutations that inactivate *ced-9* cause increased activity of *ced-3* and *ced-4* which killed cells that normally survive. Thus the function of *ced-9* seems to be to prevent cells that should survive from undergoing programmed cell death

2.6 REGULATION OF APOPTOSIS IN MAMMALIAN CELLS

It is now widely accepted that apoptosis is a gene-directed process and can be seen as part of the repertoire available to the cell which enables response to external and internal stimuli (Williams and Smith, 1993). The cell death genes described in *C. elegans* have structural and functional homology with genes expressed in mammalian cells. The *C. elegans* gene *ced-9* protects against cell death and appears to be homologous to the proto-oncogene *bcl-2* (Hengartner *et al.*, 1992; Hengartner and Horvitz, 1994). Miura *et al.* (1993) found that *ced-3* also appears homologous to the mammalian gene for interleukin-1β converting enzyme (ICE) which can actively lead to cell death. Bissonnette *et al.* (1992) found the pathways that mediate cell death and proliferation may be closely linked, thus implicating the genes *c-myc* and p53, in the regulation of apoptosis. Finally, a group of specific cell death receptors (Fas/Apo-1) exist which when triggered rapidly stimulate cell death.

2.6.1 THE *BCL-2* FAMILY

The *bcl-2* gene was identified over 10 years ago by the analysis of a chromosomal defect found in the malignant cells of most patients with follicular B cell lymphoma (Tsujimoto *et al.*,1985; Bakhshi *et al.*, 1985). *Bcl-2* was the first gene to be implicated in human cancer that did not share homology with the "classic" proto-oncogenes found in animal studies of retrovirus-induced neoplasia. The Bcl-2 polypeptide was unrelated to any proteins known at that time.

During the ensuing years evidence for the role of *bcl-2* in apoptosis emerged from gene transfer experiments in which it was over-expressed in a variety of interleukin (IL)-dependent cell lines of hematopoietic origin (Vaux *et al.*, 1988; Nunez *et al.*, 1990). Using immature pre-B cells that are dependent on the lymphokine IL-3 for their growth and survival in culture, these investigators noticed that transfection with vectors that produced stable *bcl-2* expression permitted prolonged cell survival in the absence of IL-3, but without concomitant cell proliferation. These results suggested that *bcl-2* was capable of blocking apoptosis *in vitro*.

Subsequent studies have shown that the *bcl-2* gene prevents many, but not all forms of apoptotic death. While the expression of the *bcl-2* gene prevents radiation- and calcium ionophore-induced apoptosis in thymocytes it does not prevent negative selection (Sentman *et al.*,1991). Similarly, microinjected Bcl-2 can prevent apoptosis in sensory neurons that are dependent on neurotrophic factors (e.g. nerve growth factor) but not in ciliary neurotrophic factor-dependent ciliary neurons. These results suggest either the existence of multiple independent intracellular mechanisms of apoptosis, some of which can be prevented by Bcl-2 and others that are unaffected by this gene product, or that the additional pathways involve proteins that differentially regulate Bcl-2 function. Bcl-2 also protects against apoptosis mediated by *c-myc* expression (Vaux and Weissman, 1993).

Bcl-2 protein is a 25-kDa integral membrane protein that has been localised to mitochondria, nuclear envelope/perinuclear membrane and endoplasmic reticulum (Hockenbery *et al.*, 1990; Monaghan *et al.*, 1992; Krajewski *et al.*, 1993). Although its precise biochemical role remains unclear, Bcl-2 protein appears to function in an antioxidant pathway which prevents cellular damage by reactive oxygen species (Hockenbery, *et al.*, 1993). However, it is known that Bcl-2 protein appears to block a relatively early event associated with apoptotic cell death because none of the characteristic morphological changes such as cell shrinkage, chromatin condensation and nuclear fragmentation occur in its presence. Marked reductions in Bcl-2 protein levels render cells more prone to apoptosis but are insufficient in isolation to cause cell death (Reed *et al.*, 1990).

Several *bcl-2*-related genes have been identified. Oltvai *et al.* (1993) isolated a Bax protein that shares amino acid homology with Bcl-2. The Bax protein homodimerises and forms heterodimers with Bcl-2 *in vivo*. Overexpression of *bax* accelerated apoptosis induced by cytokine deprivation in an IL-3-dependent cell line and countered the repressor effect of Bcl-2 on apoptosis. The *bax* gene is expressed in a broad range of tissues but the role in apoptosis and its localisation within particular cell types remains to be determined.

The gene *bcl-x* described by Boise *et al.* (1993) is another member of the *bcl-2* family that encodes two functionally different proteins: Bcl-x_L and Bcl-x_S. Bcl-x_L inhibits apoptosis after deprivation of growth factor in IL-3-dependent prolymphocytic cell lines in mice. In contrast, *bcl-*x_s encodes a protein that prevents *bcl-2* expression from inhibiting apoptotic death after deprivation of growth factor. *Bcl-x* has been detected in a range of tissues, particularly in the central nervous system and thymus.

Unlike Bax, Bcl-x_S does not form dimers with Bcl-2 but acts as a dominant negative mutant form of Bcl-2 by competing for substrates or regulators to prevent Bcl-2 from suppressing apoptosis.

The expression of *bcl-x* can therefore explain why there are several forms of apoptosis that appear to either bypass, or be regulated independently, of *bcl-2* (Sentman *et al.*, 1991).

Veis-Novack and Korsmeyer (1994) performed an extensive study of the pattern of *bcl-2* expression during embryonic development in the mouse. Their observations that *bcl-2* expression correlates exceptionally well with cell survival in certain tissues suggests that such survival factors may be required to preserve cell viability during early development. While it has long been recognised that cell death plays a role during normal embryogenesis, particularly in neural tissues, newer evidence suggests that apoptosis plays a major and widespread role in early development. With this in mind it seems likely that factors that suppress apoptosis also play a critical role in regulatory morphogenesis.

2.6.2 INTERLEUKIN-1β-CONVERTING ENZYME (ICE)

The discovery of structural and functional similarities between the product of the *C. elegans* cell-death gene *ced-3* and mammalian interleukin-1β-converting enzyme (ICE) provides an important insight into the molecular mechanism of apoptosis. ICE was first isolated from monocytic cells, where it was shown to cleave the IL-1β precursor to generate the mature form of IL-1β, a cytokine which plays a key role in inflammation and a variety of other physiological and pathological processes.

ICE is a cysteine protease with a protein sequence 28% identical to that of *ced-3* (Yuan *et al.*,1993). Miura *et al.* (1993) have shown that high levels of ICE expression can induce apoptosis in mammalian cells. Bcl-2 can prevent the activation of ICE as can the CrmA protein from cowpox virus which binds to and inactivates ICE. As with *bcl-2* there exists a family of ICE homologs including Nedd2, ICH-1, CPP32 and prICE. At present it is not clear whether these cysteine proteases mediate apoptosis induced by all types of signals or only those in which *bcl-2* provides protection against cell death (Kumar, 1995).

Until recently, efforts to identify the molecular mechanisms of apoptosis focused on identifying endonucleases capable of cleaving DNA at internucleosomal sites, as this type of DNA degradation was a characteristic feature of apoptosis in most, but not all forms of apoptosis. However, it is becoming apparent that proteases, particularly those of the ICE family, may play a key role in the regulation of events leading to collapse of the cell structure in a manner either related or unrelated to the activation of endonucleases (Martin and Green, 1995).

Proteases were found to be important triggers for apoptosis following the studies of the proteins found in the cytoplasmic granules of cytotoxic T lymphocytes and natural killer cells, both of which kill by binding to target cells and inducing apoptosis. Further evidence that proteases were centrally involved in the regulation of cell death followed with the studies of *C. elegans* and the discovery that the ICE family of proteases were homologous to the death gene *ced-3*.

The presence of several *ced-3*-like proteins in mammals provides evidence that the cellular regulation of ICE-like proteases in apoptosis is likely to be complex but at present there is limited information available on this. It seems apparent that ICE-like cysteine proteases act as effectors in a cascade involving the generation of active enzymes in a target cell leading to cell death (Vaux *et al.*, 1993) much like the protease cascades of clotting and complement activation. Proteolytic cleavage of specific substrates may further contribute to the process of apoptosis in several ways; through structural changes, by activation of other effector molecules such as nucleases or by removal of an inhibitor (Martin and Green, 1995). As the morphological characteristics of apoptosis are similar among different cells, these authors predict that the protease substrates are ubiquitous and have been conserved through evolution.

The initial activation may lead to further autoactivation to drive the pathway into cell death and the negative regulation of these proteases may be achieved by interaction with proteins such as Bcl-2. This protein and others may inhibit the activation of the cysteine proteases by blocking proteolytic cleavage, or by sequestering the downstream targets. The so-far undiscovered mammalian homolog of *C. elegans*, *ced-4*, may either interact directly with, or act as a target for, the cysteine proteases (Miura *et al.*, 1993; Hengartner and Horvitz, 1994). However, the actual role and location of *ced-4* in the apoptotic pathway remain to be established.

2.6.3 *C-MYC* AND P53

These two genes are important in controlling cell proliferation but have more recently been implicated in the regulation of apoptosis (Bissonnette *et al.*, 1992). The nuclear proto-oncogene *c-myc* is found in higher levels in growing cells than in quiescent cells, indicating that the expression of *c-myc* is necessary for cell proliferation. In addition, *c-myc* expression is frequently deregulated in neoplasia and it is implicated in tumour progression. However, it has recently been shown that the *c-myc* gene plays a role in apoptotic cell death and that its expression under conditions of growth arrest, such as growth factor deprivation, can induce apoptosis (Evan *et al.*, 1992).

The p53 gene encodes a transcriptional activator whose targets may include genes that regulate genomic stability, cellular response to DNA damage, and cell cycle progression (Chang *et al.*, 1993). Yonish-Rouach *et al.* (1991) showed that wild-type p53 could also induce apoptosis. Once the DNA is damaged, p53 accumulates and switches off replication to allow for repair mechanisms. When the repair of DNA fails, p53 may trigger cell suicide by apoptosis (Chang *et al.*, 1993).

It appears that the decision between proliferation and death is determined by other regulatory genes that may provide a second signal. It is known that gene transfer-mediated over-production of *bcl-2* protein can partially suppress p53-induced apoptosis in some haemopoietic cell lines (Wang *et al.*,1993).

Similarly, concomitant activation of *bcl-2* with *c-myc* nullifies the apoptotic influence of *c-myc*, allowing the proliferative effects to dominate (Bissonette *et al.*, 1992).

2.6.4 SPECIFIC CELL DEATH RECEPTORS

The best studied cell death receptor is Fas/APO-1. This receptor was identified by two groups and given two different names: APO-1 by Krammer's team at the German Cancer Research Centre, Heidleberg (Trauth *et al.*, 1989) and Fas by Yonehara's group at the Department of Tokyo Metropolitan Institute of Medical Science, Japan (Yonehara *et al.*, 1989). It is a member of the tumour necrosis factor-α receptor family. The Fas/APO-1 receptor initiates a sequence of events that is finally mediated by intracellular ICE-like proteases (Los *et al.*, 1995; Nagata and Golstein, 1995). The receptor itself is not toxic to cells until it interacts with the FAS ligand or is cross-linked by a stimulatory antibody. Los *et al.* (1995) described the Fas/APO-1 receptor as one of the major regulators of apoptosis.

2.7 APOPTOSIS IN DEVELOPMENT

As the process of apoptosis can eliminate cells without harming their neighbours, it is able to participate in developmental processes, in normal cell turnover and in homeostasis of organs under physiological conditions. The widespread occurrence of cell death in embryonic systems was recognised by early developmental biologists as playing a major role in the sculpting of organs and tissues, known as morphogenesis (Glücksmann, 1951; Saunders, 1966). There are many known examples of apoptosis in development. Considerable cell death occurs during the metamorphosis of insects and amphibians (Lockshin, 1981). Cell death in mammalian embryogenesis starts as early as inner cell mass differentiation (El-Shershaby and Hinchliffe, 1974; Copp, 1978)

In embryogenesis, apoptotic cell death functions to eliminate unwanted cells during developmental processes (Garcia-Martinez *et al.*, 1993). A classic example of this kind of morphogenetic death is found in the maturation of a limb where columns of cells die in the interdigital spaces to form fingers and toes. Mammalian limb development is an exquisitely regulated system of cell movement, division, differentiation and death. The initial formation of a foot or hand palette, which consists of a core of mesenchymal cells that form condensations of cartilaginous primordia, is subsequently remodelled to produce the digits. The remodelling process involves extensive cell death in the interdigital mesenchymal tissue located between the chondrifying digits (Ballard and Holt, 1968).

During murine digit formation, *bcl-2* is expressed at high levels in digital zones and at decreasing levels in interdigital zones (Novack and Korsmeyer, 1994). It was considered that high level expression of *bcl-2* in the chondrocytes which form the digits may be an important factor in their survival. Cells in the interdigital zones do not express *bcl-2* and may therefore be susceptible to signals for cell death. However, *bcl-2* deficient mice have grossly normal limbs, arguing that *bcl-2* expression is not solely responsible for the maintenance of digital architecture. In fact, most organs display relatively normal development in *bcl-2* deficient mice. One possible explanation for the viability of *bcl-2* deficient mice relates to an emerging family of Bcl-2-related molecules which might provide a redundancy in death repressor activity during embryogenesis (Boise *et al.*, 1993).

The role of cell death in the pattern formation of limbs suggests that the process may be regulated by pattern forming genes. There are several pattern forming genes known to act in the limb. Members of the *hox* gene family including *hox d* are expressed in both the forelimb and hindlimb and it has been postulated that they specify the precursors of the limb (Morgan and Tabin, 1993).

Another class of genes with potential to regulate pattern formation in the developing limb, by controlling the transcription of other genes, is the *Msx* gene family. *Msx-1* and *msx-2* direct pattern formation, of which cell death is an important part. These genes which are related to the *Drosophila* muscle segment gene, are expressed in the regions of limbs that undergo cell death (Brown *et al.*, 1993).

Retinoic acid (RA) is known to influence limb morphogenesis. Treatment of pregnant animals with RA results in a variety of neural tube and limb abnormalities (Yasuda *et al.*, 1990; Alles and Sulik, 1989). RA directly or indirectly affects the pattern formation of the limb, perhaps by regulating gene expression. RA receptors are members of the family of nuclear receptors affecting transcriptional responses to steroid and thyroid hormones (Evans, 1988). When activated by RA, the receptors bind to upstream regulatory sequences of DNA, which are putative response elements of target genes.

In order to address the issue of what determines that one cell will die when its neighbour survives, Zakeri and Ahuja (1994) carried out studies on normal and *Hm* mutant mice. The *Hm* mutant has webbing between digits 2, 3, 4 and 5. The authors used RA to study the relation of this agent to the patterns of cell death in the interdigital regions. The authors found that RA increased cell death in specific areas of the limb where apoptosis occurred naturally in normal mice and introduced cell death between digits 2, 3, 4 and 5 of the *Hm* mutant where there was no cell death to begin with. From their observations, they concluded that there is a direct relation between RA and cell death and that this interaction may be required for correct pattern formation.

In a similar manner to limb pattern formation, a recent study by Mina *et al.* (1995) has shown that the *msx-1* and *msx-2* genes play a role in the morphogenesis of the chick mandible. However, in contrast to limb patterning where the expression of both *msx-1* and *msx-2* is correlated with cell death, only *msx-2* expression is correlated with cell death in the mandibular arch.

Msx-1 expression in the chick mandible was mapped to areas of highly proliferative cells in the mesial tips corresponding to the chondrogenic proliferating zones found at the distal tip of developing limbs which also express *msx-1*. These parallel patterns of expression of the *msx-1* and *msx-2* genes in the developing limb and mandibular process raise the possibility of involvement of these genes in mandibular morphogenesis and also suggest that they may have similar morphogenetic roles in regulating proliferation and cell death in both organs.

During embryogenesis in vertebrates, approximately half of the developing neurons undergo cell death. The mechanism of cell death appears to occur through competition for trophic factors produced by target tissues (Raff *et al.*, 1993). Once established, the majority of post-mitotic neurons in the adult animal are long-lived. The survival of neurons is thought to depend on neurotrophic factors (Raff *et al.*, 1993) and in their absence, they undergo apoptosis.

Previously the *bcl-2* gene was implicated as a potent regulator of neuronal survival because of its ability to prevent the death of primary neurons following trophic factor deprivation (Garcia *et al.*, 1992; Allsopp *et al.*, 1993; Kane *et al.*, 1993). However, more recent evidence shows that it appears not to be essential for the survival of primary neurons, as adult mice in which the *bcl-2* gene has been disrupted exhibit normal development and maintenance of the CNS. Investigations into the role of other genes that could control the survival of neurons during adult life have discovered *bcl-x*, a *bcl-2* related gene (Boise *et al.*, 1993; Gonzalez-Garcia *et al.*, 1995). These researchers found that two forms of *bcl-x* are expressed in embryonic and adult neurons. Bcl-x_L is active against neuronal cell death and may regulate neuronal survival *in vivo*. In addition, Bcl-x_β can also protect neurons from cell death and when acting together with Bcl-x_L, an additive effect is achieved. This suggests that, at least in part, these proteins counter cell death by different mechanisms or function at different cellular sites. Thus, the Bcl-x proteins appear to play an important role in the regulation of neuronal survival in the adult central nervous system.

The role of apoptosis during the development of the unique rhombomeric pattern of cranial neural crest cell migration will be discussed in the following chapter and evidence for apoptosis during the disruption of the medial edge epithelium of the newly fused palate will be discussed in Chapter 3. It is now evident that there are many examples of the correlation of cell death with morphogenetic events. However, despite considerable progress in this area, there is still much to learn about the specific components and pathways that lead to apoptosis.

3

OVERVIEW OF MAMMALIAN EMBRYOLOGY

3.1 INTRODUCTION

In order to make the use of a rodent model relevant to the study of embryology in humans, an understanding of the equivalent ages in rodent and human embryos is essential. The literature available on rodent embryology is sparse and studies on the equivalent ages in rodent and human embryos (Otis and Brent, 1954; Kaufman, 1990) are difficult to interpret and at times conflicting.

In the following description of embryology an attempt will be made to discuss the various developmental stages with reference to both human and rodent staging. Some stages that have not been described in the available literature will be estimated by the relation to stages of known timing. The timing of these stages will be flagged as approximate by the placement of an asterisk (*) following the time stated.

A description of the current hypotheses of palatogenesis is provided. Despite the large number of teams involved in this area of craniofacial research, to date there is still no consensus on the exact mechanisms that operate during fusion of the palatal shelves. It is the premise of this study that apoptosis plays a significant role in palatogenesis, however, it is possible that other mechanisms co-exist to bring about fusion of the palatal shelves.

3.2 EMBRYOLOGY

3.2.1 DEVELOPMENT OF THE BLASTOCYST

The fertilised ovum undergoes a series of divisions as it travels down the oviduct towards the uterus. These mitotic divisions result in cells (or blastomeres) that become progressively smaller with each division. On entering the uterus (Human - Day 5; Rat - Day 4) it consists of a solid mass of about 16 blastomeres called the morula (see Figure 3.1). Fluid begins to accumulate between the blastomeres transforming the morula into a fluid-filled cavity (blastocyst) containing a clump of cells at one pole called the epiblast (see Figure 3.2).

It is at this stage of development that the blastocyst embeds into the uterine wall (Human - Day 6*; Rat - Day - 5). This process is facilitated by the breakdown of the surface membrane (zona pellucida). The blastocyst becomes attached to the uterine epithelium at its embryonic pole, where the embryoblast is located. At this pole the large trophoblast cells that cover the surface of the blastocyst cavity form an invasive cytoplasmic mass and by this stage (Human - Day 7*; Rat - Day 6*) the process of implantation is well under way.

3.2.2 DEVELOPMENT OF A BILAMINAR EMBRYONIC DISC

As implantation continues in humans, the cells of the embryoblast become arranged into a two-layered embryonic disc (see Figure 3.3). The upper layer (or epiblast) consists of columnar cells and eventually gives rise to the ectoderm and the mesoderm. The lower layer (or hypoblast) becomes the endoderm (Human - Days 8-9*).

FIGURES 3.1 - 3.4: Early developmental stages up to the development of a bilaminar embryonic disc.

FIGURE 3.1: The Morula

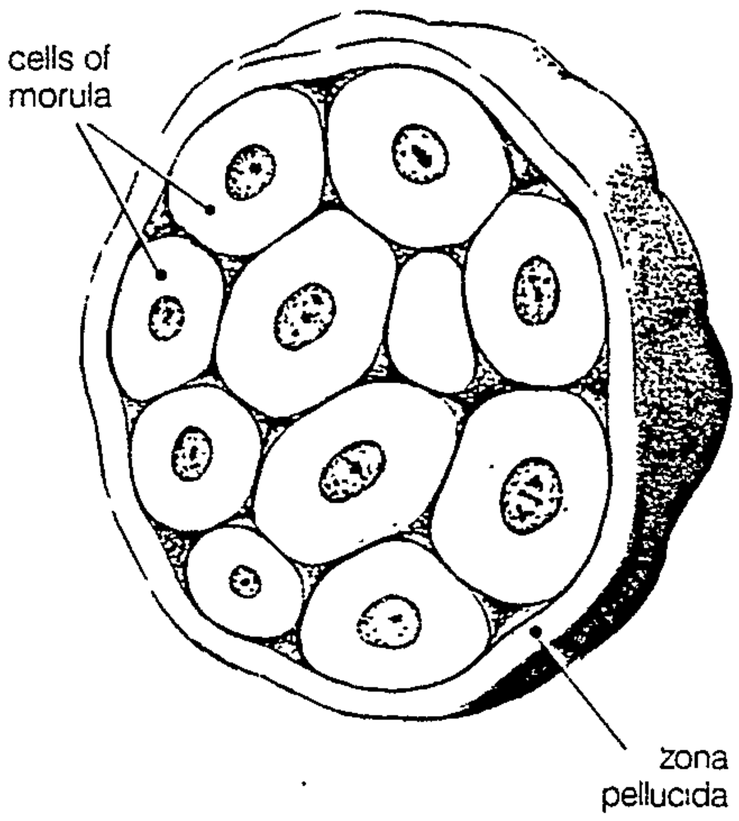


FIGURE 3.2: The Blastocyst

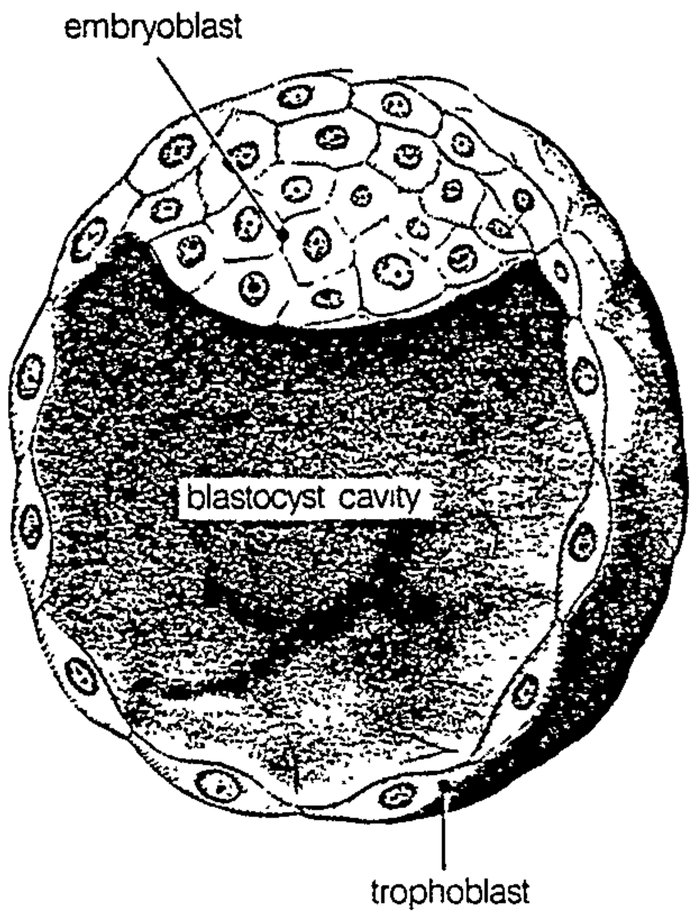


FIGURE 3.3: The Bilaminar Embryonic Disc

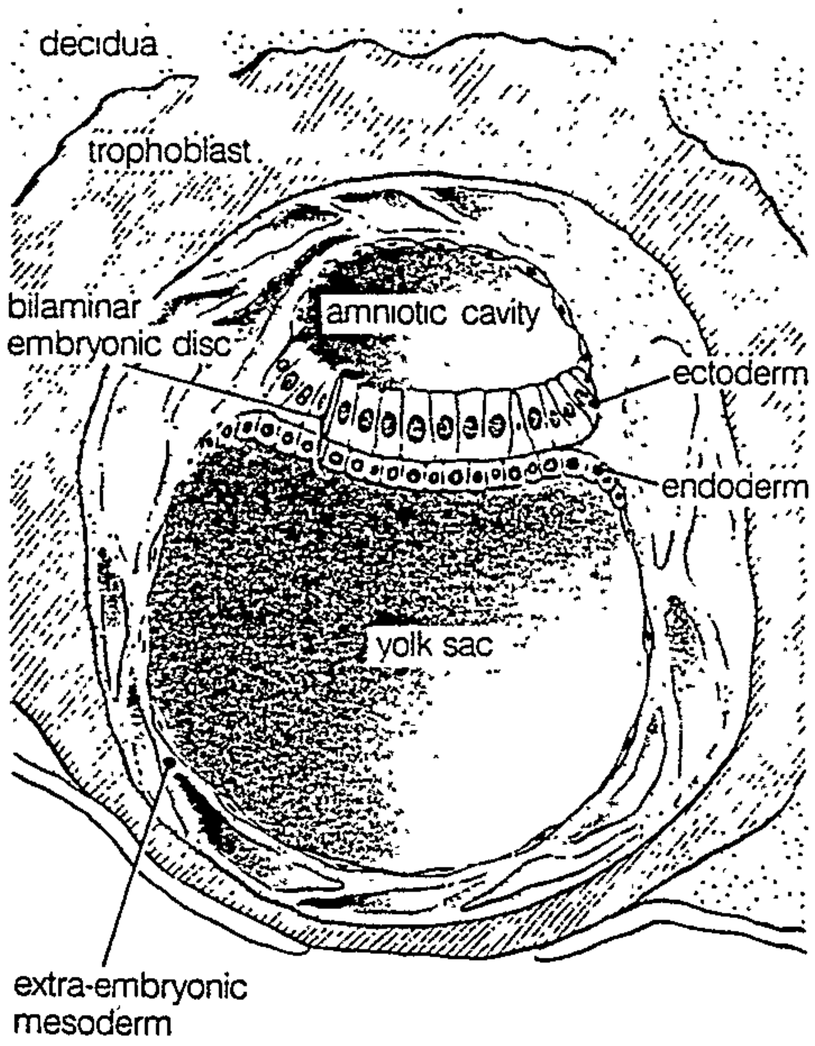
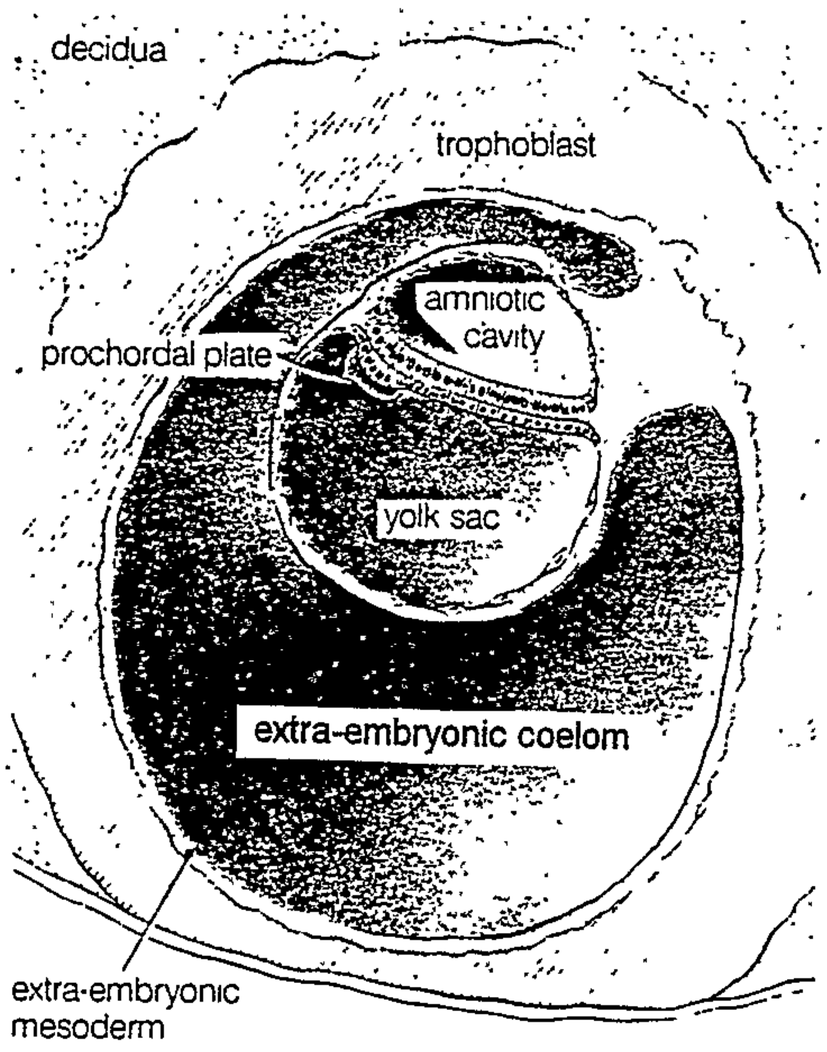


FIGURE 3.4: The Extra-embryonic Coelom



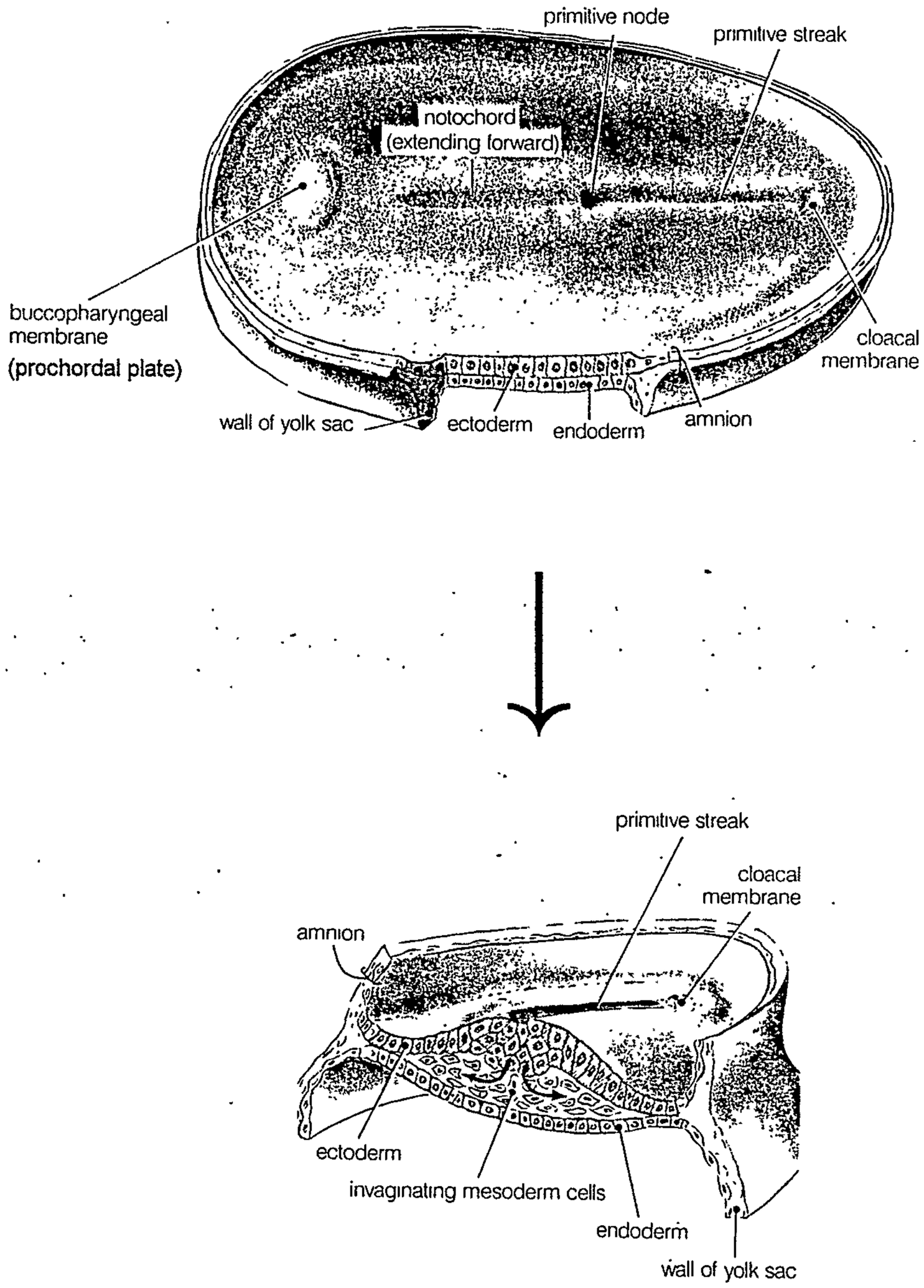
(From Mjör and Fejerskov, 1986)

The amniotic cavity in humans is formed at around the 10th Day* above the epiblast by the action of the trophoblast and this cavity is eventually lined by a thin layer of epithelium. In a similar manner the yolk sac cavity is formed beneath the hypoblast. These two cavities are initially surrounded by the extra-embryonic mesoderm but as development continues (Human - Days 10-13*) another cavity is formed (the extra-embryonic coelom) that completely surrounds the amnion and yolk sac, pushing the extra-embryonic mesoderm out to form an inner lining around the trophoblast layer (see Figure 3.4). A thickened area of extra-embryonic mesoderm is maintained at the posterior end of the embryonic disc that connects it to the trophoblast. This connecting stalk marks the area of future development of the placenta.

The development of the embryonic bilaminar disc in rats differs quite markedly from that of humans. During days 6 and 7 in the rat, a process of rapid differentiation within the trophoblast layer results in the formation of the ectoplacental cone which projects above the embryonic pole. Within the embryonal cell mass a central group of cells forms the ectodermal node, while an underlying cuboidal cell layer adjacent to the trophoblast becomes the endoderm.

This bilaminar layer in rats, known as an egg-cylinder, is U-shaped following the curvature of the extraembryonic membranes (see Figure 5.1). Initially, the germ layers are inverted and therefore must go through a process of inversion known as entypy. These changes that take place during days 10-10.5. As the rat embryo undergoes entypy, it invaginates into and becomes surrounded by, the amnion and yolk sac (Kaufman, 1990).

FIGURE 3.5: Dorsal view of the embryonic disc showing the primitive streak and the process of gastrulation.



(From Mjör and Fejerskov, 1986)

3.2.3 GASTRULATION

A longitudinal thickening called the primitive streak appears in the midline of the caudal part of the epiblast (from Human - Day 14; Rat - Day 7.5). The primitive streak extends posteriorly but anteriorly is bounded by a thickening called the primitive node in humans (the primitive node is not developed in the rat - Hebel and Stromberg, 1986). During further development the primitive streak becomes depressed forming the primitive groove. Epiblast cells migrate medially into the primitive groove from where they subsequently migrate laterally beneath the epiblast forming the intra-embryonic mesoderm (see Figure 3.5).

In humans, once this process of gastrulation is complete, a trilaminar embryonic disc exists - comprising ectoderm, mesoderm and endoderm. The lateral portions of the intra-embryonic mesoderm at the margins of the embryonic disc merge and become continuous with the extra-embryonic mesoderm of the amnion and yolk sac. In the rat the process of gastrulation takes place just prior to entypy. There are two areas within the embryo in which the ectoderm and endoderm remain in direct contact without the interposition of mesoderm. The anterior area is called the prochordal plate and the posterior area, at the posterior limit of the primitive streak, is called the cloacal membrane (see Figure 3.5).

Following the development of the trilaminar disc in humans, cells from the primitive node migrate anteriorly to the prochordal plate forming a longitudinal rod called the notochord. In rats a notochordal plate is formed in the posterior midline. Beyond this point, mesoderm cells pass on either side of the prochordal plate and will eventually form the primordia of the heart. The prochordal plate, which eventually becomes the buccopharyngeal membrane, and the cloacal membrane, mark the anterior and posterior limits of the developing gastrointestinal tract.

The intra-embryonic mesoderm on either side of the notochord thickens to form a longitudinal column, the paraxial mesoderm. Near the end of the third week in humans and during the 9th or 10th day in rats this tissue becomes segmented into a series of distinct blocks called the somites. Within each somite there are three regions, the sclerotome which gives rise to the vertebrae, the myotome which gives rise to the skeletal musculature and the dermatome which gives rise to the dermis of the skin.

3.2.4 SHAPING OF THE EMBRYONIC BODY

During the following two weeks the flat trilaminar embryonic disc in humans is transformed into a fundamentally cylindrical body by a series of foldings. Around the entire circumference the sides of the embryonic disc are folded ventrally. This process acts to tuck the prochordal plate and the pericardial cavity underneath the developing head fold (see Figure 3.6). In the same manner, posteriorly, the folding process causes the cloacal membrane to lie ventrally beneath the developing tail fold (see Figure 3.6). The inherently curved nature of the trilaminar disc in the rat allows the primordial heart to develop caudally to the head. During shaping of the embryo the stomodeum is created. It remains separated from the pharynx by the buccopharyngeal membrane until about the fifth week in humans and the eleventh day in rats.

The neural plate is formed on the dorsal surface of the embryo by a process of induction influenced by the underlying notochord (Human - Day 16, Rat - Day 9*). A second process of folding which involves the neural plate results in the formation of the neural tube and subsequently the primitive brain and spinal cord (see Figure 3.7). A specialised population of cells develops at the junction of the neural plate and the surface ectoderm. These neural crest cells are liberated during the folding process and undergo extensive migration to various parts of the body where they are involved in the differentiation of a wide variety of cell types including spinal and sympathetic ganglion cells, Schwann cells, pigment cells of the skin, odontoblasts and the skeletal structures of the craniofacial complex.

FIGURE 3.6: Schematic representation of the shaping of the embryonic body.

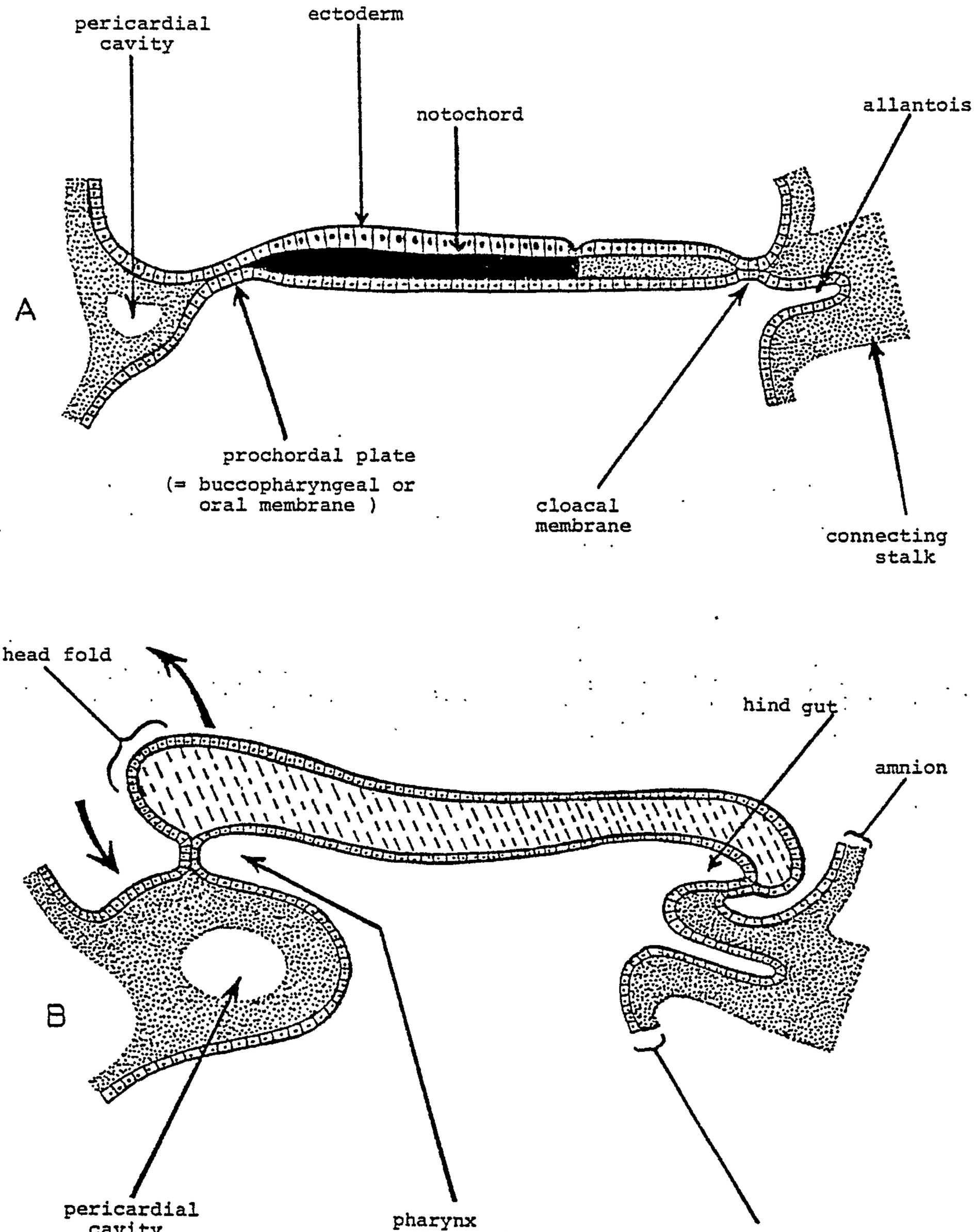
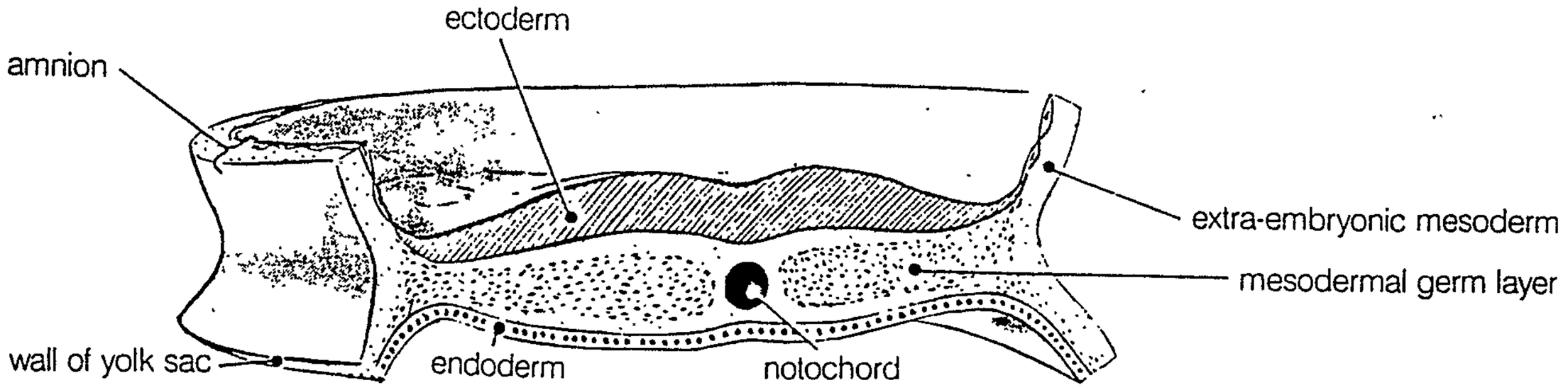
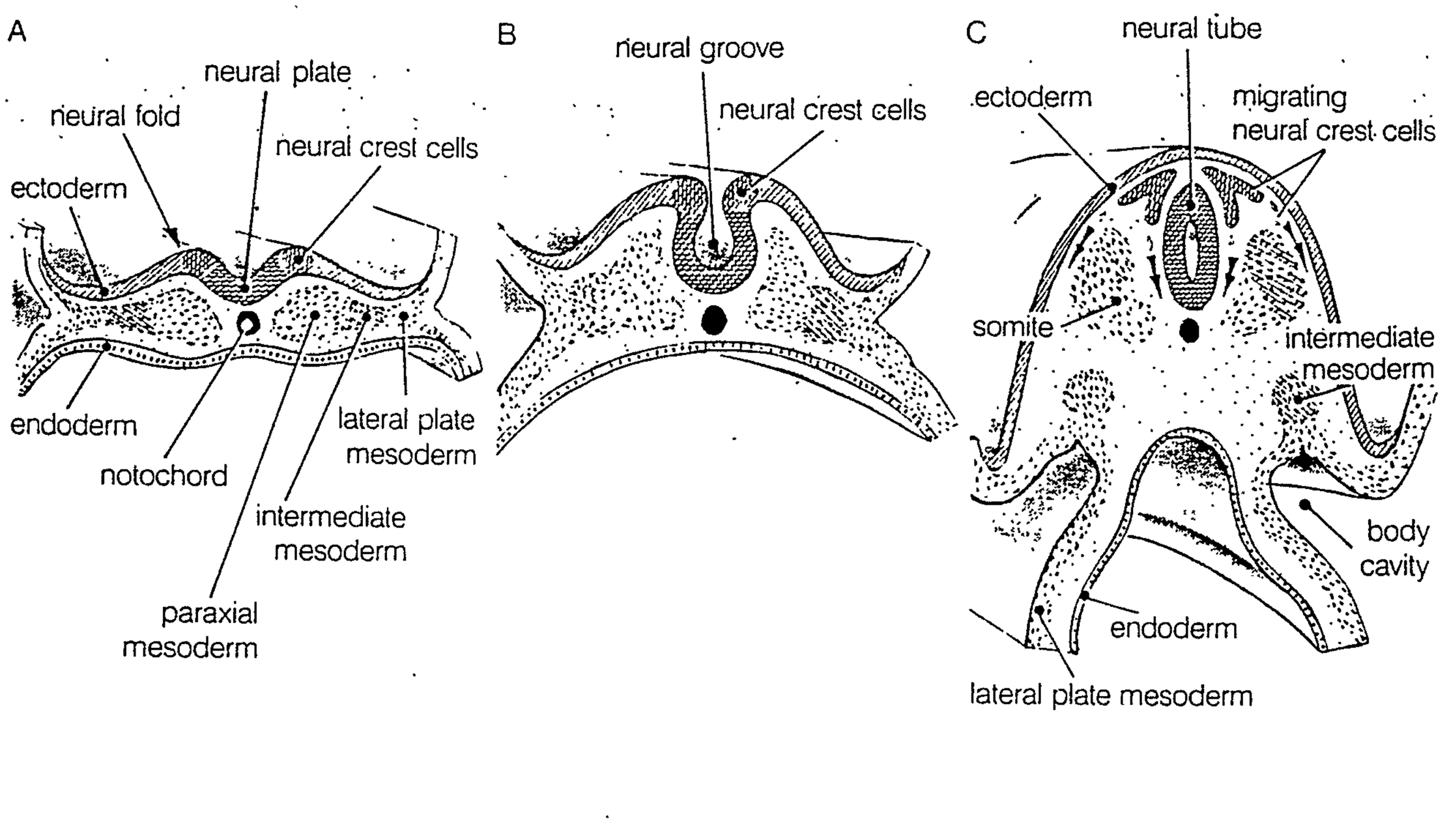


FIGURE 3.7: Transverse section through the trilaminar disc illustrating the process of neurulation.





From Mjör and Fejerskov, 1986)

3.2.5 FORMATION OF THE PHARYNGEAL ARCHES

A second stream of neural crest cells migrates towards the lateral aspect of the head where they surround the mesodermal cores in the developing pharyngeal arches (Human - During the 4th Week; Rat - Beginning Day 12). Both the frontonasal process and the pharyngeal arches play a large role in the formation of the face and the oral cavity.

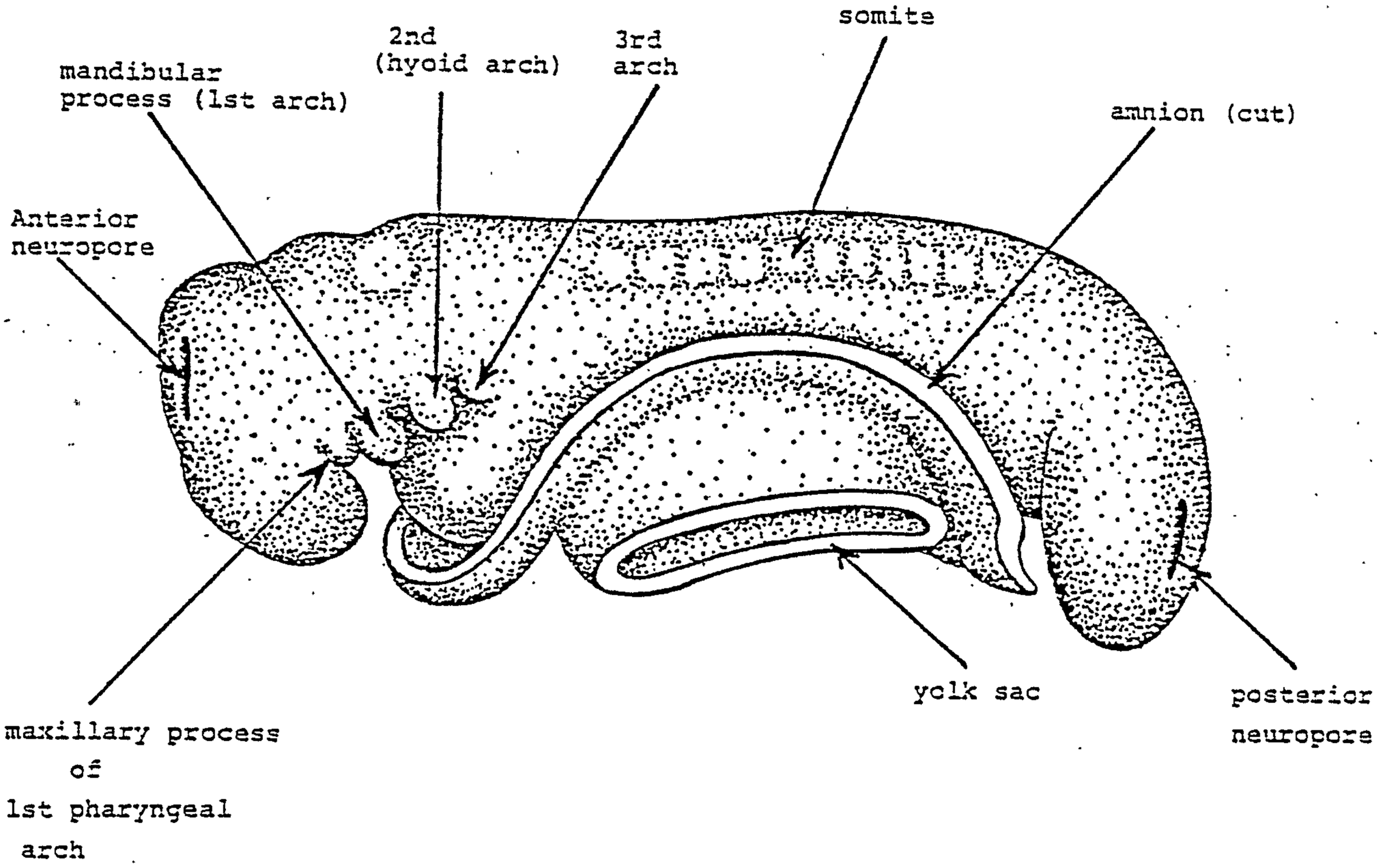
The pharyngeal arches are six paired thickenings formed within the lateral plate mesoderm. They expand ventrally between the stomodeum and the developing heart meeting in the midline. The first pharyngeal arch is the mandibular arch and the second is the hyoid arch. The third and fourth arches do not have specific names and the fifth and six arches in mammals are rudimentary (see Figure 3.8).

The pharyngeal arches are separated by deep furrows in which the ectoderm and endoderm are in contact. The internal surfaces of these furrows are the pharyngeal pouches whose derivatives include the tympanic cavity, the tonsillar fossa, the parathyroid tissue and the thymus.

The first and second arches, that is the mandibular and hyoid arches respectively, are the most prominent arches. Tissues from the mandibular arches form all the lower face and most of the midface while the other pairs of arches with their associated pouches are involved mainly in the formation of the neck region.

Each arch contains a cartilaginous and muscular component, a nerve and an artery. The skeletal element is derived from the neural crest mesenchyme while the mesoderm gives rise to striated muscle cells. Cranial nerves pass directly into the arches from the adjacent hindbrain. The nerve supply to the first arch is the trigeminal nerve. The facial nerve supplies the second arch, the glossopharyngeal nerve the third arch and the vagus nerve the rest of the arches.

FIGURE 3.8: Lateral view showing the development of the pharyngeal arches.



The tongue develops from four independent swellings which enlarge and merge with each other in the ventral wall of the primitive pharynx (see Figure 3.9). The anterior two-thirds of the tongue remains distinct from the posterior third with regards to both the epithelial lining and nerve supply which are bounded by the sulcus terminalis.

The epithelium and connective tissue of the tongue are derived from the pharyngeal apparatus but the striated musculature has a different origin. It is thought that the lingual muscles arise from the occipital somites, migrate ventrally round the pharynx and invade the tongue, carrying the hypoglossal nerve with them. This migration would explain why the innervation of the lingual musculature is not of pharyngeal arch origin.

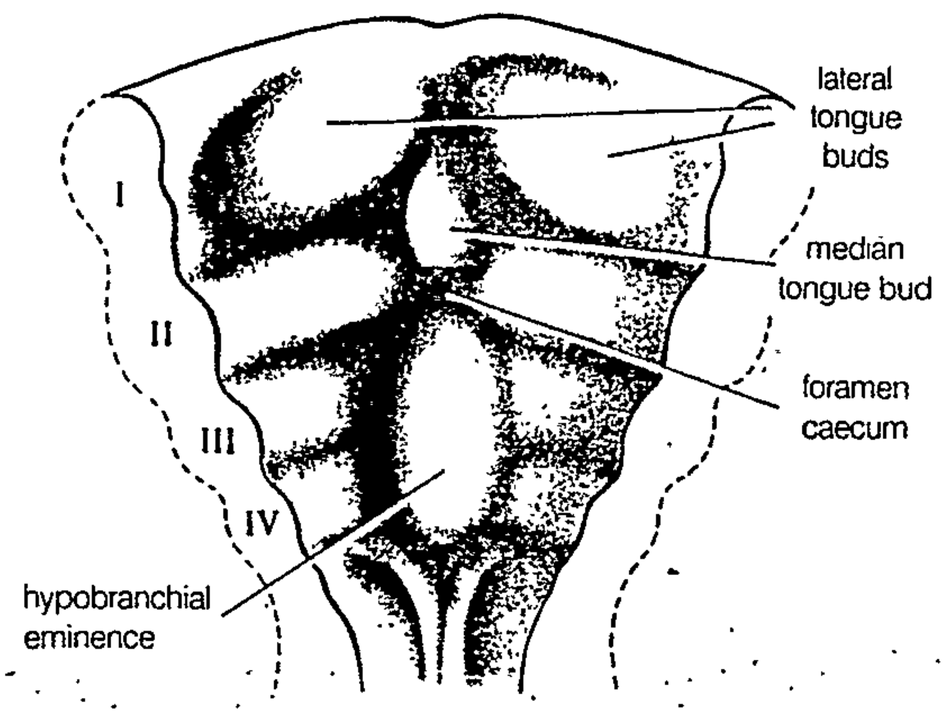
3.2.6 THE DEVELOPMENT OF THE HUMAN FACE

The face is developed from a number of mesenchymal processes that surround the stomodeum (see Figure 3.10). The frontonasal process is situated cranially. It gives rise to two ectodermal thickenings called the nasal placodes which develop into olfactory epithelium. The ectoderm surrounding them becomes raised due to proliferation of the underlying mesenchyme, forming lateral and medial nasal processes.

The first pharyngeal arch gives rise to the mandibular processes that lie caudally to the stomodeum. The maxillary processes, lying lateral to the stomodeum are also formed from the first pharyngeal arch. The formation of the maxilla is brought about by an extension of the maxillary processes to fuse with the inferior borders of the lateral nasal processes and the inferior and lateral borders of the medial nasal processes. The median nasal processes also merge in the midline to form the premaxillary region, giving rise to the philtrum of the upper lip and also the medial portion of the upper alveolar process carrying the incisor teeth and the primitive palate.

FIGURE 3.9: The pharyngeal apparatus seen from within showing the development of the tongue. A: The tongue arises from four independent swellings. B: Arrows indicate the direction of growth. C: The final anatomy of the tongue. The sulcus terminalis forms the dividing line between the anterior and posterior parts of the tongue.

A



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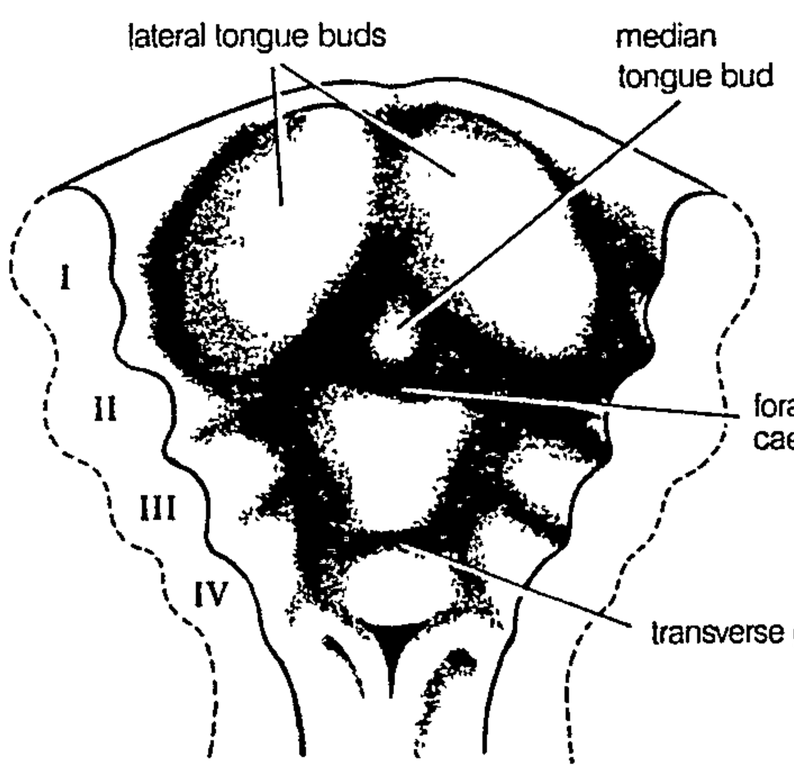

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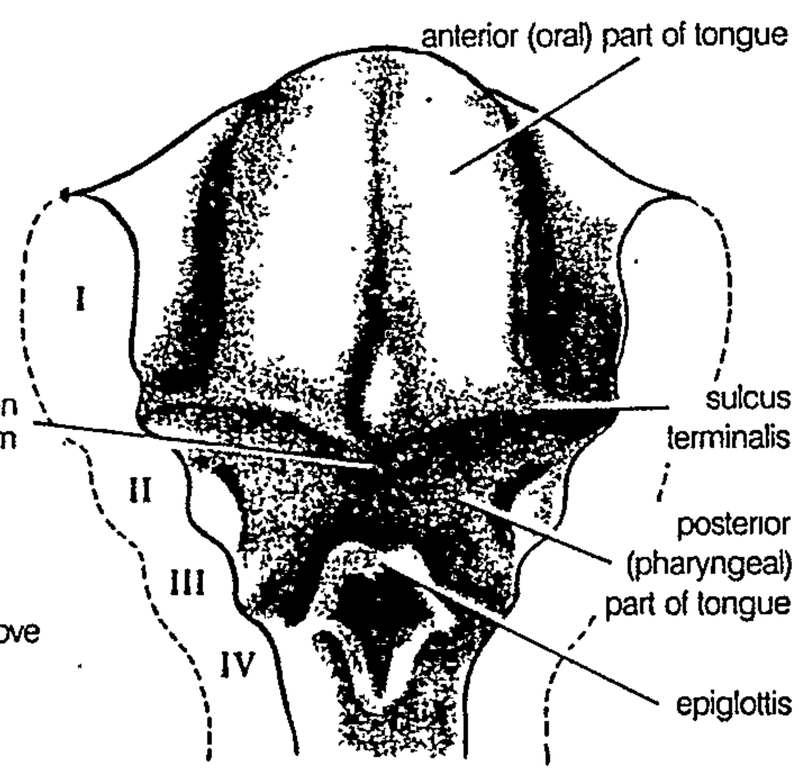
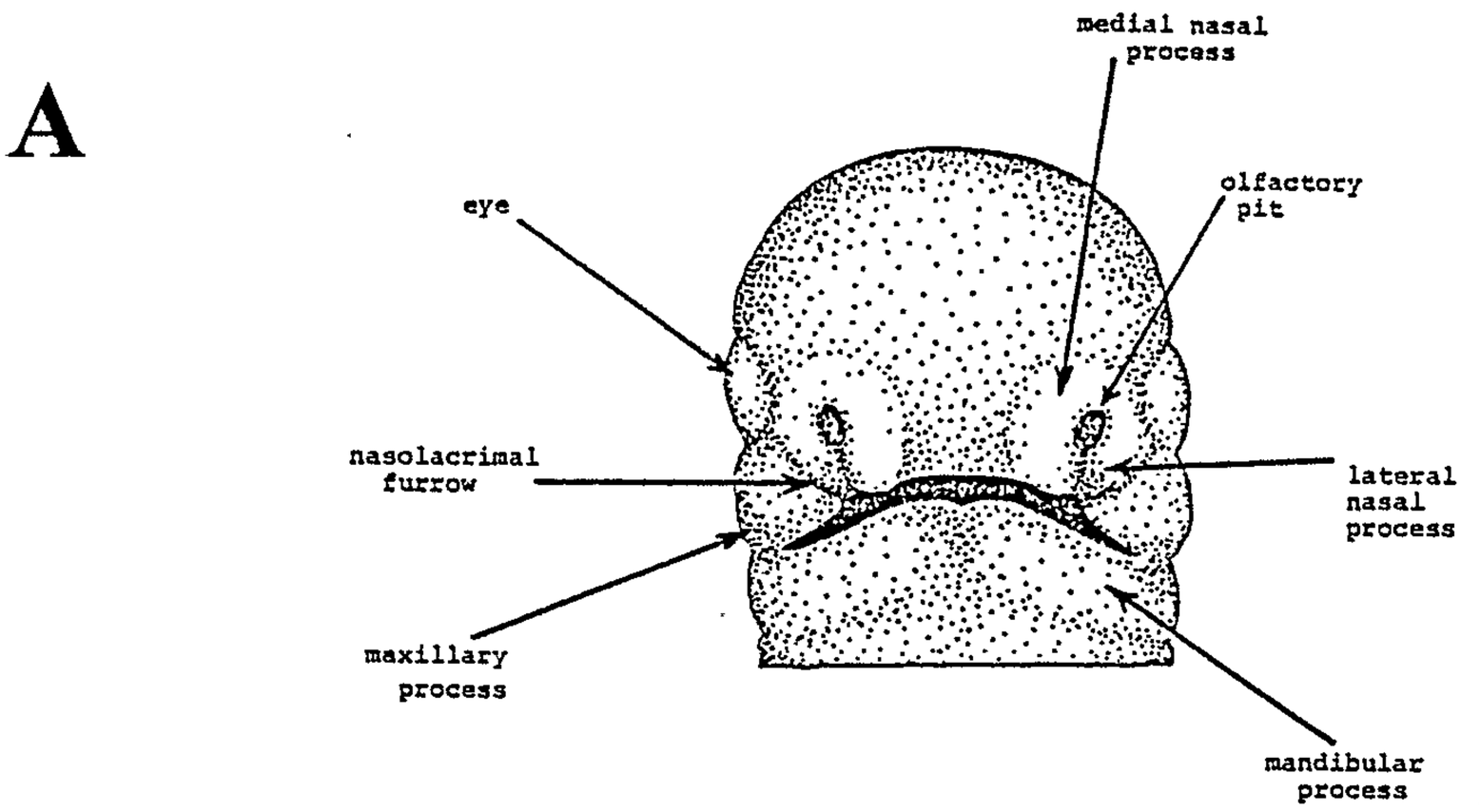


(From Mjör and Fejerskov, 1986)

FIGURE 3.10: Development of the human face. A: 5 weeks *in-utero*. B: 6 week *in-utero*. C: 7 week *in-utero*.



A groove extending from the developing eye to the mouth, is formed bilaterally, between the lateral nasal process and the maxillary process. This groove goes on to form the nasolacrimal duct. During continued development in humans the eyes gradually move medially to take up their ventral position in the face. The mandibular processes merge in the midline forming the lower lip and the lower parts of the cheeks.

3.3 DEVELOPMENT OF THE PALATE

The development of the palate in man is a complex event considered to be determined by both genetic signals and environmental influences. This event is frequently disturbed leading to the deformities of cleft lip and palate which affect approximately 1 in 1000 live births (Vanderas, 1987). Ferguson (1987) summarised the many developmental perturbations that can lead to cleft palate into five major disruptions (see Table 3.1). In order to understand the multifactorial disturbances that may lead to facial clefting it is essential to know the developmental mechanisms in normal palatal formation.

During craniofacial development, the nasal pits, bounded by the lateral and medial nasal processes, deepen forming two blind-ended sacs bounded by an epithelial wall. The epithelial wall of the nasal pit forms a contact with the epithelium of the roof of the stomodeum forming the oronasal membrane. This membrane ruptures creating a communication of the nasal cavity and the stomodeum through the primitive choana.

Anteriorly, there is a division between the nasal and oral cavities created by the primitive palate. The primitive nasal septum is formed by a vertical downgrowth of the frontonasal process. Posterior to the primitive palate its inferior edge is free and in contact with the developing tongue.

TABLE 3.1: Developmental mechanisms causing cleft palate.

INHIBITION OF CELL DIVISION AND/OR MIGRATION	• Palatal shelves too small to meet
FAILURE OF SHELF ELEVATION AT CORRECT TIME	• Altered synthesis of GAG's and Collagens • Inhibition of palatal cell contraction • Interference with neurotransmitter synthesis or receptors • Vascular insufficiency or haemorrhage • Excessive resistance factors e.g. Pierre Robin sequence • Distribution and affinity of binding sites for teratogens and growth factors
EXCESSIVE HEAD WIDTH	• Asynchrony of growth plateau in head width and timing of shelf elevation • Sex differences • Racial differences
FAILURE OF SHELF FUSION	• Defective mesenchymal signalling of critical epithelial-mesenchymal interactions • Failure of MEE cell adhesiveness - surface coat and desmosomes • Failure of MEE cell death • Failure of differential gene expression • Mechanical obstruction or excessive shelf movement
POST FUSION RUPTURE	• Causes yet to be identified

(From Ferguson, 1987)

The palatal processes arise during the sixth week of human development (Rat - 15th Day) behind the primitive palate from the deep surface of each maxillary process. They are initially directed in a vertical plane, in contact with the sides of the tongue. As the tongue descends, the palatal processes swing upwards and appose each other in the midline (Human - Week 7; Rat - Day 16). The free edges of the palatal processes fuse firstly with the posterior margin of the primitive palate and then with each other in an anterior-to-posterior direction (see Figure 3.11).

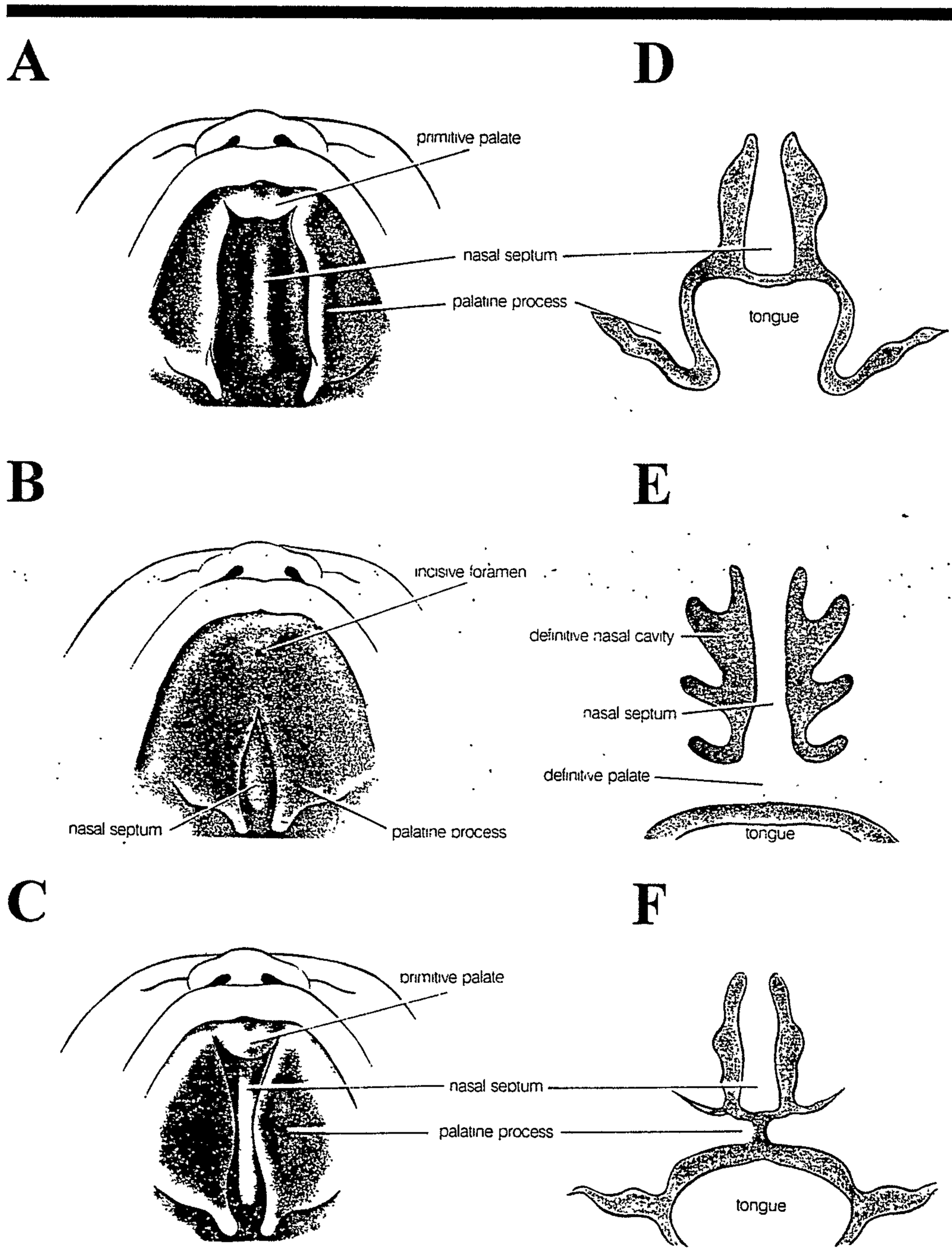
Anteriorly, along the midline, the superior surface of the palatal processes attain fusion with the free inferior border of the nasal septum. At the junction of the primitive and secondary palates the incisive foramen is formed. With the formation of the nasal septum and the palate, the original stomodeum becomes subdivided into the definitive nasal cavities and the mouth. The completion of palatogenesis is achieved by the 12th week in humans (Rat - Day 18).

3.3.1 PALATAL SHELF ELEVATION

The mechanism of palatal shelf elevation is still not fully understood. It appears to involve a combination of mechanical and intrinsic forces. The palatal processes are initially directed vertically, lying adjacent to the tongue, which at this early stage fills the whole stomodeum. As the cranium rotates away from the cardiac prominence and the mandible enlarges, the tongue descends from between the palatal shelves.

These mechanical events make elevation of the palatal shelves possible. Other mechanical factors that are thought to contribute to shelf elevation are continued growth, in cranial height and size of the oronasal cavity (Diewert, 1978), and myoneural maturation of the tongue (Wragg *et al.*, 1972).

FIGURE 3.11: Development of the human palate. A, B and C: View of the forming palate from below at 6.5, 7 and 9 weeks respectively. D, E and F: Coronal section at 6.5, 7 and 9 weeks respectively showing (D) the horizontal palatine processes, (E) the horizontal palatine processes and (F) fusion of the palate and nasal septum.



(From Mjör and Fejerskov)

The intrinsic forces involved in palatal shelf elevation are multifactorial. Ferguson (1987) showed that glycosaminoglycans (GAG's) may have a role in causing osmotic changes within the extracellular matrix of the palatal shelf mesenchyme. The GAG within the palatal shelf mesenchyme is predominantly hyaluronic acid which is highly electrostatic. The hydration and expansion of the hyaluronic acid may result in an erectile force directed by bundles of Type 1 collagen which run down the centre of the vertical palatal shelves (Ferguson and Fyfe, 1987).

Another intrinsic factor that may play a role in palatal shelf elevation is the contraction of individual mesenchymal cells within the palatal shelves. Zimmerman and Wee (1984) showed that mesenchymal cells contract under the control of neurotransmitters, including serotonin and acetylcholine, which are released by nerves and palatal mesenchyme.

3.3.2 PALATAL SHELF FUSION

The palatal shelves consist of mesenchymal tissue covered by a thin layer of epithelium. As the shelves attain a horizontal position the outermost layer of epithelial cells, the peridermal cells, are shed (Waterman and Meller, 1974). The remaining epithelium covering the potential sites of fusion is named the medial edge epithelium (MEE). Once the palatal shelves have elevated and become apposed in the midline, fusion of the MEE results in the formation of a midline epithelial seam. Greene and Pratt (1976) identified a surface glycoprotein coat on MEE which imparts specific adherence properties to these cells. It has been postulated by Ferguson *et al.* (1984) that these specific adhesion molecules, particularly desmoplastin, are assembled within the MEE just prior to and upon shelf contact. Following initial contact of the shelves, the opposing MEE are firmly joined by desmosomes (Chaudhry and Shah, 1973).

During palatal fusion, the midline epithelial seam must become discontinuous in order for the core mesenchyme of the palatal shelves to become confluent. Currently three different mechanisms have been proposed to account for the disappearance of the MEE from the midline position of the palate (See Figure 3.12).

3.3.2.1 Apoptosis of the MEE

In 1972, Kerr *et al.* described a specific form of cell death, named apoptosis, that involved precise intracellular events. This process was distinct from necrosis in its physiological quality, the morphological characteristics and the programmed nature within embryological development. Its role in palatal development remains the subject of much research.

Palatal epithelium consists of three distinct regions, namely nasal, medial edge and oral which have different developmental fates. The nasal epithelial surface differentiates into pseudostratified ciliated columnar cells and the epithelium on the oral surface become keratinised stratified squamous. The long-standing theory on the fate of the MEE has been that it undergoes apoptotic death allowing for mesenchymal confluence.

The original evidence for apoptosis of the midline epithelial seam came from three separate observations made by many different researchers. Firstly, it was found that DNA synthesis ceased 24-36 hours prior to palatal fusion leading to the loss of mitotic potential (Hudson and Shapiro, 1973; Pratt and Martin, 1975; Greene and Pratt, 1976). Secondly, the ultrastructural changes observed in the MEE, *in vitro*, were consistent with cell death (Farbman, 1968; Shapiro and Sweney, 1969; Hudson and Shapiro, 1973; Chaudhry and Shah, 1973; Pratt *et al.*, 1984). Finally, evidence of increased lysosomal enzymes in the MEE was presented (Hayward, 1969; Smiley, 1970; Pratt and Greene, 1976).

FIGURE 3.12: A schematic representation of the three current hypotheses of the fate of the medial edge epithelium - A: Apoptosis. B: Epithelial-Mesenchymal transformation. C: Migration.

Ferguson *et al.* (1984) continued the research into cell death in palatogenesis, performing organ culture of mouse, alligator and chick palatal shelves either in isolation or in homologous pairs. This work revealed that the terminal differentiation of the MEE (cell death in mammals) occurred in single palatal shelves and was therefore independent of shelf contact. Regional differentiation of the nasal epithelia into pseudostratified ciliated columnar cells and the oral epithelia into stratified squamous cells also occurred in single palatal shelves of all three species.

In addition, Ferguson and Honig (1984) performed an extensive series of epithelial-mesenchymal recombination experiments using combinations of mandibular, limb and palatal tissues of mouse, chick and alligator embryos to investigate the role of the mesenchyme in determining the fate of the MEE. Their work showed that nasal, medial edge and oral palatal epithelial differentiation is specified by the mesenchyme in a species-specific fashion. The authors concluded that epithelial cell death of the MEE in mammals is specified by the underlying mesenchyme and that the palatal epithelium appeared to play a passive role.

Despite Ferguson's commitment to the role of apoptosis in the disruption of the MEE, he did allude to the idea that other factors may also be involved. This idea was presented following Ferguson's experiments in 1988, where a large number (up to 50%) of MEE cells were found to migrate into the palatal mesenchyme, with loss of staining for cytokeratins and instead expressing the intermediate filament of vimentin, thus becoming indistinguishable from other palatal mesenchyme cells.

A recent cytochemical investigation of the fusing foetal mouse palate using specific labelling of DNA fragmentation has been carried out (Mori *et al.*, 1994). Using terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labelling (TUNEL) to identify apoptosis and immunohistochemical staining of keratin to identify cells of epithelial phenotype, the disappearance of the MEE was observed.

The results indicated that during shelf elevation and prior to contact, few cells showed TUNEL-positive staining. These cells were though to be sloughing peridermal cells. Even on initial contact of the palatal shelves the MEE remained negative to TUNEL staining. However, once the midline epithelial seam began to disappear by breaking up into a discontinuous seam, TUNEL-positive staining was found in the nuclei of some of the cells within the epithelial islands. Of interest was the detection of some TUNEL-positive, keratin-negative cells just outside the epithelial islands.

As the epithelial islands disappeared the TUNEL staining became less intense and was restricted to the oral and nasal "epithelial triangles" adjacent to the oral and nasal cavities respectively. A similar pattern of TUNEL-positive, keratin-positive staining was seen in the fusion line between the nasal septum and the dorsal palate. These results suggest that DNA fragmentation occurs in some cells along the palatal midline when the epithelial seam becomes discontinuous. Therefore, it seems that apoptosis is closely associated with the disruption of the MEE but may not be required for initial contact or epithelial fusion of opposing palatal shelves.

3.3.2.2 Epithelial-Mesenchymal transformation.

The first example of an epithelial-mesenchymal transformation occurs during the process of gastrulation described earlier where the mesoderm emerges from the epiblast under the inductive signals from hypoblast.

Fitchett and Hay (1989) observed ultrastructural and molecular changes *in vivo* indicating that the MEE in rats underwent an epithelial-mesenchymal transformation and remained in the connective tissue of the palate as viable mesenchymal cells. They disputed Ferguson's concept of programmed cell death of the MEE and speculated that such transformations allowed for both conservation of embryonic cell populations and a mechanism for achieving mesodermal confluence within the palatal processes.

Shuler *et al.* (1991, 1992) carried out experiments in mice that supported the concept of an epithelial-mesenchymal transformation of the MEE. Using a vital cell labelling technique and phenotypic markers, the fate of the MEE cells was traced during palatal fusion *in vivo*. The exogenous marker - 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), is irreversibly incorporated into the plasma membranes of cells providing a means of identifying specific cell populations during development. Immunohistochemical markers for keratin, vimentin and laminin were employed as phenotypic markers of epithelial cells, mesenchymal cells and the basal lamina respectively.

Using these techniques, the MEE cells were examined during palatal development. In the initial stages they were present in the midline position where they were attached to an intact laminin-containing basement membrane and contained keratin intermediate filaments. At later stages of palatal development the DiI-labelled MEE were not separated from the mesenchyme by an intact basement membrane and did not contain keratin. By the completion of palatal fusion , the DiI-labelled cells remained as viable cells but were observed to have a mesenchymal morphology, showing staining for vimentin intermediate filaments, thus making the case for an epithelial-mesenchymal transformation.

3.3.2.3 Migration of the medial edge epithelium.

Carette and Ferguson (1992) proposed that the MEE cells may survive and retain their epithelial phenotype but migrate out of the seam to merge with the adjacent oral and nasal epithelia. Performing parallel experiments to Schuler *et al.* (1991), their conclusions were quite different. In addition to conventional histology they utilised confocal laser scanning microscopy. This offers significant advantages with its increased resolution and enhanced contrast, but most importantly allows optical sectioning of vital specimens.

With the aid of this new technology, they showed that the MEE cells migrated nasally and orally where they constituted epithelial triangles on both the oral and nasal aspects of the palate and subsequently merged into the epithelium covering the nasal and oral surfaces of the palate.

The persistence of these three hypotheses to the present time may indicate that all three processes, apoptosis, epithelial-mesenchymal transformation and epithelial migration, have a role to play in the fate of the MEE. While several factors may be involved in the thinning and disruption of the MEE including expansion in palatal height, epithelial-mesenchymal transformation and the migration of epithelial cells, it appears that apoptosis is involved in the final breakdown of cells once the seam has been disrupted. Mori *et al.* (1994) concluded with the hypothesis that apoptosis, epithelial-mesenchymal transformation and cell migration are all involved at various stages of palatal fusion and that their occurrence is spatially and sequentially regulated.

Much research continues in the field of palatal fusion and in the morphogenesis of craniofacial clefting. Further studies on the mechanisms of cell death should provide an insight into the role of apoptosis in normal palatogenesis. With this knowledge and continuing teratological studies it is hoped that an understanding of the events in palatal clefting will eventuate and allow the development of better preventive and therapeutic techniques.

3.4 SUMMARY

Thorough knowledge of normal embryological development is essential for an understanding of the aetiology and pathogenesis of congenital malformations. Intensive research into craniofacial embryogenesis has made possible an almost complete description of the changes which take place during facial development. However, the controlling factors and induction of processes that lead to these changes are still largely unknown. Malformations of organs or parts of organs can occur at all stages of development and anomalies can be traced to well-defined defects occurring at early stages of intrauterine life.

In order to achieve further knowledge of the complex process of palatal development, a study of the morphological events occurring during the first fusion process, that is closure of the neural tube, was carried out. Although a great deal of work has been published on neural induction, shaping and bending of the neural folds and neural crest cell migration, the events leading to confluence of the two neural folds in the formation of a patent neural tube have not been described. Without a better understanding of this fusion process, further consensus as to the fate of the MEE and the mechanisms of palatal fusion may not be possible.

4

NEURULATION AND THE NEURAL CREST

4.1 INTRODUCTION

Neurulation is the initial process in the development of the central nervous system in chordates. The study of neurulation in mammals has only become commonplace in recent times. Most early work in the field utilised amphibian and avian models because of their ease of manipulation during early embryogenesis. However, it is known that while some of the features of neurulation are common amongst differing species there are many features that vary significantly amongst amphibian, avian and mammalian models.

Neural tube defects are among the most common human congenital defects. The incidence of these defects worldwide is estimated to range from 2 - 8 per 1000 live births (Nevin, 1981; Stein *et al.*, 1982; Baird, 1983). In addition, neural tube defects have been found to be the most frequent abnormality seen in spontaneously aborted foetuses, with incidences reported to be ten times higher than those noted in live births (Stocks, 1970; Byrne and Warburton, 1986). However, despite their high prevalence and significant morbidity, little is known of their aetiology.

Anencephaly, a lethal condition in which the brain tissue is exposed and spina bifida, a condition involving regionalised disruption of spinal nerve pathways are the most prevalent forms of neural tube defects.

These defects result in an abnormal communication between the cerebro-spinal and amniotic fluids, resulting in a higher than normal level of α-foetoprotein in the amniotic fluid. Although measurement of α-foetoprotein levels allows prenatal diagnosis of these conditions, there is little to offer in the way of therapeutic options at present.

Genetic and environmental factors (intrauterine environment or teratogen exposure) are known to influence neural tube defects in human populations and rodent model systems (Campbell *et al.*, 1986). For this reason animal studies have been useful in both the evaluation of the role of suspected human teratogens and in the determination of the mechanism of normal and disordered neurulation in different species.

In order to develop an understanding of neurulation and the perturbations leading to neural tube defects, it is necessary, initially, to look back at the early work carried out on various amphibian and avian models. Traditional views of how neurulation occurred were developed beginning over a century ago with the pioneering work of His (1889) and FC Sauer (1935) but advances in recent years have discovered inadequacies in some aspects of these principles. The evolution of these early hypotheses into the contemporary concepts that are believed to be true today will be discussed in the ensuing chapter.

An understanding of the events of early embryology is essential for the study of neurulation. The development and migration of the neural crest cells will also be discussed in order to highlight the intimate relation of these cells with those of the neural folds at the time of fusion. Studies of the neural crest have identified the origins and complex migratory pathways of these cells but the mechanisms of cell migration and differentiation are still not completely understood.

4.2 STRUCTURE AND MITOTIC BEHAVIOUR OF THE EARLY NEURAL TUBE

The newly formed neural tube in vertebrate embryos shows a characteristic concentration of mitotic figures adjacent to the lumen. In his review of the structure and mitotic behaviour of the early neural tube, Watterson (1965) described the classical concept of His. In 1889, His referred to the mitotic cells adjacent to the lumen as "germinal cells" and believed that they were special stem cells that remained adjacent to the lumen and divided repeatedly to produce daughter cells which migrated out of the germinal zone immediately to become the peripherally located "spongioblasts". For this theory to be correct, the orientation of the mitotic spindle would have to lie perpendicular to the lumen so that the outermost daughter cell would literally be squeezed out of the germinal layer, leaving the innermost daughter cell to remain in the germinal zone and function as a stem cell.

F C Sauer (1935) was the first to the describe the neural plate and neural tube in terms of a single pseudo-stratified layer of columnar epithelium whose cells were attached to each other at the free (or lumenal) surface by terminal bars. He disputed the presence of an internal limiting membrane as described by His, concluding that the internal limit of the neural tube was formed only by the cytoplasmic membranes of the lumenal layer of neuroepithelial cells.

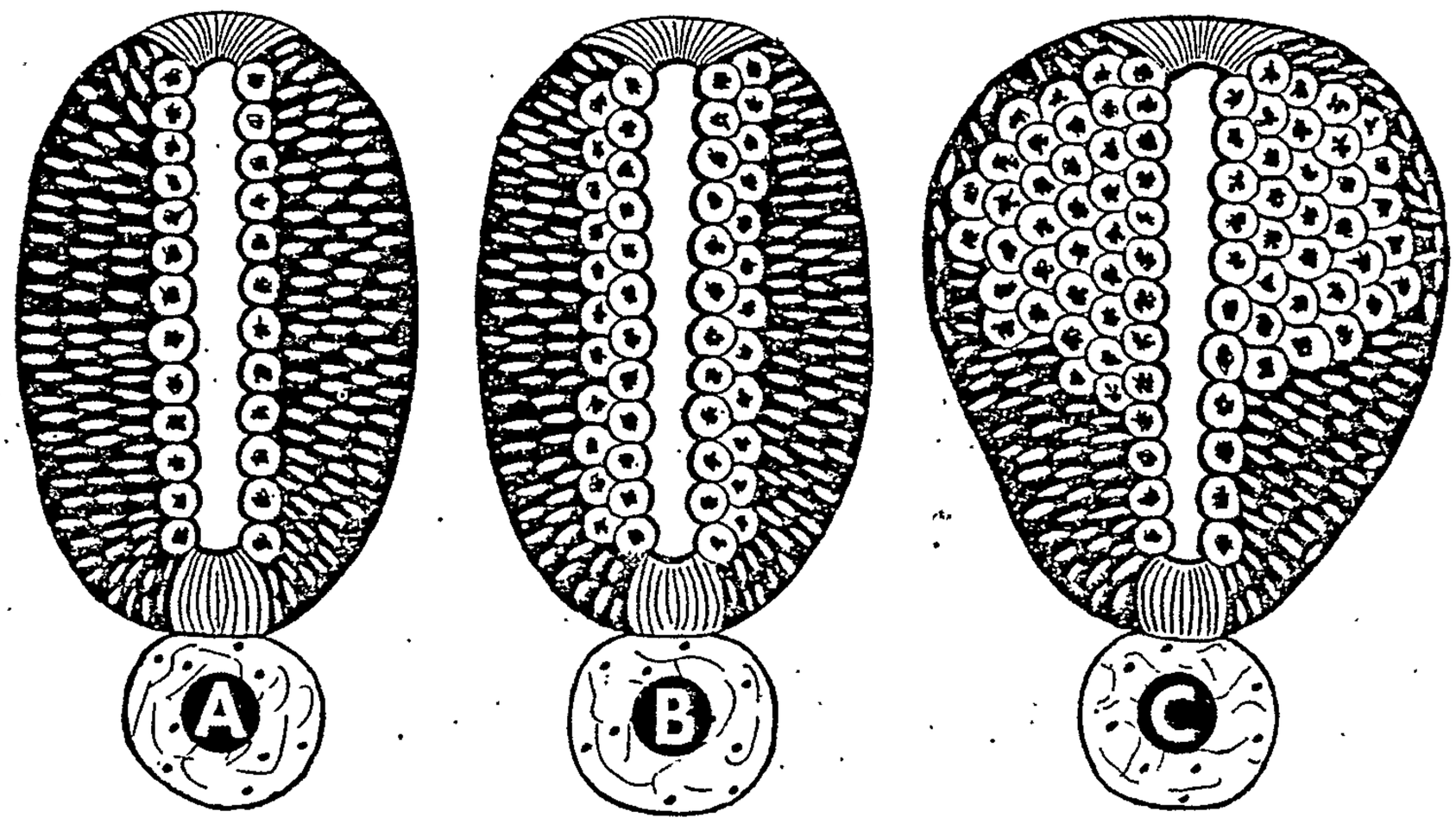
As the neural plate thickens and develops into a tube, the effect of a stratified layer of cells is created by an elongation of the epithelial cells such that their nuclei become arranged into multiple layers. The explanation of this process followed F C Sauer's observation of pig and chick embryos during the appropriate stages of neurulation.

By taking measurements of the dimensions and distances from the lumen of interkinetic nuclei and of nuclei in the various stages of mitosis he concluded that cells entering the prophase of mitosis underwent a change of form in which their nuclei migrated towards the lumen of the neural tube and their cytoplasm assumed a rounded form in preparation for nuclear division. The metaphase, anaphase and early telophase occurred only while the nuclei were adjacent to the lumen and following these stages the nuclei migrated back to a deeper position within the wall of the tube and entered the interphase again. Thus dividing cells visible in the "germinal zone" at any given time were not permanent residents of the zone but a population of mitotic cells that changed constantly.

Although F C Sauer presented this interpretation in a convincing way it was based solely on descriptive evidence and was largely ignored for the following 20 years until experiments utilising a mitotic block at the metaphase stage were carried out. Colchicine solution was used by Watterson *et al.* in 1956 to confirm F C Sauer's observations. Colchicine solution was deposited onto the surface of a developing chick embryo and after 48 hours of incubation the embryos were sectioned and examined under light microscopy (see Figure 4.1).

The existence of a true germinal layer of cells adjacent to the lumen of the neural tube would be shown as a single layer of blocked mitosis in this region regardless of the length of time the mitotic block was imposed (A). According to this concept, all other cells of the neural tube wall lose their mitotic capacities once they leave this zone. However, if F C Sauer was correct in his interpretation, a single layer of blocked mitoses adjacent to the lumen should only be encountered in those embryos exposed to colchicine for a very short time. This would occur because with a brief exposure to the agent only those few cells approaching metaphase at the time of colchicine application would be arrested in metaphase.

FIGURE 4.1: Diagrams showing changing patterns of blocked mitoses expected in the neural tube wall after increasing intervals following application of a given amount of colchicine. Diagrams A, B and C are discussed in the text.



(From Watterson, 1965)

As the length of time the chick embryo was exposed to colchicine increased, other mitotic cells entering prophase stage should have begun to round up and withdraw toward the lumen because the agent does not interfere with the passage of neural tube cells through prophase changes. These cells would presumably be prevented from withdrawing all the way to the lumen because of the presence of the turgid layer of blocked cells and so they would form another layer of blocked metaphase cells (B). After several hours of exposure almost every neuroepithelial cell in the lateral regions of the neural tube would be blocked (C), strongly confirming F C Sauer's interpretation.

A greater number of blocked mitoses were found in the lateral aspects of colchicine treated neural tubes compared with the basal regions. Although this suggested the possibility that the mitotic duration may have been less in the lateral regions than in the basal regions, it was later found that the greater accumulation of blocked mitoses in the lateral regions simply indicated that many cells of the basal region began to differentiate at an early stage and then lost the capacity for proliferation even though they may still have been present in the neural epithelium.

Thus, observations of the mitotic behaviour of the early neural tube wall of embryos treated with colchicine led to the conclusion that most, if not all cells of the lateral regions are capable of division whereas many of the cells of the basal region have this capacity only at an early stage.

Unfortunately, within an hour after its application colchicine causes a radical shrinkage of the blastoderm, a reduction in the length of the embryo and formation of convolutions in the neural tube. Transverse sections through different levels of such a distorted neural tube would give conflicting pictures of the number of layers of blocked mitoses in a single specimen. Although the colchicine experiments represented the first validation of F C Sauer's interpretation of the structure and mitotic behaviour of the neural epithelium it became evident that better methods of testing the concept were needed.

M E Sauer and Chittenden (1959) used measurements of individual nuclear DNA contents to further prove that the mitotic cycle in the early neural tube of chick embryos involved nuclei at all depths. Their measurements of DNA content in nuclei of the peripheral two-thirds of the neural tube wall was intermediate between the mean 2n value of telophase stages and the mean 4n value obtained in metaphase and anaphase stages of the inner portion of the neural tube.

To further validate F C Sauer's theory, M E Sauer and Walker (1959) studied neuroepithelial cells labelled with tritiated thymidine, which becomes incorporated into chromosomes of cells preparing for division and is retained by these chromosomes and their progeny. Sequential radioautographs were prepared from chick embryos that had been sacrificed at short intervals following their treatment with tritiated thymidine. Initially, (after 2 hours) labelled nuclei were visible only in the outer half to two-thirds of the wall of the neural tube.

Mitotic figures at the lumen were not labelled after this short exposure time. After four hours the radioactivity extended throughout the wall, with a layer of greatest intensity being located at the periphery. By 8 hours strongly radioactive nuclei extended uniformly from the periphery to the lumen with little change thereafter.

These results demonstrated that the interphase stages which began to incorporate tritiated thymidine into newly synthesised DNA were found peripherally in the wall of the tube. Partially labelled nuclei then continued to incorporate tritiated thymidine as they continued to migrate towards the lumen with the result that mitotic figures adjacent to the lumen were then heavily labelled. By 8 hours labelled nuclei were seen migrating toward the lumen where they underwent division and migrated back towards the periphery. They again incorporated tritiated thymidine and repeated the mitotic cycle as judged by the increased intensity of labelling in the periphery. These results provided a convincing confirmation of F C Sauer's interpretation of the structure and mitotic behaviour of the early neural tube wall.

4.3 NEURULATION

Neurulation is the process of neural tube formation that results in the development of the brain and spinal cord. It can be divided into two distinct phases in avian and mammalian embryos. The first phase, primary neurulation, begins with the formation of the neural plate and ends with the closure of the neural tube. The second phase, secondary neurulation, involves the formation of the caudal portion of the spinal cord (Schoenwolf, 1985).

Once formed the tubular nature of the central nervous system is retained throughout life. Its lumen, the central canal, carries cerebrospinal fluid and its walls differentiate into discrete, concentrated zones of neurons. The process of primary neurulation will be discussed in this chapter. In higher vertebrates, including humans, primary neurulation occurs in four recognisable stages:

1. Formation of the neural plate involving ectodermal thickening as a consequence of induction.
2. Shaping of the neural plate.
3. Bending of the neural plate.
4. Closure of the neural groove with formation of the roof plate of the neural tube, the neural crest and the overlying surface epithelium.

In most vertebrates the presumptive neural tissue is first seen as a thickening of the ectoderm overlying the notochord and somitic mesoderm. This thickened ectoderm constitutes the neural plate. During the early stages of neurulation, the borders of the neural plate elevate to form the neural folds. The neural groove is formed by the continued elevation and apposition of the neural folds forming a trough. Eventually, the edges of the neural folds meet and fuse forming a hollow neural tube.

The surface ectoderm which has been drawn over the tube by the medial movement of the neural folds fuses along the midline of the embryo. The cells that come to lie between the neural tube and the overlying ectoderm make up the specialised cells of the neural crest.

The formation of the neural tube is significant for several reasons. Firstly, it constitutes the first fusion process during embryological development. Secondly, it represents the formation of the first organ, initiated by an inductive process. Finally, neurulation involves a change in morphology from a flat sheet of cells to a hollow cylinder, representing one of the most basic examples of morphogenesis.

4.3.1 THE TRADITIONAL VIEWS OF NEURULATION

Cell morphology during neurulation was initially studied in anurans, urodeles and chicks and although there were differences in the sequence of events, similar morphological transformations of the neurectodermal cells occurred. The study of neurulation in the chick embryo showed a pseudostratified ectodermal thickening lying over the notochord and the mesoderm immediately lateral to the notochord. The presumptive neural cells were polygonal in shape and were taller than they were wide. During development the cells elongated in the dorsoventral axis forming a flat columnar neural plate. As the neural plate began to fold the neural cells became strongly columnar achieving a height approximately 10 times their width.

It was the belief that with further development the most medial cells underwent constriction of their apices which proceeded further and further laterally causing the neural folds to appose each other in the midline. Finally, the right and left neural folds fused to form the neural tube.

Following neural induction, shaping and bending of the neural plate take place. Most early researchers acknowledged that forces were required to drive these processes and several different sites of origin were proposed. Some investigators suggested that these forces originated in the neurectoderm itself while others concluded they were extrinsic to the neurectoderm. The concept that the folding of the neural plate was a passive event was also considered.

An experiment first performed by Lewis (1947) was the forerunner of many studies designed to investigate the forces involved during neurulation. In his study, Lewis made an incision in the ectoderm in an anterior-posterior axis immediately lateral to the neural fold on one side of an embryo of A. *punctatum*. The consequences of this were an immediate opening of the wound with both borders moving away from their prior positions. Secondly, the neural anlage continued to neurulate and form a morphologically intact neural tube and at the same time the lateral ectoderm began to heal the wound. However, the forces that pulled the neural fold medially were greater than the forces that caused the neural fold to participate in wound healing resulting in a deviation of the fusion line of the neural tube away from the side of the wound. Any hypothesis that could not explain these observations was not considered to be an adequate interpretation of the process of neurulation.

4.3.1.1 Forces originating in the nonneural ectoderm.

It was suggested that the neural plate forms neural folds as a result of the dorsal and medial migration of the nonneural ectoderm. This migration was thought to occur as a result of the differential mitotic rate, that is the nonneural ectoderm divided faster than the neural ectoderm.

As cited by Karfunkel (1974) an experiment by Bragg in 1938 found that in anurans the mitotic index of the nonneural ectoderm was higher than that of the adjacent neural ectoderm and that the ratio of their mitotic indices was higher than in any other part of the embryo.

Bragg further reported that the mitotic indices of both the neural and nonneural ectoderm were higher at the neural plate stage than the neural groove stage. These observations were the basis for a hypothesis that in anurans neurulation might be, at least partly, produced by the growth of recently formed daughter cells within the nonneural ectoderm.

Gillette (1944) also addressed this hypothesis by examining changes in the number of cells lying in dorsal, lateral and ventral areas of nonneural ectoderm during neurulation in *Ambystoma*. He found that the total number of cells in the entire ectoderm increased and that the percentage of the total ectoderm found in the dorsal section increased during neurulation whereas that in the lateral and ventral sections decreased. Gillette was the first to quantify the movement of nonneural ectoderm in a dorsal direction during the course of neurulation.

Karfunkel (1974) concluded that although there seemed to be little question that the lateral ectoderm migrated dorsally during the course of neurulation, Gillette's experiment could not be used as evidence that the dorsal migration caused the closure of the neural tube as it did not account for Lewis' observation. That is, if the lateral ectoderm actively migrated dorsally, pushing the neural folds medially, then when a wound is made just outside the neural fold in an anterior-posterior axis, it should not result in the formation of a widening wound.

4.3.1.2 Forces originating in the neural folds

Karfunkel (1974) cited the work of Jacobson who in 1962 excised the neural plate leaving the neural folds intact and reported that "the course of neurulation was perfectly normal, apart from some retardation". Jacobson also carried out experiments in which a part of the neural plate of an *Ambystoma* embryo was inverted and reportedly still completed neurulation without abnormalities.

However, Karfunkel commented that Jacobson had not provided photographs of his work or addressed the issue of whether or not wound healing alone had produced the medial migration of the neural folds. Karfunkel reported the results of his own work in carrying out a variation of Jacobson's experiment and concluded that the medial migration of the neural folds seen in his experiment was the result of wound healing alone.

4.3.1.3 Forces originating in the neural plate

The belief that the forces involved in neurulation originated within the neural tube were founded by Roux (1885) whose experiments were cited by Karfunkel (1974). Roux explanted chick neural plates and observed them to fold normally despite their isolation from the lateral ectoderm and mesoderm. After that time investigations into the possible roles of changes in tissue mass of the neural anlage and uptake of water by the cells of the neural anlage were undertaken by Glaser (1914) but subsequently disproven by Brown *et al.* (1941) who were the first workers to suggest that cell-shape changes resulted from changes in the cytoskeleton of neuroepithelial cells. This proposal was further supported by the subsequent work of both Holtfreter (1946) using *Ambystoma* embryos and more recently by Adler using chick embryos (1971).

4.3.1.4 Forces originating in the chordamesoderm

The possibility of a force produced by the myotomes was considered over the years. It was raised again in 1970 by Schroeder who noted that the elevation of the neural folds seemed to be enhanced by a thickening of the subjacent mesoderm in *Xenopus* and correlated with the formation of the somites by that mesoderm. Karfunkel's own experiments (1971, 1972) seemed to show that the chordamesoderm may have a role to play for *Xenopus* but not for either urodeles or chick embryos.

By the 1970's it was the common belief that the forces playing a role in the process of neurulation originated from the presumptive neural ectoderm and that the processes of cell elongation and apical constriction were critical events. The involvement of microtubules in the elongation process of neural ectodermal cells was first suggested by Brown *et al.*,1941). Direct evidence of microtubules orientated along the axis of elongation of the cells followed in various models including chick embryos (Karfunkel, 1972) and *Xenopus* (Schroeder, 1970; Karfunkel, 1971).

Experiments undertaken by Karfunkel (1971) where the microtubules were disrupted using vinblastine sulphate caused previously elongated neurectodermal cells to lose their elongated shape and a flattening out of the already elevated neural folds. Unfortunately, vinblastine sulfate disrupts the microfilaments as well as the microtubules in *Xenopus* and so the effects of the microtubules alone were difficult to interpret.

The hypothesis that microfilaments caused the apical constriction of the neural epithelial cells which in turn led to the curvature of the neural plate was originally based on morphological evidence alone. In Karfunkel's vinblastine sulfate experiments (1971) it was noted that cells that were apically constricted prior to treatment lost their apical constriction following treatment. In the chick, microfilaments can be disrupted using cytochalasin B without affecting the microtubules. Cells treated with cytochalasin retained their elongated shape and microfilaments; however, cells that would have become apically constricted failed to do so. Karfunkel (1974) concluded that these findings confirmed a direct correlation between the presence of microfilaments and the apical constriction of neural epithelial cells, the elevation of the neural folds and formation of the neural tube.

In summary, Schoenwolf and Smith (1990) defined the three fundamental principles that encompassed the traditional view of neurulation:

(1) All the forces involved in neurulation were intrinsic to the neuroepithelium.

(2) Neurulation was driven by changes in the shape of neuroepithelial cells and

(3) These changes in cell shape were generated by the cytoskeletal elements (microtubules and microfilaments).

Having now defined the principles that constituted the traditional view of neurulation the scene is set to explore their inadequacies in the light of the current research and arrive at the contemporary viewpoint.

4.3.2 THE CONTEMPORARY VIEW OF NEURULATION

The current concept of the process of neurulation also consists of three fundamental principles concerning both neural plate shaping and bending.

The process of neurulation shows some regional variation along the rostrocaudal axis of the embryo. Early in the process of shaping of the neural plate the notochord becomes anchored to the midline of the neural plate establishing a medial hinge point (MHP). This point furrows and the lateral neural plate on either side elevates forming the V-shaped neural folds. In the region of the future brain there are two further hinge points formed at the junction of the surface ectoderm and the neural plate. These two hinge points are termed the dorsolateral hinge points (DLHP's) - one located on either side of the neural plate.

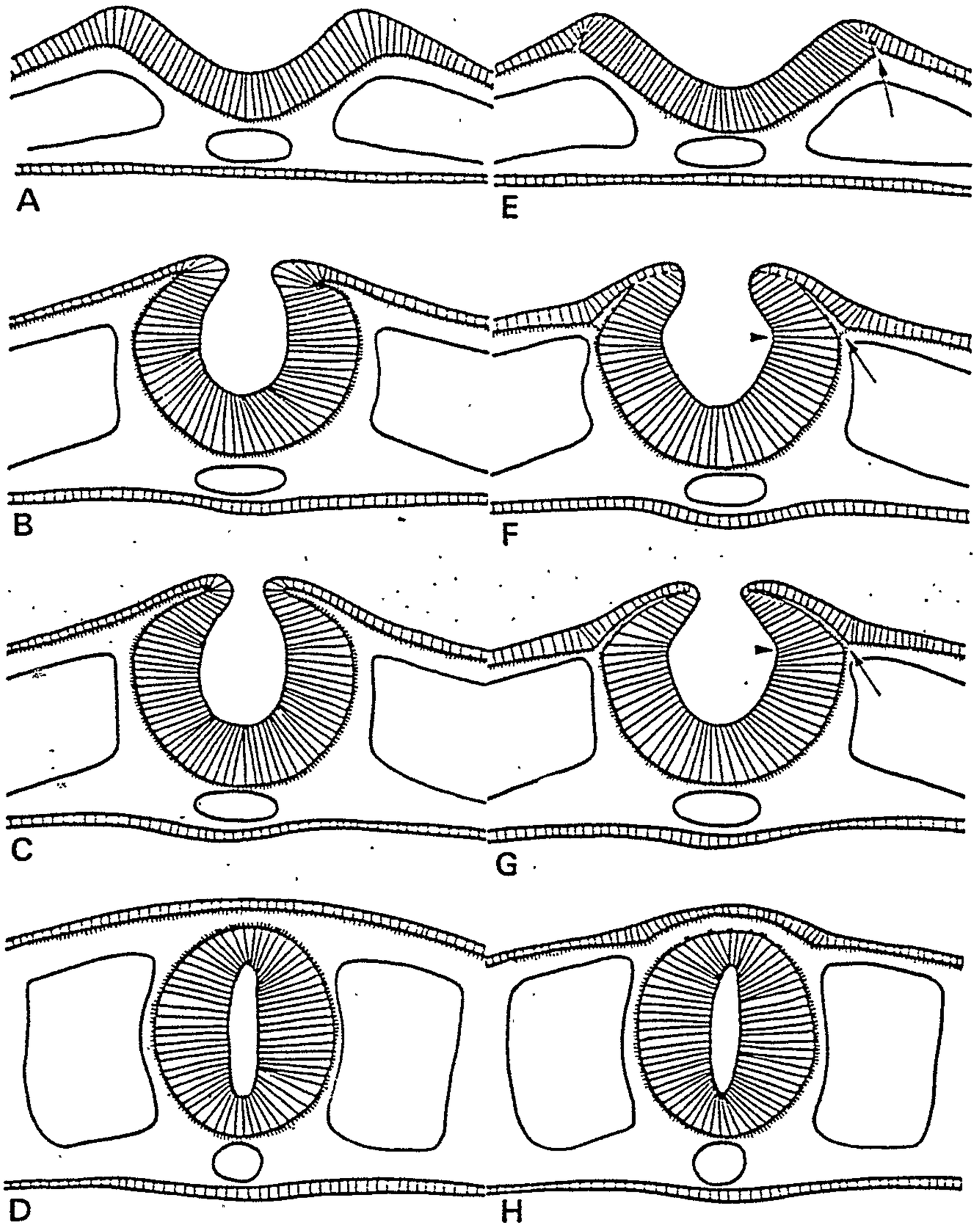
Martins-Green's (1988) work in chick embryos has shown that as the cranial neural folds elevate the surface ectoderm and the neural ectoderm delaminate progressively at the location of the DLHP. During the early stages of this process the two tissues remain connected across the zone of delamination by their previously existing basal lamina.

However, once apposition of the neural folds has occurred this connection breaks down leaving only a sparse basal lamina on the dorsal surface of the neural tube, the region from which the neural crest cells migrate. The basal lamina on the surface ectoderm overlying the dorsal portion of the neural tube becomes complete shortly after fusion (See Figure 4.2).

In the future brain the DLHP's form a point around which the neural folds rotate medially to appose each other in the midline. However, caudally, in the region of the future spinal cord, DLHP's do not form and the process of neurulation varies slightly. As the neural folds elevate in this region each lateral half of the neural plate comes into midline apposition with its counterpart resulting in temporary occlusion of the neural tube lumen. Finally, neurulation in the region of the sinus rhomboidalis (region of the closing caudal neuropore) involves the formation of both MHP and paired DLHP's in the same manner as in the cranial level.

The first fundamental principle of the contemporary view of neurulation is that the forces involved are both intrinsic and extrinsic to the neuroepithelium. The original experiment by Roux in 1885 using explanted chick neural plate folds, described previously, was thought to prove conclusively that the forces for neurulation originated solely from the neuroepithelium. However, it is widely known (Burnside, 1972; Schoenwolf and Smith, 1990) that isolated pieces of epithelium in culture quickly roll up into tube- or vesicle-like structures, depending on their initial shape. Based on this, Roux's experiment loses credibility and certainly cannot be cited to exclude the possibility of the role of extrinsic forces in neurulation.

FIGURE 4.2: Comparison of the conventional model for neural fold elevation and fusion (A-D) with the new observations (E-H) presented by Martins-Green (1988).



(From Martins-Green, 1988)

The conclusions reached from Lewis' classical study (1947) that were used as the benchmark by which all other studies were evaluated were also challenged by Schoenwolf and Smith (1990). They argued that in Lewis' experiment, the closure of the wound within one hour would not be expected to occur if all forces for shaping and bending were intrinsic to the neuroepithelium because the neuroepithelium, in isolation from more lateral 'restraining' tissue should narrow and bend earlier than normal thereby pulling away from the wound.

Furthermore, they noted that the observation that the 'freed' neural fold is displaced medially cannot be taken as evidence of faster neurulation because wound gaping alone would be expected to cause such a displacement. Finally they concluded that Lewis' experiment could not distinguish between intrinsic and extrinsic neurulation forces because, due to the rapid healing, the surface ectoderm was intact during most of the period of neural plate shaping and bending and could have played a role in this process.

Schoenwolf (1988) produced direct evidence that the bending of the neural plate is an autonomous event at the level of the future forebrain but that extrinsic forces generated by the lateral tissues (i.e. the surface ectoderm) are required at more caudal levels. An experiment was carried out using early stage chick embryos in whole-embryo culture. Either uni- or bi-lateral longitudinal cuts were made through the entire thickness of the blastoderm at the approximate boundary between the prospective surface ectoderm and neural plate. In the bilaterally cut embryos, the forebrain level formed a closed vesicle but more caudal levels of the neuraxis underwent normal shaping and MHP formation but failed to undergo further elevation and apposition. When the neural plate was separated unilaterally, shaping occurred normally.

These microsurgical experiments, therefore, provide direct evidence in support of a role for extrinsic forces from surface ectoderm during bending of the neural tube. In contrast to bending, Schoenwolf *et al.* (1989) suggested that shaping is largely or exclusively the result of intrinsic forces although they did not rule out the potential role of extrinsic forces.

The second fundamental principle of the contemporary view is that neurulation is driven by both changes in neuroepithelial cell shape and other form-shaping events. According to the traditional views neural plate shaping resulted solely from neuroepithelial cells becoming taller and bending results solely from the elongated cells becoming wedge-shaped. However, this model has been considered two-dimensionally and when applied three-dimensionally would result in the formation a vesicle rather than a tube. Furthermore, it does not address the process of longitudinal lengthening of the neural plate.

While it is agreed that cell elongation acts in transverse narrowing of the neural plate during its shaping (Jacobson and Gordon, 1976; Schoenwolf, 1985) it can only account for about 15% of the reduction in neural plate width that occurs during normal shaping (Schoenwolf, 1985), the remaining reduction and the rapid longitudinal lengthening must, therefore, be driven by other forces. Two other events have recently been implicated in neural plate shaping, both of which generate intrinsic forces. These are rearrangement and cell division of neuroepithelial cells.

The evidence for the role of cell rearrangement in neural plate narrowing and lengthening came from Schoenwolf and Alvarez, who, in 1989 transplanted circular plugs of quail neural plate into chick embryos and followed the displacement of cells within the grafted plug over time. The plug was observed to narrow transversely and lengthen longitudinally concomitant with neural plate narrowing and lengthening.

Although amphibian embryos experience little cell division and no cell growth during neurulation (Gillette, 1944), higher vertebrates undergo substantial cell division during this process (Tuckett and Morriss-Kay, 1985; Smith and Schoenwolf, 1987, 1988).

The neuroepithelial cells of the flat neural plate of the chick were shown to have a cell cycle of about 8 hours, thus, over the 24 hour period in which neurulation occurs, between 2-3 rounds of cell division would be expected (Smith and Schoenwolf, 1987, 1988). Schoenwolf (1985) suggested that the direction of cell division (ie. whether division is orientated to insert daughter cells into the longitudinal or transverse axis) differs along the rostrocaudal axis. Again, using quail grafts, he concluded that it seemed likely that cell division was directed mainly within the transverse plane in the brain and within the longitudinal plane in the spinal cord.

Having established that changes in cell number and cell position have a role to play in shaping of the neural plate, it is necessary to consider the factors important in the bending of the neural plate. The bending process involves two principal events; furrowing of the neural plate and subsequent folding around established hinge points. These two processes result in elevation and convergence of the neural folds toward the dorsal midline of the embryo.

Furrowing is generated by neuroepithelial cell wedging, an intrinsic cell behaviour. Traditionally, this process was thought to be the sole driving force of bending, however, it is now realised that not all neuroepithelial cells undergo wedging during neural plate bending (Schoenwolf, 1985). The notochord induces most of the cells above it to become anchored and wedge-shaped, thus forming the MHP.

This cell wedging involves both apical constriction, presumably assisted in part by the contraction of apical bands of microfilaments (Lee and Nagele, 1985) and basal expansion, presumably due to the repositioning of each cell's nucleus to the base of the cell through cell cycle regulated, interkinetic nuclear migration.

Similarly, the neuroepithelial cells of the DLHP become anchored to the adjacent surface ectoderm of the forming neural folds and many of them become wedge-shaped. In contrast to these specific areas within the neural plate, most of the cells of the lateral areas of the neural plate remain spindle-shaped throughout the process of bending.

In an experiment carried out by Smith and Schoenwolf (1989), in which notochordless chick embryos were observed, there was lack of formation of MHP characteristics. However, despite the absence of cell-wedging in the midline, some neural folds elevated, converged and fused forming a closed neural tube with an abnormally thick ventral midline region and a small eccentric lumen. These results demonstrated that elevation and convergence of the chick neural folds can occur in the absence of both the notochord and wedging of MHP cells. This suggests that wedging of MHP cells drives furrowing and is necessary to establish the characteristic cross-sectional morphology of the floor plate of the neural groove and tube, but that it is not the driving force behind neural fold elevation and convergence (Schoenwolf and Smith, 1990).

Schoenwolf (1994) discussed the changes in cell behaviour outside the neural plate that contribute to its bending. There are at least three extrinsic cell behaviours that drive bending. Changes in cell shape involve the surface ectodermal cells undergoing spreading and apicobasal flattening. These surface ectodermal cells also divide at 10 hour intervals during neurulation, which accounts, at least partially, for the increase in the volume and surface area of the surface ectoderm during neurulation. Finally, the surface ectodermal cells intercalate in the transverse plane in concert with similar intercalations occurring within the neural plate.

Changes in the extracellular matrix probably contribute to the extrinsic forces required for bending because depletion of the matrix results in a severe inhibition of this process (Schoenwolf and Fisher, 1983). Schoenwolf (1994) suggested that changes in the matrix may act in a mechanical way to cause bending (eg. from hydrostatic pressure resulting from hydration) or could regulate cell behaviour thereby acting indirectly through a cascade of events.

The third and final fundamental principle of the contemporary view of neurulation is that forces for cell shape changes are generated both by the cytoskeleton and by other factors. The role of microfilaments in neuroepithelial cell wedging and neural plate bending was reexamined by Schoenwolf *et al.* in 1988. Although they found that exposure to cytochalasin D often resulted in disruption of apical microfilaments and the formation of neural tube defects, MHP cells consistently became wedge-shaped on schedule, the midline furrow formed and elevation of the neural folds around the MHP still occurred. This demonstrated that apical microfilaments were not required for wedging of MHP cells, midline furrowing and neural fold elevation during bending of the chick neural plate.

The role of basal expansion of neuroepithelial cell-wedging was disregarded traditionally, firstly, because the hypothesis that basal expansion resulted from increased water uptake by the bases of neuroepithelial cells (Glaser, 1914) was never able to be demonstrated and secondly because attention became focused on apical narrowing. It is now clear that basal expansion occurs in conjunction with apical constriction to transform neuroepithelial cells from spindle-shaped to wedge-shaped during bending. As shown by F C Sauer in 1935, the nuclei of neuroepithelial cells undergo interkinetic migration during the cell cycle, whereby their nuclei migrate apically during mitosis and the nuclei of the daughter cells migrate back to reside at the base of the neural plate. The position of the nucleus determines the widest part of the cell with the cell tapering to a slender process apically and/or basally.

In this context, a spindle-shaped cell is one whose nucleus resides within the middle portion of the cell with apical and basal tapering, while a wedge-shaped cell is one with an expanded basal region containing the nucleus and a much narrower apex. Consequently as nuclei migrate from the apex of the neural plate to the base and then back to the apex again to undergo division, neuroepithelial cells sequentially transform from inverted wedge-shaped to spindle-shaped to wedge-shaped. It follows, then, that in order to create wedge-shaped cells by basal expansion it is necessary to regulate the cell cycle in such a way that the nucleus is maintained within the basal region of the cell.

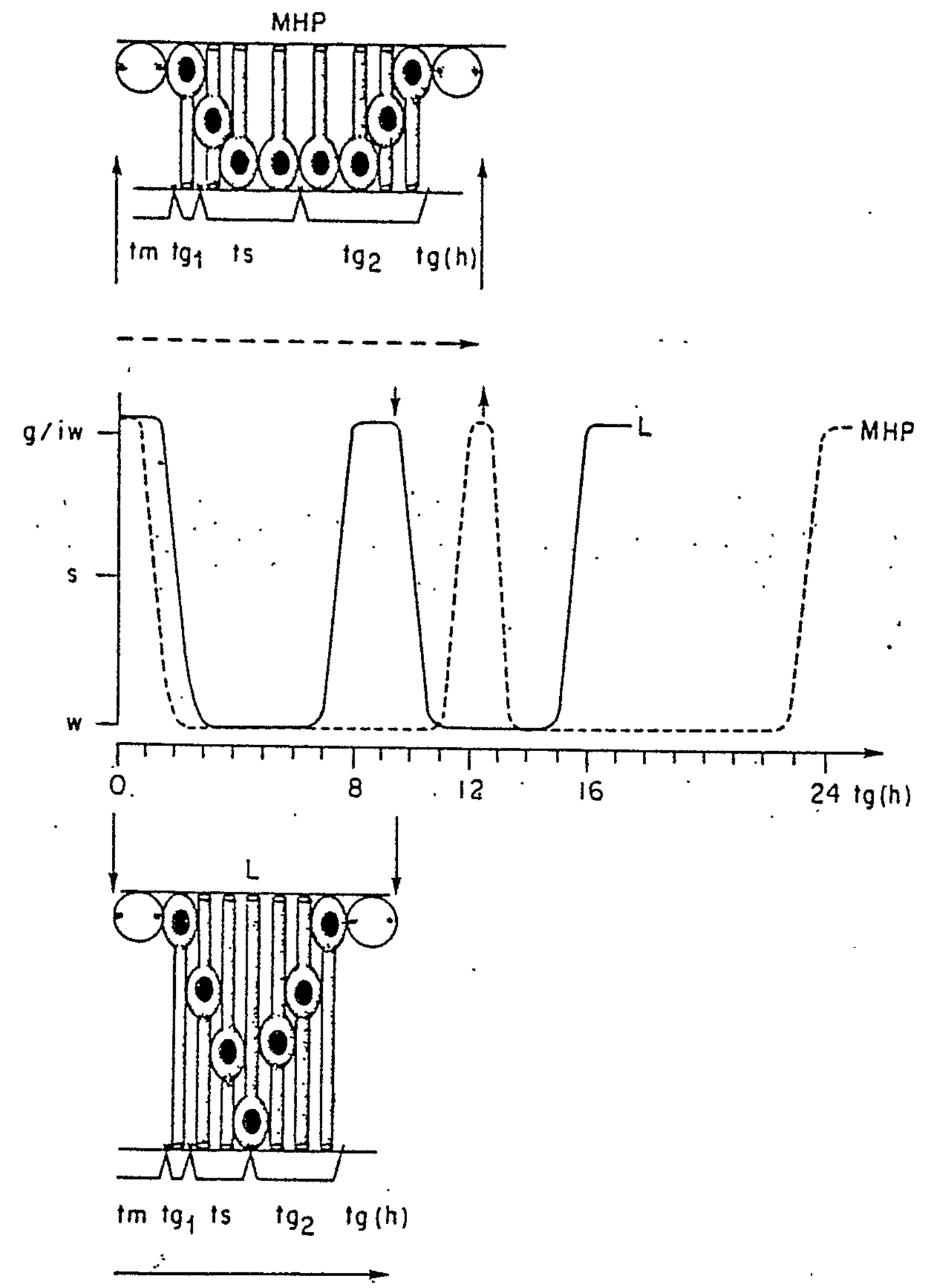
Evidence to support this theory was provided firstly by Smith and Schoenwolf (1987) who showed that the generation time of MHP cells is lengthened as these cells become wedge-shaped during neural plate bending. Secondly, in 1988 the same authors demonstrated that both the DNA synthetic (S) phase and non-DNA synthetic (non-S) phase of the cell cycle were significantly longer in the MHP than in the lateral neuroepithelial cells (L), whereas the mitotic (M) phase is significantly shorter in the MHP than in the L during stages of neural plate bending (See Figure 4.3).

This study also revealed that wedge-, spindle- and inverted wedge-shaped cells within the MHP can be in either the S phase or non-S phase of the cell cycle. Thus DNA synthesis is not restricted to the base of the neural plate during bending as previously reported and nuclear position and cell cycle phases are not coupled totally.

Smith and Schoenwolf (1989) have demonstrated that in the absence of the notochord the midline cells in the chick neural plate do not develop typical MHP characteristics, failing to furrow and become wedge-shaped. Furthermore transplanted notochords are capable of inducing typical MHP characteristics in L. This evidence strongly suggests that the notochord plays an inductive role in the formation of MHP characteristics through alterations in the cell cycle. By lengthening the phases during which their nuclei of these neuroepithelial cells reside at the base of the neural plate these cells become wedge-shaped for longer periods of time (see Figure 4.3)

FIGURE 4.3: Model illustrating how alteration of the cell cycle of neuroepithelial cells might generate cell wedging within the hinge points.

[MHP - median hinge point; L - lateral neuroepithelium; iw - inverted wedge-shaped; g - globular ; s - spindle-shaped; w - wedge-shaped; tg - total generation time of cells; tm, tg_1, ts, tg_2, - duration of M, G_1, S and G_2 phases of cell cycle, respectively.]



(From Schoenwolf and Smith, 1990)

Patterning of the neuraxis begins during shaping of the neural plate and continues throughout the process of neurulation. The neural plate becomes patterned in both the transverse and rostro-caudal axes. Initially, during shaping of the neural plate, transverse patterning results in the formation of two populations of cells - those of the hinge-points and those of the lateral neurectoderm (Schoenwolf and Franks, 1984).

This patterning facilitates bending of the neural plate through the wedging of MHP cells. Once formed these cells undergo cell wedging by the action of both apical constriction (involving the contraction of apical bands of microfilaments) and basal expansion (involving the repositioning of the cell nucleus to the base of the cell through cell cycle regulated, interkinetic nuclear migration).

Later rostrocaudal patterning of the neuraxis comes into play, with the expression of positional identity genes such as the homeobox-containing gene *engrailed-2*. The highly orchestrated morphogenic movements required during neurulation involve the precise coordination in space and time of cell behaviours within multiple tissues.

With the discovery that homologs of *Drosophila* genes, such as the homeobox-containing genes, are expressed in temporally and spatially restricted domains of vertebrate embryos during early development, it became apparent that these genes may confer positional identity. *Engrailed-2* is expressed in a specific rostro-caudal pattern in chick embryos (Gardner *et al.*, 1988). Experiments carried out by Darnell *et al.* (1992) discovered that expression of *engrailed-2* in the midline region of the most ventral part of the neural tube is normally suppressed by the notochord through induction of MHP cells.

Final closure of the neural groove is thought to be mediated by cell surface glycoconjugates, but this aspect of neurulation has received comparatively little attention. Shaping and bending of the neural plate brings the neural folds into apposition along the dorsal midline, but complete closure requires subsequent adherence and fusion. Takahashi and Howes (1986) and Takahashi (1988, 1992) showed that adherence and fusion involve the synthesis of cell surface coats (lectins) in avian embryos but little else is known about the processes (Schoenwolf, 1994)

In summary, the process of neurulation as studied in amphibian and avian models has led to the view that both intrinsic and extrinsic forces are involved in bending of the neural plate. Schoenwolf and Smith (1990) have formulated a hinge-point model to describe the process. They propose that the neural plate is firmly anchored to adjacent tissues at the hinge points (MHP and DLHP's) and that the forces for bending are generated outside these hinge points. Thus, while each hinge point is an area that directs and facilitates folding, the forces involved arise outside of these points.

Schoenwolf (1994) has provided the most up-to-date review of neurulation in avian embryos, concluding that the traditional viewpoint of neurulation is no longer tenable. Bending of the neural plate cannot be driven principally by the microfilament-mediated cell wedging intrinsic to the neural plate. While the traditional view point is partially correct with respect to cell wedging causing the furrowing of the MHP, Schoenwolf postulated that such wedging does not drive subsequent bending, and that the mechanism of cell wedging involves both apical constriction and basal expansion.

4.4 NEURULATION IN MAMMALS

Neural tube closure had always been described as a continuous bidirectional process starting in the cervical region and proceeding in both rostral and caudal directions like a zipper. However, more recent evidence (Geelen and Langman, 1977; Sakai, 1989; Golden and Chernoff, 1983,1993) showed that in mammals the fusion of the neural tube is not an uninterrupted process but displays an intermittent pattern with four distinct areas of closure. Although all three researcher groups agreed that an intermittent pattern of closure exists in mice, there are variations in the documented sites of initiation and timing of closure.

The findings of Golden and Chernoff (1993), are shown in Table 4.1 and Figure 4.4. Their work suggests that the rhombencephalon is a distinct area not only in its timing of closure but also the method of closure. Instead of a fusion of the neural folds to form the neural tube as in Closure sites I - III, in the rhombencephalon (Closure site IV) neural folds remain separated and closure is accomplished by growth of a membrane over the region. Golden and Chernoff (1993) suggest, however, that a common mechanism of closure may exist in humans.

The applicability of this model of neural tube closure in humans is unclear. The literature continues to advocate the 'zipper' model in human development, however, the rostral neural folds have been described as closing from opposite directions to form the rostral neuropore (Müller and O'Rahilly, 1986). It may be that due to the scarcity of early human embryos available for examination of closure of the rostral neural folds that the exact nature of the closure process is yet to be documented.

Neural crest cell emigration is part of the normal process of neurulation in the developing mammalian head. It plays an essential role for normal neurulation in the midbrain/rostral hindbrain level where it appears to be necessary for flexibility of the lateral edges of the neural folds, allowing them to curve medially and fuse in the dorsal midline (Morriss-Kay *et al.*, 1994).

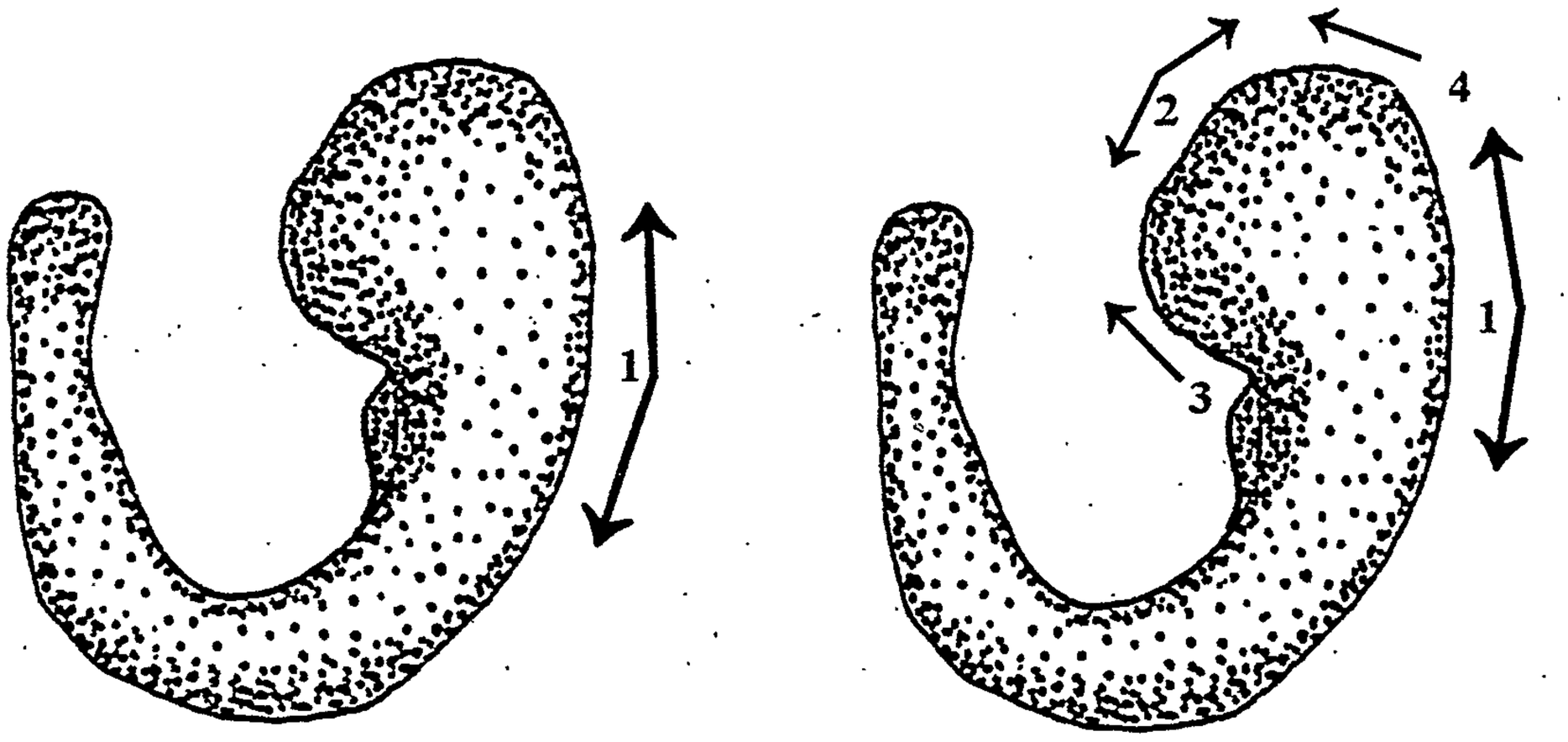
TABLE 4.1: Sites of initiation and direction of closure of the neural tube in mice.

	SITE OF INITIATION	DIRECTION OF CLOSURE
CLOSURE I	Between 2nd and 4th Somites	Bidirectional
CLOSURE II	Prosencephalic-mesencephalic border	Bidirectional
CLOSURE III	Level of stomodeum	Unidirectional - Caudal
CLOSURE IV	Caudal end of rhombencephalon	Unidirectional - Rostral

(Adapted from Golden and Chernoff, 1993)

FIGURE 4.4: Schematic diagrams representing the traditional "zipper" model of neural tube closure and the contemporary intermittent model.

ZIPPER MODEL **INTERMITTENT MODEL**



(Adapted from Golden and Chernoff, 1993)

In the trunk, neural tube formation in mammals is essentially the same as in chicks. The formation of the median hinge-point by anchoring of the midline neuroepithelium to the notochord leads to bending of the neural plate. Throughout the mammalian neural tube, the contraction of apical microfilaments is important for the generation and maintenance of concave curvature of the neuroepithelium prior to neural tube closure (Morriss-Kay *et al.*, 1994).

This contraction of apical filaments results in the extrusion of apical cytoplasm from between the junctional complexes forming apical protrusions along the lumenal surface of the neural tube (Freeman, 1972).

The extracellular matrix molecules, including heparan and chondroitin sulphate proteoglycans (HSPG and CSPG) are also implicated in the formation and maintenance of concave neural folds (Morris-Kay and Tuckett, 1989; Tuckett and Morriss-Kay, 1989). While HSPG appears to have a direct role in maintaining neural fold morphology, CSPG is essential for neural crest cell emigration and therefore plays a secondary role in the closure events of the midbrain/rostral hindbrain region.

By the end of neurulation, there is a distinct pattern of gene expression in the mammalian neural tube (Kessel and Gruss, 1990). The unique genetic code at each segmental level is reflected in the paraxial mesoderm (Kessel and Gruss, 1991) and in the cranial region at least, in the neural crest (Hunt *et al.*, 1991). While the role of segmental gene expression in control of morphological development of the neural tube is, at present unknown, correlations between normal and altered gene expression patterns and normal and abnormal patterns of neurulation are clearly beginning to provide evidence that will lead to significant insights into the roles of specific genes in the mechanisms underlying mammalian neurulation (Morriss-Kay *et al.*, 1994).

4.5 THE NEURAL CREST

4.5.1 FORMATION AND MIGRATION

The neural crest cells (NCC's) are a population of cells found only in vertebrates and higher chordates. During neurulation the flat neural plate thickens and invaginates to form the neural tube. Around the time of its closure the NCC's are liberated from the neural tube and undergo extensive migration. The precise time of neural crest cell (NCC) migration differs between species and at differing axial levels. For example, in contrast to the cranial level (Nichols, 1981), the NCC's of the trunk of the mouse embryo do not begin to migrate until well after the neural folds are fused (Erickson and Weston, 1983).

Much of the pioneering work on the neural crest was carried out by Hörstadius (1950). A great deal of his work centred on the neural crest derived pigment cells which provided excellent markers for neural crest cell migration. He also presented data on the contribution of the neural crest to the amphibian cranial skeleton. Since that time the origin, mechanisms of migration and segregation of cell lineage in the neural crest have also been studied in avian embryos and most recently in mammalian models.

There are distinct differences in the derivatives formed by NCC's along the rostrocaudal axis. Grafting studies in chimaeric quail and chick embryos have shown that neural crest (NC) from every level has the potential to form components of the normal peripheral nervous system including sensory and autonomic ganglia, Schwann cells, adrenomedullary cells and pigment cells (Le Douarin, 1980). However, only NCC's from the cranial region have the ability to produce cartilage and bone.

In the same manner as closure of the neural tube, initiation of NCC emigration occurs in a rostral to caudal direction commencing in the head region (Bronner-Fraser, 1994). Under normal circumstances NCC's at or near the dorsal midline of the closing neural tube, undergo an epithelial-mesenchymal transition and emigrate away from the neuroepithelium. This emigration continues for 24 to 36 hours. The properties of the dorsal midline of the neural tube during closure making it an accessible area for NCC migration are firstly, the discontinuity of the basement membrane (Martins-Green and Erickson, 1987) and secondly, the changes in cell-to-cell adhesiveness (Akitaya and Bronner-Fraser, 1992).

The NCC's begin their migration in the cell-free zone surrounding the tube. Within this zone which is rich in extracellular matrix, the NCC's are easily identifiable but as they continue their migration they intermix with, and become morphologically indistinguishable from the surrounding tissues (Bronner-Fraser, 1993a). Researchers have been able to follow the migration of NCC's by using cell labelling techniques in chick embryos.

The migration and subsequent differentiation of the NCC's is a highly patterned process that varies according to the axial level. Weston (1963) and Le Douarin (1980) transplanted neural tubes labelled with either radioactive or species-specific markers into unlabelled host embryos. Rickmann *et al.* (1985) and Bronner-Fraser (1986) used antibody staining techniques that recognised migrating neural crest cells such as monoclonal antibody HNK-1 and Serbedzija *et al.* (1992) utilised the lipophilic dye (DiI) for vital staining of the neurectoderm.

4.5.2 NEURAL CREST MIGRATION IN THE TRUNK

The NC in the trunk region of the vertebrate embryo first appears as a strand of cells on the dorsal surface of the neural tube along the lines of fusion of the neural folds (Tosney 1978, 1982). Erickson and Weston (1983) described four processes that act as stimuli for the initiation of NCC migration in the trunk:

(1) Weston *et al.* (1978) found that NCC's create space by secreting large amounts of hyaluronic acid prior to migration. While this extracellular polymer may be responsible for forming the space above the cells it is probably not sufficient to stimulate the migration on its own.

(2) Presumptive NCC's may lose adhesive properties, allowing them to detach easily from the neurectoderm.

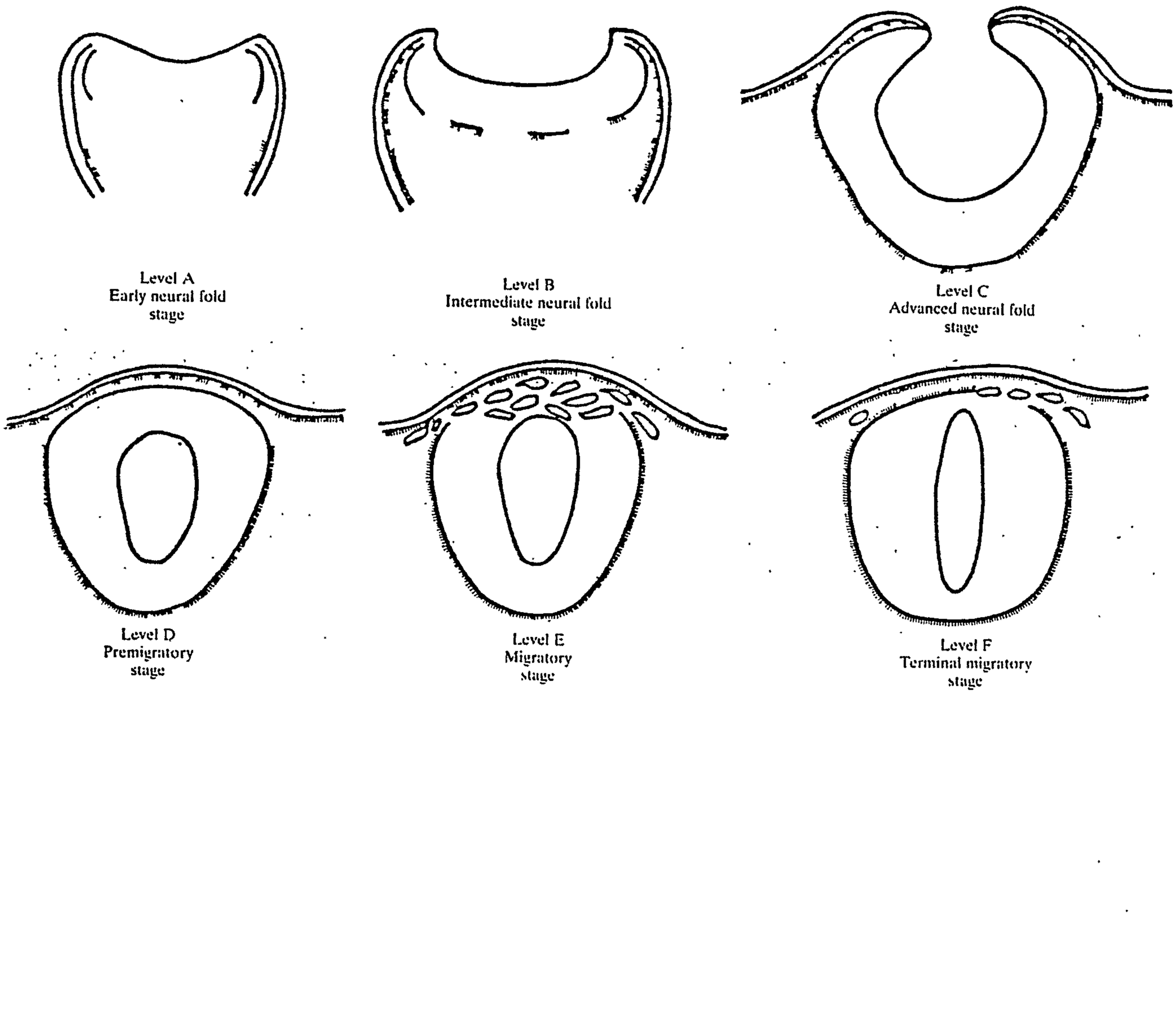
(3) Activation of previously latent locomotor capabilities may initiate the migration of NCC's.

(4) Following fusion of the neural folds, the ectoderm separates from the neurectoderm leaving the dorsal surface of the neural tube devoid of an intact basal lamina. It is possible that this discontinuity permits cells of the neurectoderm to emigrate. The cessation of migration coincides with the formation of an intact basement membrane over the neural tube.

Erickson and Weston (1983) conclude that while all of these processes may have a role in the initiation of NCC migration, the disruption of the basal lamina appears to be the major factor.

Martins-Green and Erickson (1986) performed an ultrastructural study to evaluate the condition of the basal lamina during neural fold elevation and NCC emigration. Their results showed that the basal lamina becomes progressively more extensive from neural fold to migratory stages. It initially forms on the lateral portion of the neuroepithelium of the neural folds and then extends ventrally into the region adjacent to the notochord. The basal lamina only becomes continuous beneath the surface ectoderm in the terminal stages of NCC migration and over the dorsal portion of the neural tube once NCC migration has completely finished (See Figure 4.5)

FIGURE 4.5: Schematic representation showing the development of the basal lamina during various stages of neurulation in the mouse.



(From Martins-Green and Erickson, 1986)

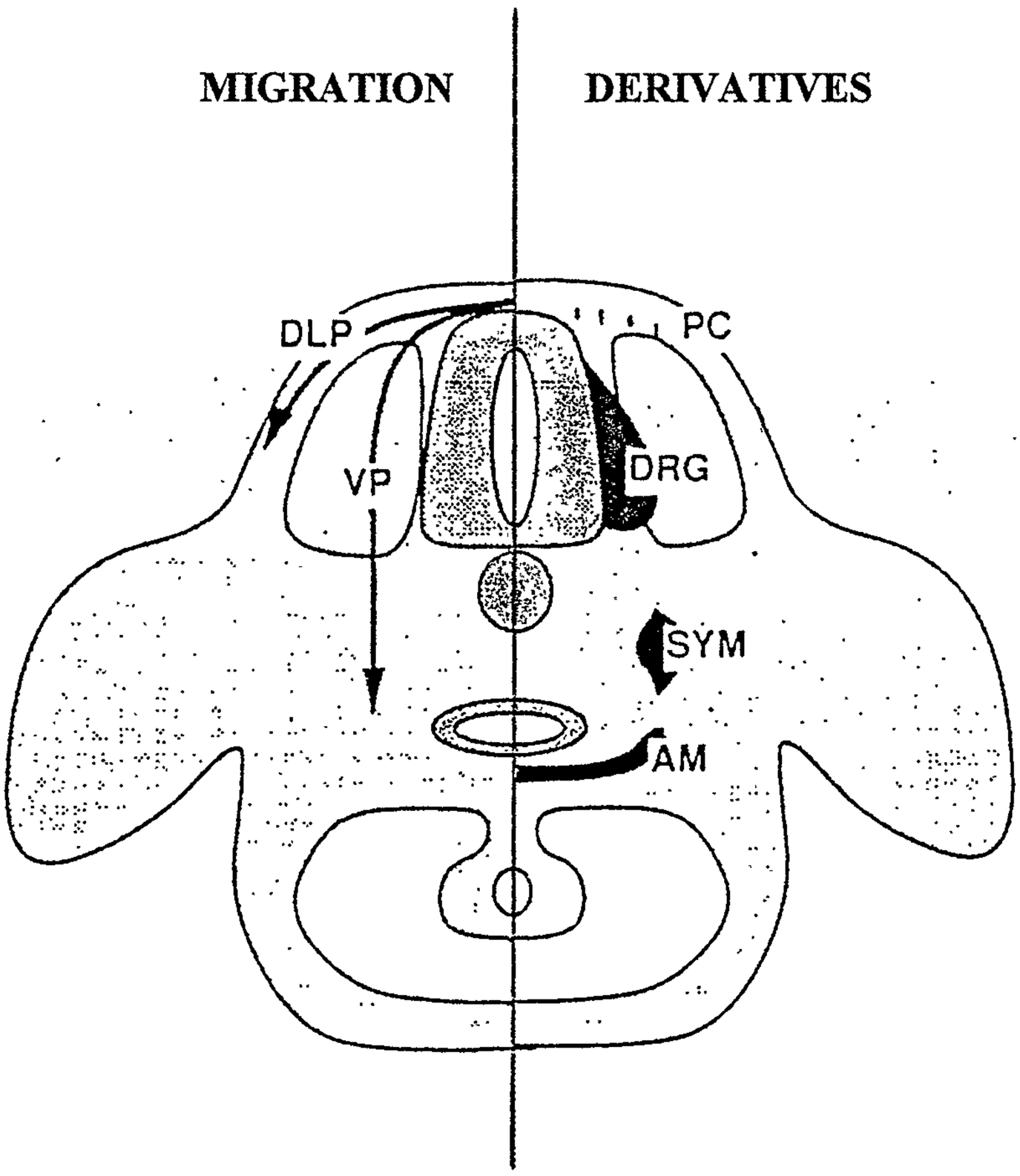
The morphology of the basal lamina also changes during the stages of neurulation. At earlier stages it is thin (40nm) and poorly organised while during the later stages it becomes thicker (60nm), finally reaching its full potential (80nm) during the postmigratory period (Martins-Green and Erickson, 1986).

The migration patterns of caudal NCC's were first studied by Weston in 1963. ^{3}H-thymidine labelled neural tubes including premigratory NCC's were transplanted into similar regions of unlabelled hosts. Emigration followed two pathways: firstly, a ventral pathway (VP) between the neural tube and somites and subsequently a dorso-lateral pathway (DLP) underneath the ectoderm (see Figure 4.6). Cells derived from the DLP were thought to differentiate into pigment cells and those derived from the VP into sensory and sympathetic ganglion cells, Schwann cells and adrenomedullary cells. Unfortunately, the use of ^{3}H-thymidine is limited due to its dilution in rapidly dividing embryonic cells, allowing the technique to be used for only short periods of time.

The work of Weston (1963) was subsequently confirmed by Le Douarin (1980) using quail/chick chimaeras. Neural tubes from quail embryos were transplanted into the same or different axial levels of chick embryos. Because quail cells contain a condensed mass of heterochromatin in their nucleolus they are easily distinguishable from chick cells which are euchromatic. However, the time required for healing of neural tube grafts and the possibility of scarring effecting the experiments has led to use of monoclonal antibodies in more recent studies.

Rickmann *et al.* (1985) and Bronner-Fraser (1986) used the monoclonal antibodies NC-1 and HNK-1 which recognise a carbohydrate epitope present on the surface of ventrally migrating NCC's. Their findings have shown that NCC's in the ventral pathway appear to migrate ventrally through the sclerotomal portion of the somite.

FIGURE 4.6: The migratory pathways and derivatives of neural crest cells in the trunk. Cell marking techniques have revealed that trunk neural crest cells migrate along two pathways: a dorsolateral pathway (DLP) where cells form pigment cells (PC) and a ventral pathway (VP) where cells form the dorsal root ganglia (DRG), sympathetic ganglia (SYM) and adrenomedullary cells (AM).



(From Bronner-Fraser, 1993)

Within the somite the migration is segmental, with NCC's observed within the rostral but not the caudal half of each sclerotome. Bronner-Fraser and Stern (1991) have shown that this segmental pattern of migration through the somites is the result of cues inherent to the somite.

Furthermore, experiments using HNK-1 antibodies (Stern *et al.*, 1991) suggest that the neural tube prescribes the dorsoventral patterning of NC derivatives. The notochord is also responsible for inhibitory signals resulting in an area of approximately 85µm diameter surrounding the notochord that is devoid of NCC's . These findings suggest that tissue interactions are critical for establishing the pattern of migration of NCC's in the trunk.

Even the use of antibodies in the identification of the migratory pathways of NCC's has some pitfalls. An example is attempted identification by probing for cell adhesion molecules. Although antibodies are available that recognise epitopes specific to particular cell adhesion molecules, these are associated with a variety of cell types apart from NCC's. Also not all NCC's express common cell adhesion molecules and finally, the patterns of cell movement can only be inferred by studying antibody-stained sections of fixed embryos.

The most recent technique employed to examine NCC pathways involves the use of DiI labelling (Serbedzija *et al.*, 1992). This hydrophobic dye intercalates into the cell membranes of the cells it contacts. The results obtained with this technique are similar to that of monoclonal antibodies, however, with DiI labelling the time and location of injection can be controlled with much more precision. Unlike HNK-1 antibody which is useful in avian and rat embryos only, DiI can be used to label NCC's in a large number of species. While there are a number of common features between species, such as the segmental pattern of NCC migration, some of the details in early migratory patterning differ between organisms. Where chick embryos show a delayed entry of NCC's along the DLP, mouse NCC's enter the DLP and VP simultaneously.

4.5.3 CRANIAL NEURAL CREST CELL MIGRATION

The cranial NCC migration consists of several populations that differ in their migratory pathways, patterns of gene expression and types of derivatives (Bronner-Fraser, 1994). This population of NCC's can be subdivided into the regions; forebrain, midbrain, rostral hindbrain and caudal hindbrain. In chicks and rats NCC's do not emerge from the forebrain neural tube except for the most caudal portion (Tan and Morris-Kay, 1986; Bronner-Fraser, 1994). However, in mouse embryos, NCC's arising at the level of the forebrain migrate ventrally between the developing eye and the diencephalon (Serbedzija *et al.*, 1992).

In the midbrain of chicks, NCC's emerge and migrate superficially as a broad unsegmented sheet of cells under the ectoderm. These cells contribute to the periocular skeleton, connective tissue of the eye, membrane bones of the face, ciliary ganglion, part of the trigeminal ganglion and Schwann cells (Le Douarin, 1982). In the mouse and rat, the midbrain NCC's migrate ventro-laterally through the mesenchyme as dispersed cells and populate the region overlying the mesencephalon (Serbedzija *et al.*, 1992).

The hindbrain can be further subdivided into 8 rhombomeres (r) that represent compartments which restrict cell migration. The migration pattern of the hindbrain NCC's is similar in chick and mouse embryos. It is segmented, with three broad streams of cells arising lateral to rhombomeres r1/2, r4 and r6. The first of these streams populates the trigeminal ganglion and mandibular arch, the second the hyoid arch and geniculate and vestibular ganglia and the third stream populates the third and fourth pharyngeal arches and associated ganglia. In the rat, NCC's from the level of the hindbrain appear to migrate through the mesenchyme as dispersed cells (Tan and Morriss-Kay, 1986).

Although Lumsden *et al.* (1991) reported that no NCC's emerged from r3 or r5 focal DiI injections into individual rhombomeres demonstrated that all rhombomeres contribute to the neural crest (Birgbauer *et al.*, 1995). These authors disagreed with the studies of both Jeffs *et al.* (1992) and Graham *et al.* (1993) who demonstrated regional depletion, by apoptotic death, of the NCC's associated with r3 and r5. Instead Birgbauer *et al.* (1995) and Sechrist *et al.* (1993) attributed the lack of NCC's in these regions to a rostral or caudal migration of the cells to join one of the three main streams (r1/2, r4 or r6).

Despite this disagreement, it seems apparent that apoptosis has a role to play in the elimination of NCC's in the hind brain of chicks. The results from both Jeffs *et al.* (1992) and Graham *et al.* (1993), who used nile blue sulphate and acridine orange staining respectively, show convincing evidence of a spatial and temporal correlation between the absence of NCC's and discrete zones of cell death in the dorsal midline of the hindbrain at the developmental stage when neural crest cells would be expected to emerge. Jeffs *et al.* (1992) found that while the region of apoptosis associated with r5 correlated precisely with the absence of NCC's in the adjacent mesoderm, the rostral region of apoptosis was wider than r3. They proposed that this additional cell death may represent either a response to over-production of the cranial NCC's or indicate an early selection of NCC precursors.

A more recent paper by Graham *et al.* (1994) found that the signalling molecule, bone morphogenetic protein-4 (BMP-4), upregulated *msx-2* expression (Chapter 2) in the rhombencephalic neural crest. Clearly, these TGF-β related molecules have a role to play, not only in the induction of neural tissue (reviewed in Chapter 1) but also in the regulation of apoptosis by upregulation of *msx-2* in the neural folds.

There are two possible mechanisms that could account for the axial patterning of cranial NC migration. Firstly, it may be established by signals within the neural tube prior to NCC emigration or secondly, the pattern may depend on interactions from the environment through which the NCC's pass.

It seems evident now that environmental influences play the major role in NCC patterning along the rostrocaudal axis.

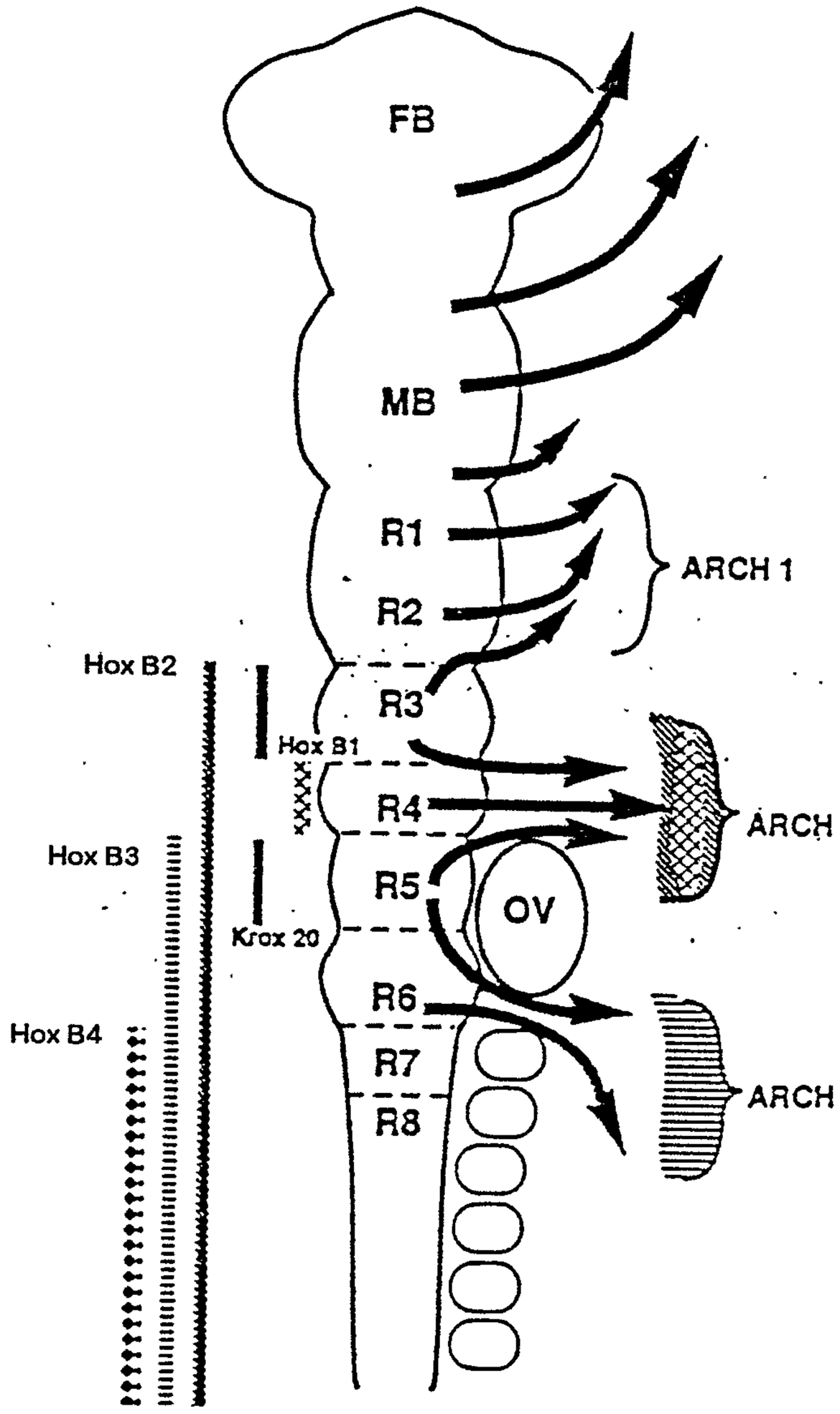
Bronner-Fraser (1994) described experiments where her team performed rostrocaudal rotations of either the cranial neural tube or adjacent ectoderm/mesoderm and grafts of the otic placode (which normally develops laterally to rhombomeres 4, 5 and 6). They found that 180° rotation of the r3/r4 section of neural tube did not alter the segmental pattern of NC migration. However, this grafting process often resulted in the formation of a small, ectopic otic vesicle which did influence the pattern of NC migration.

In most embryos containing an ectopic otic vesicle, labelled NCC's were seen to move directionally toward the vesicle, thus demonstrating that signals from other tissues can influence the pattern of NC migration in the hindbrain.

Patterns of gene expression in the hindbrain are thought to contribute to the segmental arrangement of NCC's. The vertebrate *hox* homeobox genes within the hindbrain are arranged in four clusters: *hox B1 - hox B4* (see Figure 4.7). In general, *hox* genes expressed in the in r2, r4 and r6 are identical to those in NC derivatives within pharyngeal arches 1, 2 and 3 respectively (Hunt and Krumlauf, 1991). This correlation suggests that axial identity is set within the neural tube and that NCC's arising from particular rhombomeres carry the appropriate *hox* genes to their final sites. However, this picture is complicated by r3 and r5. *HoxB2* is expressed within r3 but does not appear to be expressed by the neural crest cells migrating to the first pharyngeal arch. Similarly, r5 cells express *hoxB3* but NCC's migrating into the second arch do not.

Birgbauer *et al.* (1995) offered two possible explanations for the mismatch in the gene expression patterns between the odd-numbered rhombomeres and their associated pharyngeal arches. Firstly, there may be heterogeneity in gene expression within premigratory NCC's. Secondly, there may be rapid gene regulation once NCC's depart from the neural tube or during the early stages of migration.

FIGURE 4.7: Schematic representation of the rhombomeres, pharyngeal arches and the expression patterns of known genes. Arrows indicate the directions of migrating neural crest cells.



(From Bronner-Fraser, 1994)

Another gene that may be involved in segmental patterning in the hindbrain is *krox-20*. This gene is expressed in alternating domains in the neural plate that later correspond to r3 and r5 (see Figure 4.7). It acts as a transcriptional regulator of *hoxB2* expression (Sham *et al.*, 1993). *Krox-20* is also detectable in NC precursors in the dorsal midline of the neural tube extending from caudal r5 into r6. The expression of *krox-20* is transient, only detectable from shortly before to shortly after the time at which the r5 and r6 cells migrate caudally around the otic vesicle (Bronner-Fraser, 1994).

Graham *et al.* (1993) found that the regions in the hindbrain showing elevated levels of apoptosis were marked by the expression of members of the *msx* family of homeobox genes with *msx*-2 expression preceding apoptosis in a precisely localised pattern. They maintained that while *krox-20* and members of the *hox* gene family seem to be important in the patterning of the hindbrain region, the expression patterns do not suggest an obvious role for producing crest-free and crest-productive regions. Instead they proposed that the *msx* family were more likely to play a role in the organisation of hindbrain patterning.

4.5.4 THE ROLE OF EXTRACELLULAR MATRIX MOLECULES

Extracellular matrix molecules including fibronectin, laminin and tenascin, have been detected along NCC migratory pathways where they are suspected to play a role in the adhesion and motility of NCC's. Studies, *in vitro*, using avian models have shown that NCC's migrate extensively on fibronectin and laminin (Newgreen, 1984) but chondroitin sulfate proteoglycans tend to inhibit NCC migration (Tucker and Erickson, 1984). It is possible that NCC's migrate in the presence of these permissive molecules and in the absence of inhibitory molecules. Using this theory, the migratory pathways may be mapped by positions where permissive molecules do not overlap with non-permissive ones (Bronner-Fraser, 1993a).

It appears that different guidance mechanisms are involved at different axial levels. The attachment of NCC's to extracellular matrix molecules seems to be mediated predominantly by integrin receptors. Studies using antibodies against the β_1 subunit of integrin (Lallier and Bronner-Fraser, 1991) have shown that the attachment of NCC's to fibronectin and laminin is completely blocked by the antibodies. However, different NC populations may differ in their cell surface properties as it seems that cranial NCC's use a functionally distinct set of integrins from those of the trunk NCC's. Although both trunk and cranial NCC's possess integrin receptors, the reaction to antibodies to these receptors is quite different. An explanation for this may be that tissue interactions predominate in the trunk whereas cell-matrix interactions play a predominant role in the cranial region. In the trunk the somites appear to control the rostrocaudal patterning of NCC's, the neural tube plays a large role in controlling the dorsoventral patterning and the notochord maintains bilateral symmetry by preventing NCC's from crossing the midline. Further, although there are numerous extracellular matrix molecules present along trunk NC pathways, these molecules may play a permissive, rather than an instructive role, in patterning (Bronner-Fraser, 1993a).

4.5.5 SEGREGATION OF CELL LINEAGE

Serbedzija *et al.* (1989) used a fluorescent marker molecule to label small groups of cells to follow their progeny during development of chick embryos. Their work has shown that neural crest cells arise from the dorsal portion of the neural tube. Bronner-Fraser and Fraser (1988, 1989) used an intracellular injection technique to label individual dorsal neural tube cells *in situ* to study the lineage of single cells in the developing nervous system.

Their results show that single cells within the dorsal neural tube can contribute progeny to both the neural crest and the neural tube derivatives. This suggests that many premigratory neural crest cells are multipotent prior to their emigration from the neural tube and that the neural crest is not a segregated population in the neural tube but shares a common lineage with some neural tube cells.

The ablation studies of Scherson *et al.* (1993) have shown that cells of the more lateral and ventral regions of the neural tube from the same axial level regulate and reconstitute a population of neural crest cells in the event of ablation of the dorsal region. Their experiments suggest that lateral and ventral cranial neural tube cells normally fated to central nervous system derivatives possess a "premigratory" potential until shortly after the onset of neural crest emigration.

An important issue concerning neural crest development is how these cells give rise to a wide range of derivatives. Stemple and Anderson (1992) demonstrated the existence of a multipotent neural crest "stem cell" with a limited ability to self-renew. Under certain environmental conditions these multipotent stem cells produce "blast cells" which give rise to only limited cell types. It appears that the local environment may play an important role in this transformation and that differentially distributed growth factors and extracellular matrix molecules are likely candidates for instructive environmental signals (Bronner-Fraser, 1993b).

4.5.6 CRANIOFACIAL MALFORMATIONS ASSOCIATED WITH DEFECTIVE NEURAL CREST CELLS

Table 4.2 summarises the mechanisms of craniofacial defects involving the neural crest cells. As with all developmental defects the earlier in development that the defect occurs, the more likely the defect is to be structural and life-threatening (Hall , 1988). Several craniofacial defects in man including agnathia, median clefting and Treacher-Collins syndrome have been interpreted as having their basis in defective migration of cranial NCC's (Poswillo; 1974, 1975). Migration of NCC's could be affected by interactions with the adjacent neurectoderm, surface ectoderm, mesenchymal tissue or extracellular matrix. A delay in the onset of migration could result in NCC's not reaching their target site or arriving at the site in reduced numbers or too late to undergo the necessary tissue interactions.

TABLE 4.2: Craniofacial defects involving neural crest cells - aetiology and outcome.

MECHANISM	AETIOLOGY	OUTCOME
Total failure of NC development	✷ Failure of inductive interactions	Lethal
Abnormalities in NC migration	✷ Defects of NCC's ✷ Abnormal extracellular matrix ✷ Abnormal cell-cell interactions	Major structural abnormalities e.g face, cranium
Abnormalities of NC differentiation	✷ Altered cell surface adhesion ✷ Defective structural genes ✷ Defective inductive interactions	Failure of individual NCC derivatives e.g teeth, bones
Abnormalities of NC interaction	✷ Abnormalities in tissues with which the NCC's interact	Minor structural defects Minor defective function

(Adapted from Hall, 1988)

Although NCC's are dividing as they migrate, the rate of division increases once they reach the final site. Such enhanced proliferation probably reflects both increasing interactions amongst NCC's and with epithelium (Hall, 1983). Embryos are particularly sensitive to perturbation when growth is rapid or at times of changes in growth rate. Disruption of the normal rate of division of NCC's can lead to abnormal development resulting in craniofacial defects (Johnston and Sulik, 1979).

Potential NCC defects can be overcome by an embryo through a process of regulation. This phenomenon encompasses the ability of an embryo to compensate for actions that would otherwise lead to abnormal development. It has long been known that amphibian and avian embryos can compensate for removal of cells from the NC. This compensation has been attributed to the recruitment of neurectodermal cells from more lateral and ventral regions of the neural tube at the same axial level (Scherson *et al.*, 1993), migration of NCC's across the midline, migration from a more rostral or caudal region of the NC or as a result of increased proliferation of the remaining NC (Hörstadius, 1950; Weston, 1970; McKee and Ferguson, 1984).

4.6 SUMMARY

The process of neurulation has been studied for over a century and it has been the subject of intense research during the last two decades. It is now clear that neurulation is a multifactorial process driven by a number factors both intrinsic and extrinsic to the neuroepithelial cells. The morphogenic cell behaviours involved in neurulation include changes in cell shape, size, position and number as well as changes in cell-cell and cell-extracellular matrix interactions.

Neurulation begins with the formation of the neural plate. Although little is known about this process it is generally accepted that the induction of neuroepithelium involves the underlying mesoderm. It is thought that cell movements bring together new combinations of cells allowing for the inductive interactions that result in formation of the neural plate (Schoenwolf, 1994).

Once formed the neural plate undergoes shaping in which the pseudostratified columnar epithelium thickens apicobasally, narrows transversely and extends longitudinally. These changes are driven by three processes intrinsic to the neuroepithelium; changes in cell shape, position and number. Bending of the neural folds begins while shaping is still taking place. The two main mechanisms that result in bending are furrowing and folding. Furrowing occurs at the hinge points (MHP and DLHP's) of the neural folds by the tethering of the neuroepithelium to adjacent tissue. Cell wedging, a process facilitated by a basal positioning of the nucleus, occurs at these points. There is evidence that the notochord plays an inductive role in the process of cell wedging in the MHP.

The forces involved in bending are largely extrinsic and are produced by the surface ectoderm. Once again the types of cell behaviour resulting in these forces are changes in cell shape, position and number. Changes in the extracellular matrix may also contribute to the extrinsic forces required for folding. As a result of shaping and bending of the neural plate, the neural folds are brought into apposition in the dorsal midline. Final closure of the neural groove is mediated by cell surface glycoconjugates coating the apical surfaces of the neural folds.

Neurulation in the cranial region of mice and other mammals occurs intermittently. There appear to be four closure sites from which neurulation proceeds either uni- or bi-directionally. The area of the rhombencephalon, which also displays a very unique pattern of neural crest cell migration, displays a different mode of closure to all other cranial and caudal areas of the tube.

Due to the lack of research carried out in human embryonic material, it is not known how similar the process of cranial neurulation in humans is to that of other well-researched mammalian models.

By the end of neurulation, distinct patterning of the neuraxis exists, both in the transverse and rostro-caudal axes. The formation of patterning probably involves inductive interactions with adjacent tissues and the expression of positional identity genes.

Despite the considerable advances that have been made in the field of neurulation, there remain many questions about the biochemical processes involved, mechanisms of gene expression, interactions of neurectodermal cells with the neural crest and the nature of factors leading to the final confluence of the apposed neural folds. With the advent of new technology allowing the *in-situ* identification of both cell proliferation and cell death and immunohistochemical markers for cell phenotype there is opportunity that the precise process of neurulation can be unravelled.

With a sound knowledge of the mechanisms encompassing normal human neurulation further work into the prevention of neural tube defects can proceed. In addition a complete understanding of this event in the context of it being the first example of a fusion process and the first example of organogenesis within the embryo, may have far-reaching effects in other areas of developmental biology.

5

A MORPHOLOGICAL STUDY OF THE CLOSURE OF THE NEURAL TUBE IN RAT EMBRYOS

5.1 INTRODUCTION

Both the neural tube and secondary palate demonstrate fusion processes that are frequently perturbed in human embryology resulting in anencephaly/spina bifida and cleft palate malformations respectively. Among the three current hypotheses for the fate of the medial edge epithelium during normal palatogenesis, is removal of the medial edge epithelium by apoptotic cell death (Ferguson, 1988; Mori *et al.*, 1994). As previously discussed the recent work by Mori *et al.* (1994) has conclusively demonstrated morphological and histochemical evidence of apoptosis in the medial edge epithelium during palatal fusion. This study employs similar methods to investigate the role of apoptosis in the region of the fusing neural folds.

Structural studies currently being carried out at the Institute of Dental Research, Sydney, also confirm the existence of apoptosis during palatogenesis. Figures 1.1C and 1.1D show Toluidine Blue stained semi-thin resin sections of a 16.5 day rat palate undergoing fusion. In Figure 1.1C the medial edge epithelium has begun to disintegrate, leaving two apparent epithelial islands within the mesenchymal tissue. Careful examination of these islands in Figure 1.1D shows many cells with the characteristic morphology of apoptosis, including cell shrinkage and nuclear chromatin condensation. Future work employing electron microscopy and *in-situ* end-labelling is expected to provide conclusive evidence for the role of apoptosis during palatogenesis. However, to date there has been no similar detailed description for fusion of the neural folds.

As the formation of a patent neural tube constitutes the first fusion process in embryology, an understanding of the cellular events relating to this process may provide a better understanding of subsequent fusion events. It was the aim of this study to define the role of apoptotic death within the fusing neural folds of rat embryos by employing a previously described *in-situ* end-labelling technique for the detection of endonuclease-induced DNA fragmentation (Wijsman *et al.*, 1993) characteristic of apoptosis. The use of immunohistochemical techniques for phenotypic marking and the identification of replicating cells were trialed in order to provide further information about the cells taking part in the fusion process.

5.2 MATERIALS AND METHODS

5.2.1 ANIMALS

Animals used in the experiments were from an outbred line of Sprague-Dawley rats bred at the Institute of Dental Research (IDR). The rats were housed in the Animal House of the IDR, Sydney, in cages with wood chip litter and allowed water and commercial pellets *ad libitum*. Timed pregnant rats were obtained in-house by breeding the rats overnight, the following morning counting as day 0.5 Gestation. The procedure was to use animals between 70 and 120 days old. Two males were added to cages containing five females at 5pm and removed next morning at 8am. Approximately 15% of females conceived using this approach.

Many investigators have suggested that interlitter variability may be greatly reduced by breeding for a short period rather than overnight. However, Fujinaga *et al.*(1990) compared the mean number of implantations and live embryos, the mean crown-rump length, the somite number and the protein content of day 11 rat embryos produced by overnight and morning short period breeding regimens. Their results showed that there were no differences in mean number of implantations, live foetuses and resorptions between the two breeding regimens. Mean crown-rump length, somite number and protein content were significantly lower for embryos in the morning short period breeding group than those in the overnight breeding group when either the litter or the embryo was used as the statistical unit. However, they concluded that the differences were small and probably of little practical importance for most experimental studies.

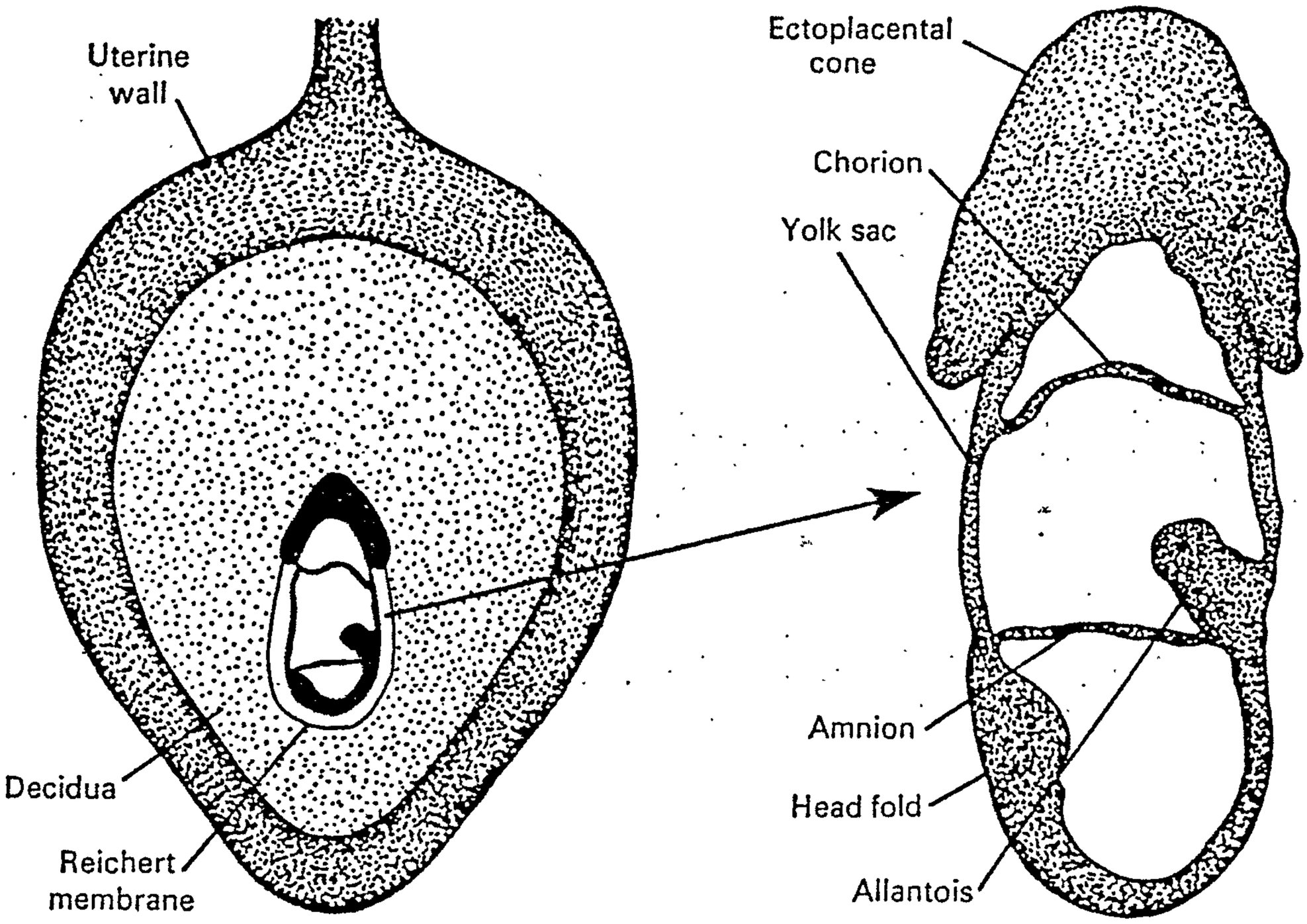
Rats were sacrificed at days 9.5 and 10.5 respectively using overdosage of halothane anaesthesia. Uteri were excised and individual implantation sites were harvested into isotonic phosphate buffered saline (PBS) - pH 7.4 (Dulbecco PBS tablets, Oxoid). The embryonic sacs are egg-shaped structures in which the pointed end contains the embryo within the extraembryonic coelom and the blunt end is mostly decidual tissue (see Figure 5.1).

During the initial stages of experimentation the embryonic sacs were dissected in half and the portion containing the embryo was processed for resin or paraffin embedding. However, during the later stages of experimentation, embryos were completely dissected out of their amniotic cavity using dissecting microscopy prior to fixation, processing and embedding.

The caudal portion of the neural tube was chosen for this study. There were several features making this area of the neural tube more suitable for the study of closure of the neural tube than the cranial region. Firstly, from the level of the second to fourth somites to the caudal neuropore, there is an uninterrupted closure in the caudal direction. Secondly, Figure 5.2 shows that the embryo can be readily oriented to provide a long segment of the neural tube in the optimal cross-sectional plane. A study of the cranial neural tube would involve many changes in orientation in order to continue the production of cross-sectional sections of the neural tube. Furthermore, with the three different closure sites and perhaps a different method of fusion in the rhombencephalon, study of the cranial region would be extremely complex.

For the purpose of control tissue, sections of ileum from mature Sprague-Dawley rats were harvested and processed for paraffin embedding.

FIGURE 5.1: Appearance of a 9.5 day rat embryo within the embryonic sac (left) and once dissection is completed (right). The diagram illustrates Reichert's membrane, the decidual tissue and the uterine wall. The 9.5 day embryo is yet to undergo entypy, that is, folding into the amniotic cavity.



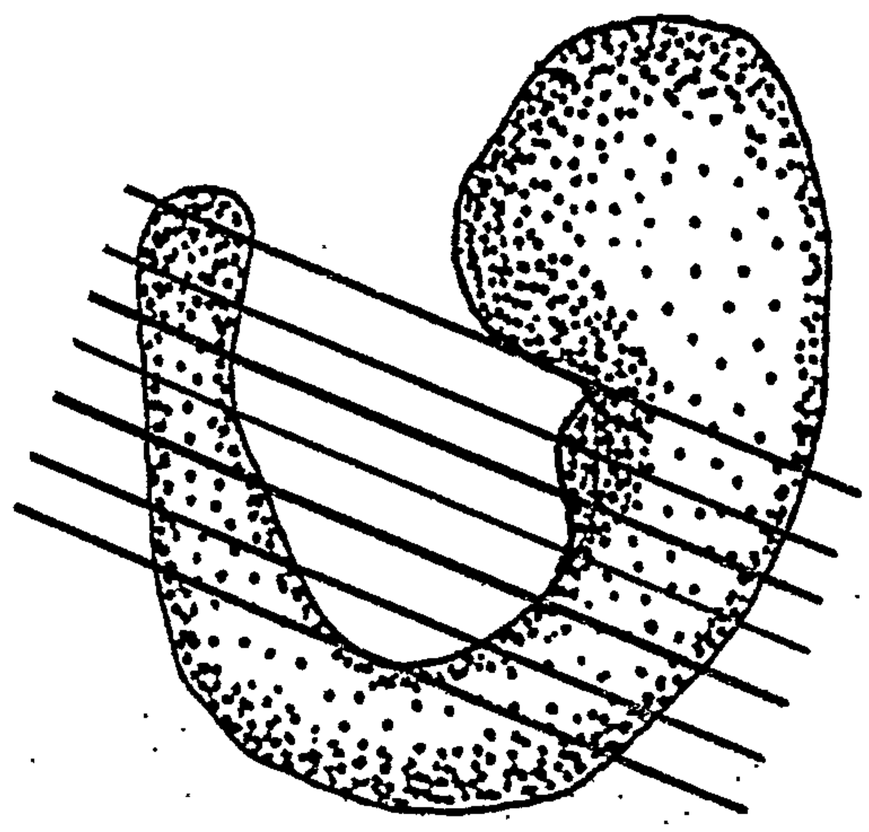
(From New, 1977)

FIGURE 5.2: Diagrammatic representation of a 10.5 day rat embryo.

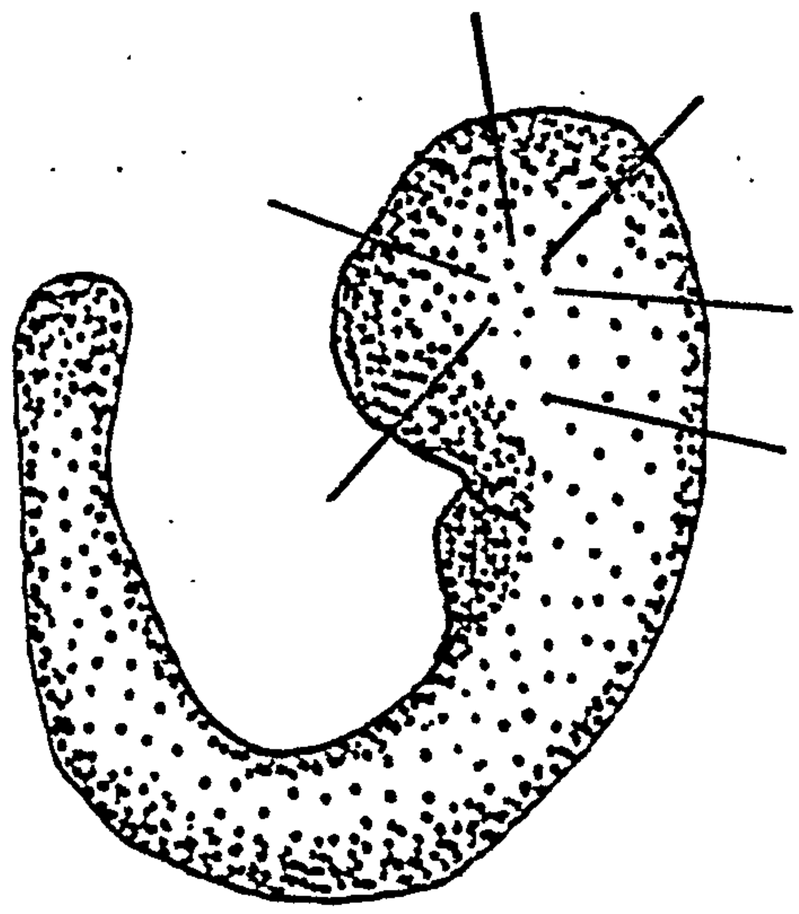
A: Orientation of embedding that allows long segments of the caudal neural tube to be cut in cross-section.

B: Producing cross-sections of the cranial neural tube would require many changes in the plane of section

A



B



5.2.2 FIXATION

Paraformaldehyde (BDH-Analar) 4% w/v PBS - pH 7.4 (Dulbecco) was the fixative used. Initially, the complete uteri were immersed in ice-cold fixative immediately following excision from the animal. This procedure was adopted after attempting fixation *in-situ* by perfusion of the maternal vasculature. The superior vena cava was cut and 40 mL of warmed PBS was introduced slowly through the left ventricle. This was followed by 40 mL of warmed fixative. It was noted, however, that on dissection the embryos hearts were beating indicating inadequate fixation.

Later, the process of complete dissection of the embryo was undertaken on unfixed tissues and the embryo was immersed into the cold fixative immediately upon retrieval. The embryo was fixed overnight at 4°C and then transferred into PBS prior to processing.

In parallel, lengths of ileum from mature rats were flushed with PBS and then fixative was introduced prior to immersion in cold fixative. Intraluminal fixation prevented smooth muscle contraction and consequent eversion of the gut. Otherwise the gut was processed under conditions identical to the embryo.

5.2.3 PROCESSING

The tissues for embedding were dehydrated through increasing concentrations of alcohol (1 X 70%, 1 X 95%, 2 X absolute alcohol) and cleared in xylol (2 X). Early tissues were embedded into resin (Glycol Methacrylate, Polysciences). The embryos were visualised under stereomicroscopy, orientated and embedded into small embedding moulds. Once cured the small resin block was mounted onto a microtome chuck. Later tissues were embedded into paraffin to enable the use of both immunohistochemical and *in-situ* end-labelling techniques.

Initially, the hemisected embryonic sac was embedded into a paraffin block at random orientation. However, once the embryos were dissected free of the embryonic sac a procedure similar to that used with resin embedding was undertaken. Embryos were orientated and embedded into paraffin within a small embedding mould. Once set, these small moulds containing embryos of known orientation were re-embedded into standard paraffin blocks to facilitate sectioning. The orientation achieved allowed the production of serial sections of the majority of the caudal neural tube in cross-section (see Figure 5.2).

5.2.4 SECTIONING

Resin sections were cut at 1*u*m and stained with 1% Methylene Blue in 1% sodium borate. Sections were examined under light microscopy.

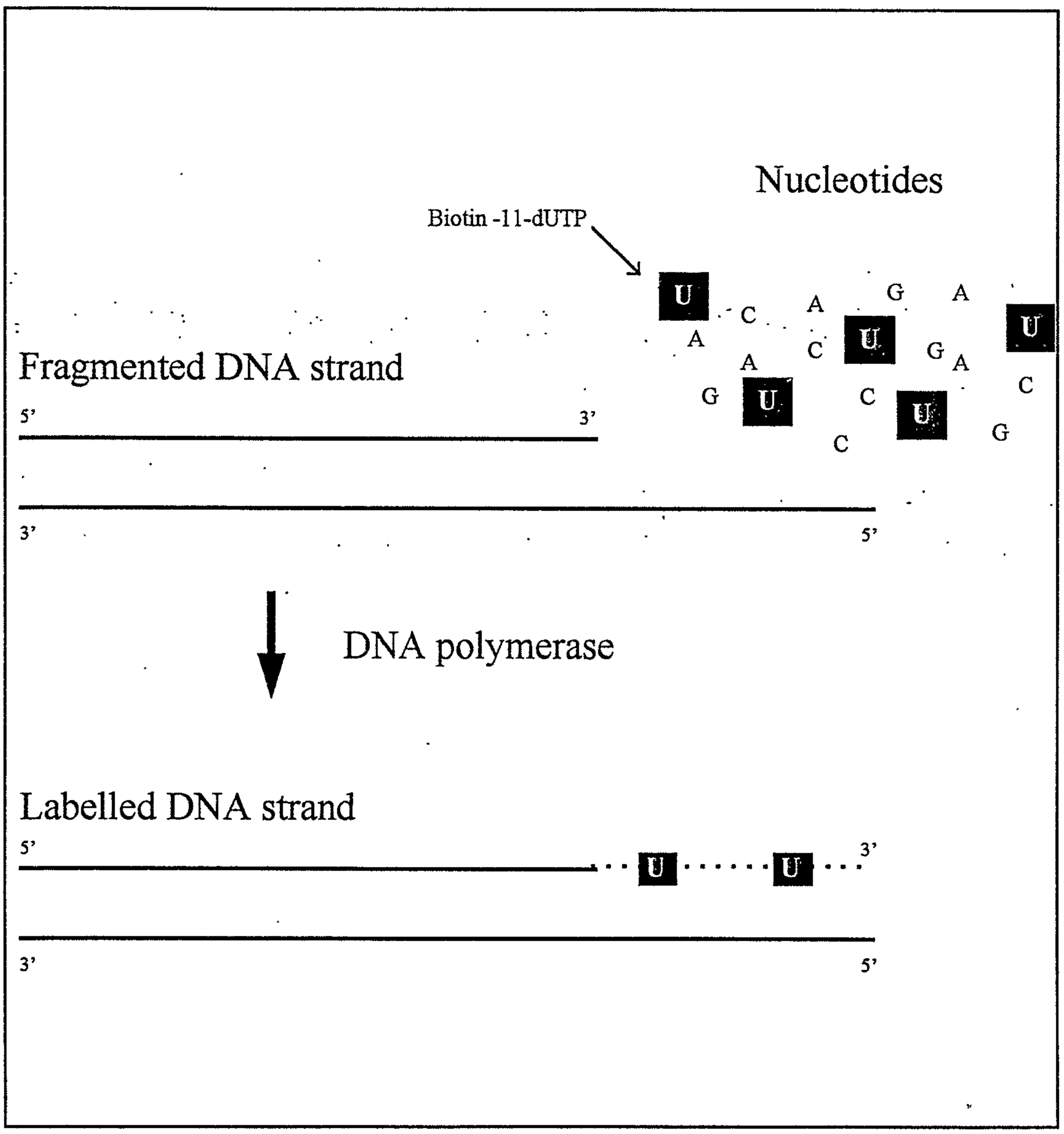
Serial paraffin sections were cut at 4*u*m and two or three sections were placed on each slide. Every tenth slide was oven-heated to 60°C for 30 minutes, deparaffinised using xylol and rehydrated through decreasing concentrations of alcohol solutions (2 X absolute ethanol, 1 X 95% ethanol, 1 X 70% ethanol). The slides were then routinely stained with Haematoxylin and Eosin. These slides served to provide orientation throughout the serial sectioning of the embryos.

5.2.5 *IN-SITU* END-LABELLING TECHNIQUE

Apoptosis can be difficult to detect in routine histological sections. Since extensive endonuclease-induced DNA fragmentation is an important characteristic of this process, visualisation of DNA breaks could greatly facilitate the identification of apoptotic cells. A staining method for formalin-fixed, paraffin-embedded tissue sections involving an *in-situ* end-labelling technique was described by Wijsman *et al.* in 1993. A schematic representation of the *in-situ* end-labelling technique can be found in Figure 5.3.

FIGURE 5.3: A schematic illustration of the *in-situ* end-labelling technique for the detection of the endonuclease-induced DNA fragmentation produced during apoptotic cell death. In the presence of DNA polymerase and nucleotides a fragmented DNA strand can be rebuilt. When using a biotin-labelled nucleotide (biotin-11-dUTP) the rebuilt DNA strand will be labelled. This technique allows the visualisation of apoptotic cells showing extensive endonuclease-induced DNA fragmentation.

In-Situ End-Labelling



Sections were oven-heated at 60°C for 30 minutes. After deparaffinising and rehydrating they underwent the following pretreatment to make the DNA accessible for incorporation of nucleotides :

(1) Sections were heated in 0.3 M NaCl and 30 mM Na-citrate, pH 7 at 80°C for 20 minutes.

(2) Sections were allowed to reach room temperature and were thoroughly washed in PBS.

(3) Incubation in 0.5% pepsin (Sigma Co; St. Louis, Missouri) in 0.05M glycine-HCl buffer pH 2 at room temperature for 10 minutes.

The digestion process proved to be extremely harsh on the delicate embryonic tissues. Trials were carried out using an alternative enzyme and varying conditions in order to achieve adequate digestion without causing tissue damage. Initially, 0.5% pepsin was used for 30 minutes at 37°C, following the technique of Wijsman *et al.* (1993) but most embryonic sections were destroyed. The use of an alternative enzyme - Proteinase K (Sigma Co; St. Louis, Missouri) was trialed under the same conditions, but it again caused tissue destruction. The optimal digestion without causing tissue damage was achieved using the technique specified in stage 3 above.

(4) Digestion was stopped by immersion in PBS, followed by thorough washing.

The incorporation of nucleotides and cytochemical detection involved the following stages:

(5) Sections were rinsed in Buffer A [50 mM Tris-HCl, 5mM $MgCl_2$, 10mM β-mercaptoethanol and 0.005% albumin (bovine) pH 7.5 (BSA) (Sigma Co; St. Louis, Missouri)] for 5 minutes at 23°C.

(6) Incubation in Buffer A containing 0.01 mM dATP, dCTP and dGTP (Deoxynucleoside triphosphate set. Boehringer; Mannheim, Germany), 0.01 mM biotin-11-dUTP (Boehringer; Mannheim, Germany) and 10 U Klenow enzyme (Boehringer; Mannheim, Germany). Approximately 50 μL was used on each tissue section at 23°C for 1 hour.

(7) Endogenous peroxidase was blocked by immersion for 15 minutes in PBS containing 0.1% H_2O_2.

(8) Sections were washed thoroughly in PBS.

(9) Sections were incubated with horseradish peroxidase-conjugated Streptavidin (Dako A/S; Glostrup, Denmark) diluted 1:100 in PBS containing 1% BSA (Sigma Co; St. Louis, Missouri) and 0.5% Tween 20 (Sigma Co; St. Louis, Missouri) for 30 minutes at 23°C.

(10) Staining was developed using the nickel-enhanced diamino benzidine reagent (Pierce; Rockford, Illinois) 10% in darkness at 23°C.

(11) Sections were counterstained with 0.1% Methyl Green and coverslipped prior to examination under light microscopy.

Both negative and positive controls were run with each series. In negative controls, the Klenow enzyme was omitted from the nucleotide solution in stage 6. The positive controls used, were transverse sections of mature rat gut that were treated in the same manner as the embryo sections. Confirmation that the procedure was working was assessed by examining the positive control for evidence of apoptotic cells at the tips of the villi of the small intestine and with no staining in the proliferating basal cells or in the lateral borders of the villi.

5.2.6 IMMUNOHISTOCHEMICAL STAINING TECHNIQUES

5.2.6.1 Phenotypic Markers

Immunohistochemical markers for cells of neural crest origin (Antibody to S_{100} - Dako A/S; Glostrup, Denmark), epithelial cells (Antibody to Keratin 8 - Dako A/S; Glostrup, Denmark) and mesenchymal cells (Antibody to Vimentin - Dako A/S; Glostrup, Denmark) were utilised to provide phenotypic information about the paraffin sections. The same procedure was used for each of these immunohistochemical markers.

Sections were deparaffinised in xylol and rehydrated through the same ethanol series as described for the Haematoxylin and Eosin staining. The rehydrated sections were incubated sequentially with the following solutions:

(1) 0.05% Trypsin (Sigma Co; St. Louis, Missouri) in PBS for 5 minutes at room temperature to retrieve antigenic groups.

(2) Blocked in 20% horse serum in PBS for 30 minutes.

(3) Monoclonal antibody diluted 1:25 - 1:50 in 10% foetal calf serum in PBS for 1 hour at room temperature.

(4) 3 X 10 minute washes in PBS.

(5) Peroxidase-labelled rabbit anti-mouse immunoglobulins (Dako A/S; Glostrup, Denmark) diluted 1:50 in 10% foetal calf serum in PBS for 1 hour at room temperature.

(6) 3 X 10 minute washes in PBS.

(7) Diamino benzidine/Metal Concentrate Peroxide (Pierce; Rockford, Illinois) 10% in darkness at 23°C until staining developed.

Immunostained sections were counter-stained with 0.1% Methyl Green, dehydrated, coverslipped and observed under light microscopy. Specificity controls were carried out by substituting an isotype-matched irrelevant monoclonal antibody for the primary antibody.

5.2.6.2 Proliferating Cell Nuclear Antigen (PCNA)

PCNA (also known as Cyclin) is a 36 kDa acidic protein molecule which is highly conserved in evolution. It functions as a co-factor for DNA-polymerase δ in both the S phase of the mitotic cycle and also in DNA synthesis associated with DNA repair (Shivji *et al.*, 1992). The cell concentration of this protein is directly correlated with the proliferative state of the cell, increasing through the pre-synthesis phase (G1), peaking at the G1/DNA synthesis phase (S) interface, decreasing through post DNA synthesis phase (G2) and reaching low levels in the mitosis phase (M) (Kurki *et al.*, 1986).

PC10 is a monoclonal antibody that recognises a PCNA-epitope. The protein is resistant to wax embedding but immunostaining is fixation dependent. Casasco *et al.* (1993) found that alcohol-fixed tissues showed no variation in intensity of immunoreaction with respect to fixation time. However, tissues fixed with formaldehyde-containing solutions were greatly influenced by the fixation time. Samples fixed for 3 hours in 4% formaldehyde solution displayed decreased immunostaining compared with samples fixed in alcoholic solutions for the same amount of time. Almost no immunoreaction was found in samples fixed with formaldehyde for 24 hours. Using flow cytometry Casasco *et al.* found that while formaldehyde fixation did not alter the quality of the DNA profile, it resulted in a significant reduction of the percentage of labelled cells and the intensity of fluorescence. Furthermore, Casasco *et al.* (1993) noted that while immunoreactivity to PC10 antibody was usually observed within the nuclei of positive cells, some cell populations in developing tissues might contain cytoplasmic PCNA. Further studies are required to elucidate whether the immunoreactivity represents PCNA synthesis, storage or breakdown within the cytoplasm.

Despite this, these authors concluded that PC10 antibody to PCNA represented a suitable reagent for the immuncytochemical detection of replicating (G1, S and G2 phases) cells in developing tissues, given adequate tissue processing.

Sections were deparaffinised and rehydrated through the ethanol series described above. The sections were then incubated in the following manner:

(1) 20 % horse serum in PBS - 30 minutes at room temperature.

(2) Monoclonal mouse anti-proliferating Cell Nuclear Antigen (PCNA) Clone PC10 (Dako A/S; Glostrup, Denmark) diluted 1:25 in 10% foetal calf serum in PBS - 1 hour at room temperature.

(3) 3 X 10 minute washes in PBS.

(4) Peroxidase-labelled rabbit anti-mouse immunoglobulins (Dako A/S; Glostrup, Denmark) diluted 1:50 in 10% foetal calf serum in PBS - 1 hour at room temperature.

(5) 3 X 10 minute washes in PBS.

(6) Diamino benzidine/Metal Concentrate Peroxide (Pierce; Rockford, Illinois) 10% in darkness at room temperature until staining developed.

Sections were counter-stained with 0.1% Methyl Green, coverslipped and studied under light microscopy. Negative controls were carried out on embryonic tissue and mature rat gut using the technique described above, but omitting the PCNA from stage 2. Positive controls using mature rat gut were processed in the same manner as the embryonic tissue. Evidence of PCNA staining in the basal cells of the crypts of the rat small intestine was taken as confirmation that the procedure was working.

5.2.7 PHOTOGRAPHY

Colour photographs were taken using an Olympus Vanox microscope and Konica Impressa colour print film (ASA 50). Magnifications at the micrograph of 40-430X where used and are specified throughout.

5.3 RESULTS

A total of thirteen 9.5 day and eighty-six 10.5 day embryos were examined under light microscopy. All thirteen 9.5 day embryos were left within the embryonic sacs and embedded in paraffin at random orientation. Table 5.1 shows the distribution of the 10.5 day embryos with regard to resin or paraffin embedding and whether they were dissected from the embryonic sac prior to fixation and processing. The initial eleven 10.5 day embryos were embedded in resin and semi-thin sections were stained with Methylene Blue and examined under light microscopy. While this method of examination was advantageous in allowing ease of manipulation for cross-sectional orientation of the embryo and preserving clear morphological detail, *in-situ* end-labelling for the detection of DNA fragmentation has not been reported using glycol methacrylate.

For this reason, it became necessary to use paraffin embedding, for which *in-situ* end-labelling and immunohistochemical staining techniques have been described. A total of seventy-five 10.5 day embryos were examined using paraffin embedding. Initially, the embryos were not dissected from the embryonic sacs and thus the embryos were embedded at random orientation within the paraffin block (see Figures 5.4A and 5.4B). As their size and fragility makes any manipulation potentially damaging to their anatomical integrity, it was felt that it was preferable to use this method and examine those that happened to be properly oriented in cross-section. However, it became clear that the yield of usable material gained by this method was very low and so it became necessary to completely dissect the embryo from the embryonic sac under stereomicroscopy prior to fixation and processing.

Table 5.1 shows the level of improvement in the percentage of useful material gained once the embryos were dissected and oriented correctly within the paraffin block. Although the procedure was more technically demanding and resulted in a greater loss of specimens during manipulation, processing and embedding, it was clearly more efficient during sectioning.

TABLE 5.1: Total number of 10.5 day rat embryos examined showing the numbers embedded in resin and paraffin. The number and percentage of embryos that yielded informative material are shown. It is evident that embryos that were dissected prior to embedding produced a higher percentage of useful information than those embedded within the embryonic sacs.

	RESIN (TOTAL)	PARAFFIN (TOTAL)	PARAFFIN (UNDISSECTED)	PARAFFIN (DISSECTED)
TOTAL NUMBER	11	75	32	43
INFORMATIVE	5	41	6	35
PERCENTAGE	45%	54%	18%	81%

FIGURES 5.4A AND 5.4B: Two different 10.5 day embryos embedded within the embryonic sac in paraffin at random orientation. Figure 5.4B appears on the following page.`

FIGURE 5.4A: The random orientation results in a section that does not provide any useful information in the study of neural tube closure [40X].

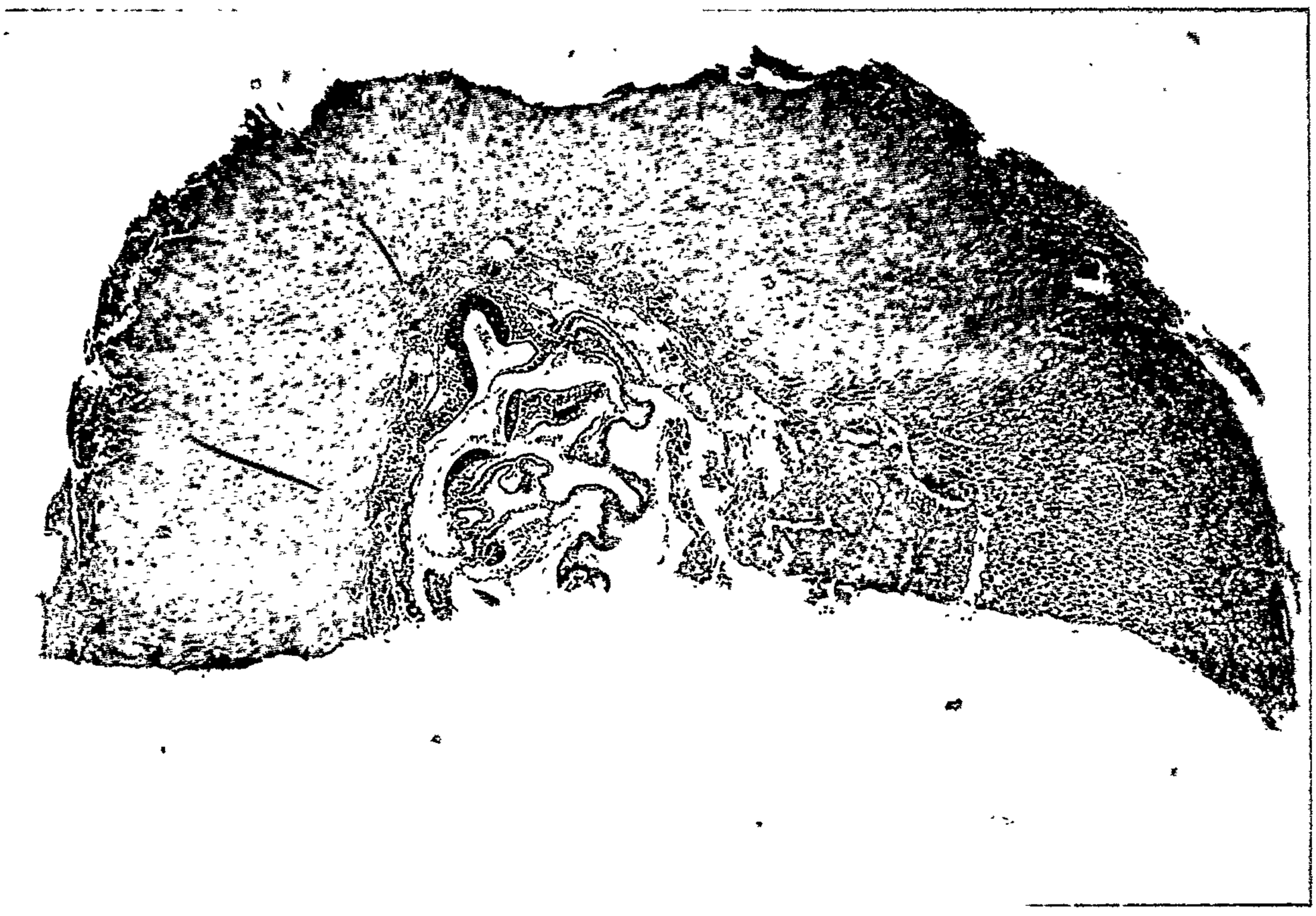


FIGURE 5.4B: Section of a randomly orientated embryo, showing cross-section of an open neural tube. By chance, the orientation of this embryo provided useful material for further study [40X].

As discussed previously, there is a wide variability in the staging of embryos even within the same litter. Thus, some 10.5 day embryos displayed wide open neural folds while in others the fusion process was already completed. As can be seen from the fusion process described in Table 4.1 and Figure 4.4 closure site I extends bidirectionally starting from between the second to the fourth somites and extending all the way to the caudal neuropore. Thus the fusion of the caudal neural tube facilitates the examination of the fusion process in serially sectioned embryos of the correct stage (approximately 10.5 days *in-utero*) and so it was this area of neural tube that was examined during the study.

5.3.1 THE MORPHOLOGY OF NEURULATION

The two series in Figures 5.5 and 5.6 are from a 10.5 day resin-embedded embryo. These two series show the subtle morphological differences in neural tube from the cranial and caudal regions. Figure 5.5A shows an open cranial neural tube that has undergone the process of bending at the median hinge point (MHP) and dorso-lateral hinge points (DLHP's). The lumenal surface of the tube displays blebbing from the extrusion of cytoplasm from the basal area of the cells. In Figure 5.5B the tips of the neural folds are beginning to appose each other. The surface ectoderm is being drawn across with the neurectoderm and the lateral neurectoderm is thickened. Figure 5.5C shows the recently fused cranial neural tube with intact surface ectoderm and many mitotic figures within the neurectoderm.

Figures 5.6A - 5.6C show a similar sequence in the caudal neural tube. One of the main differences in this process compared with that of the cranial area is the lack of formation of the dorso-lateral hinge points (DLHP's) seen in Figure 5.6C.

FIGURES 5.5A - 5.5D: CRANIAL NEURAL TUBE.
Semi-thin serial sections 20 µm apart of a resin-embedded 10.5 day embryo showing fusion of the cranial neural tube. Figures 5.5B - 5.5D appear on subsequent pages.

FIGURE 5.5A: Neural folds beginning to appose in the midline by bending at the median hinge point and the dorso-lateral hinge points. The surface ectoderm is being drawn across with the neural folds. The arrows show the characteristic blebbing of the lumenal surface that results from apico-basal wedging of the neurectodermal cells during the folding process [215X].

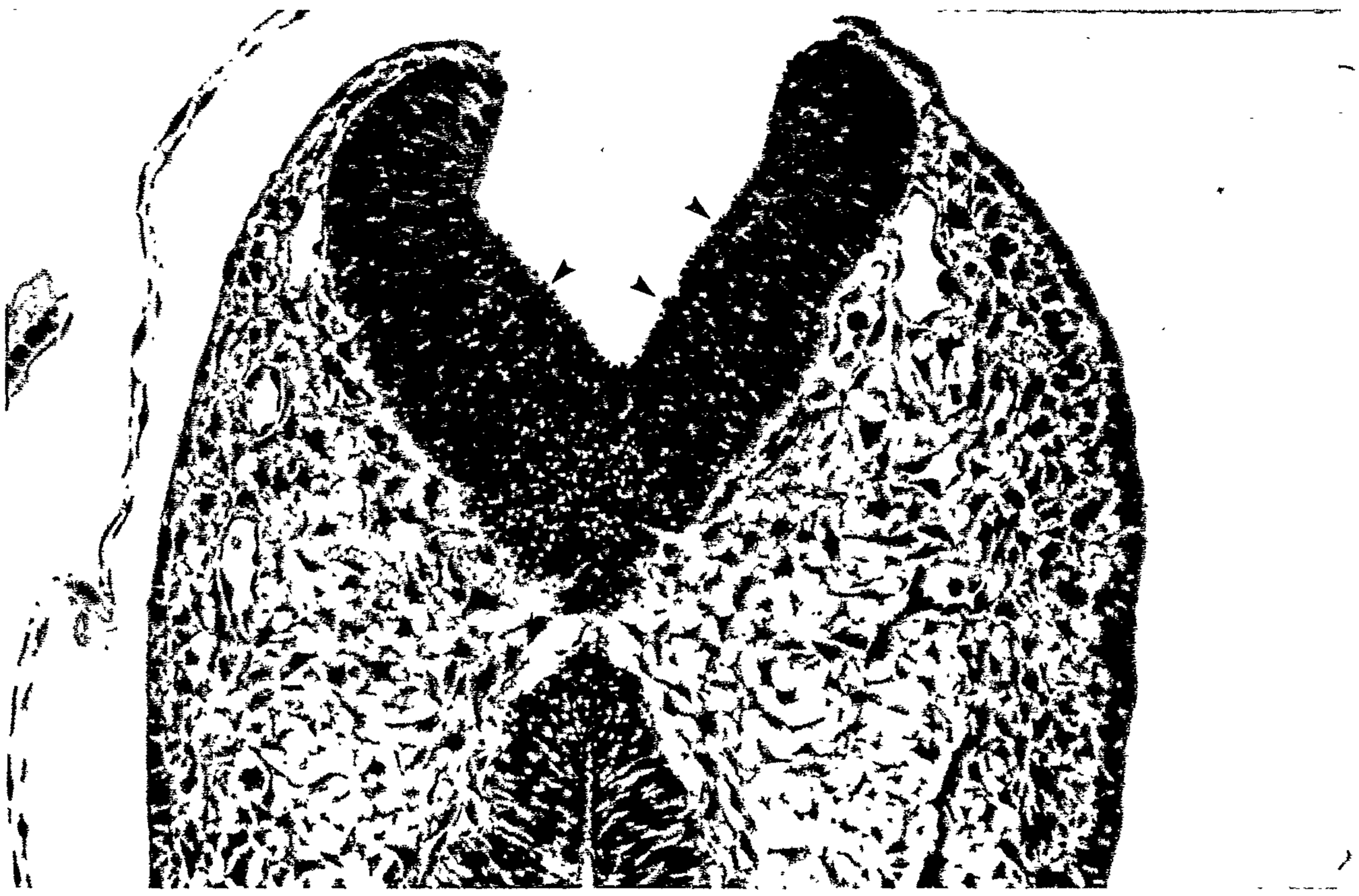


FIGURE 5.5B: The neural folds continue to come into apposition [430X].

FIGURE 5.5C: Closed neural tube showing initial adhesion achieved by a bridge of cells, 1-2 cells thick (large arrow). The high rate of cell proliferation within the neurectoderm is apparent with many cell nuclei around the lumenal surface undergoing mitosis (small arrows). The surface ectoderm has attained fusion above the neural tube (medium arrow) [430X].

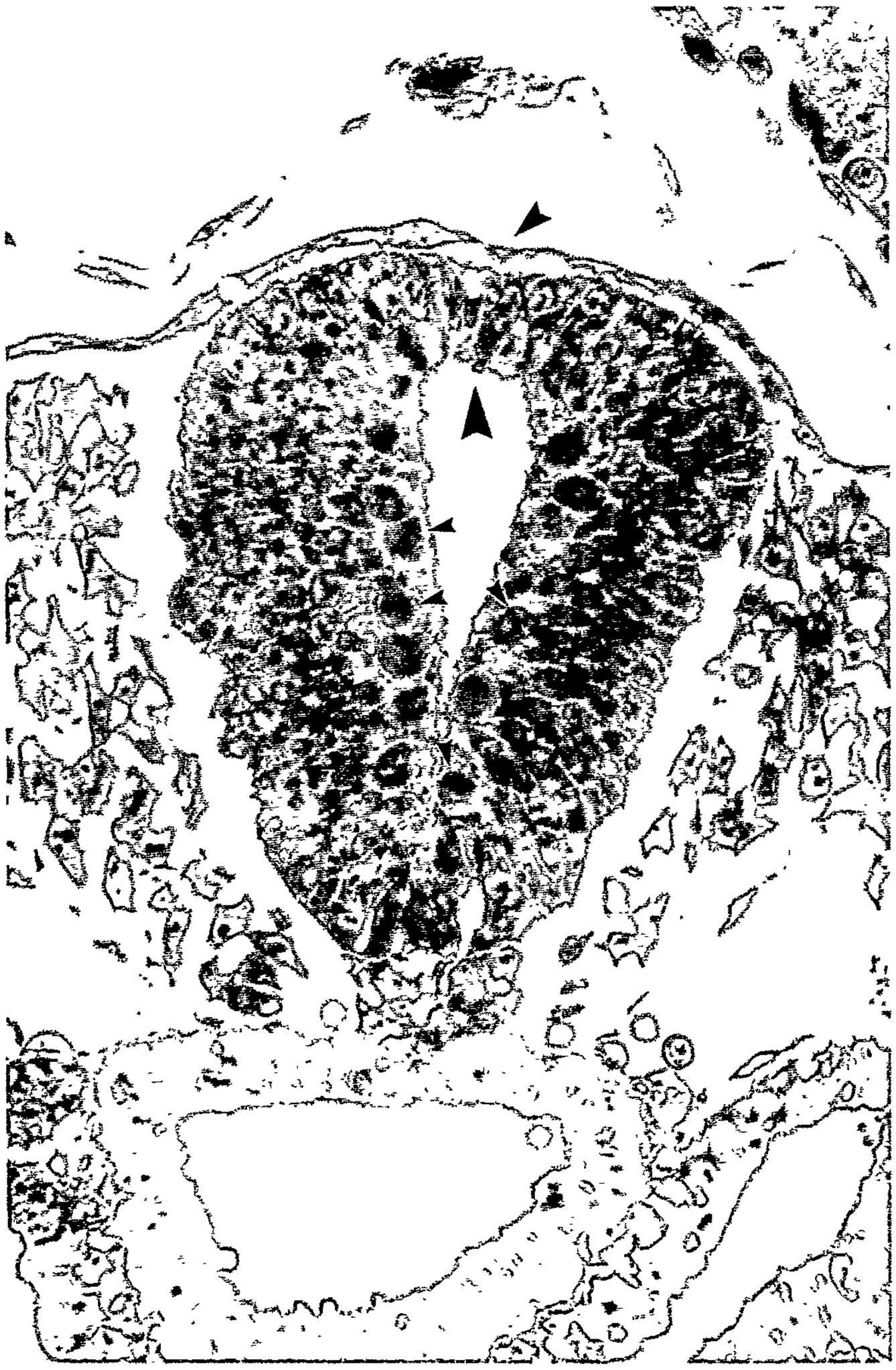


FIGURE 5.5D: Closed neural tube showing organisation of the fusion area. The surface ectoderm has become more regular. There are two cells on the lumenal surface of the fusion area (arrows) that show the morphological characteristics of apoptosis - cell shrinkage and nuclear chromatin condensation [430X].

FIGURES 5.6A - 5.6C: CAUDAL NEURAL TUBE.
Semi-thin serial sections 20 µm apart of a resin-embedded embryo showing fusion of a caudal area of neural tube. Figures 5.6B and 5.6C appear on subsequent pages.

FIGURE 5.6A: Recently closed neural tube showing intact surface ectoderm and high level of cell proliferation within the neurectoderm. Arrows indicate neural crest cells beginning to migrate into the cell-free zone. [430X].

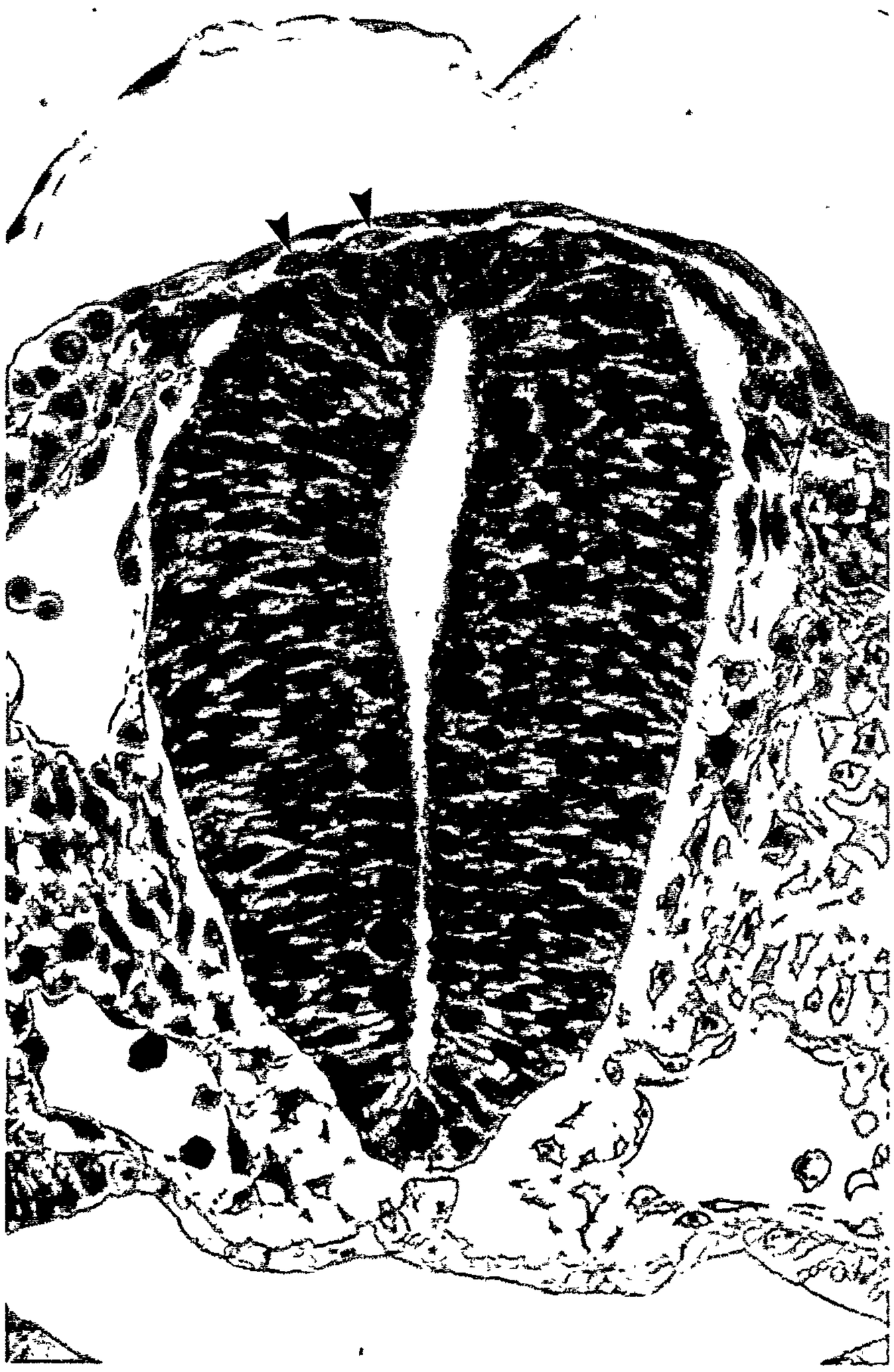


FIGURE 5.6B: Neural tube showing initial adhesion of neural folds by a bridge of cells (large arrow). There is a cell hanging from the area of fusion into the lumen of the neural tube (medium arrow) [430X].

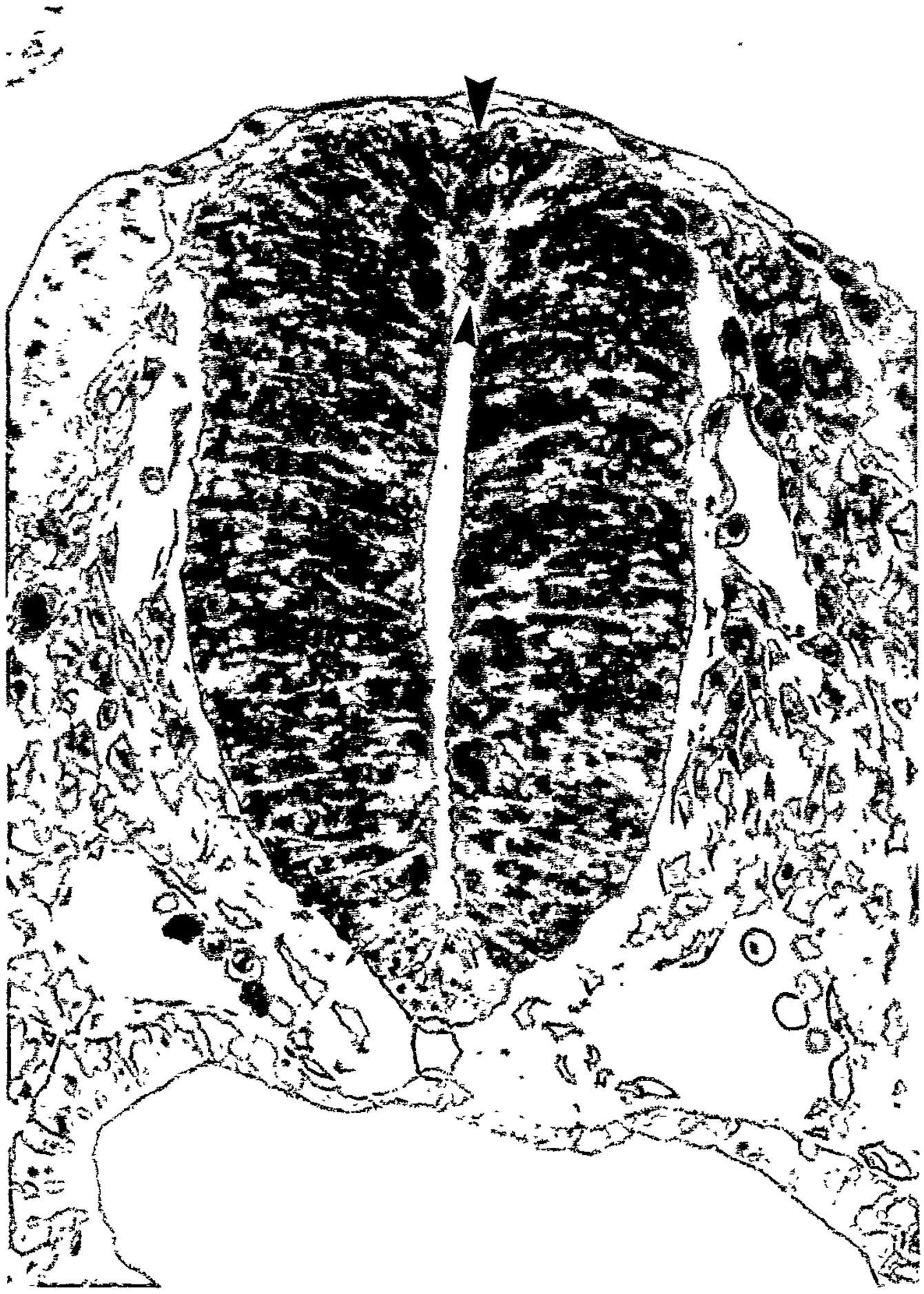
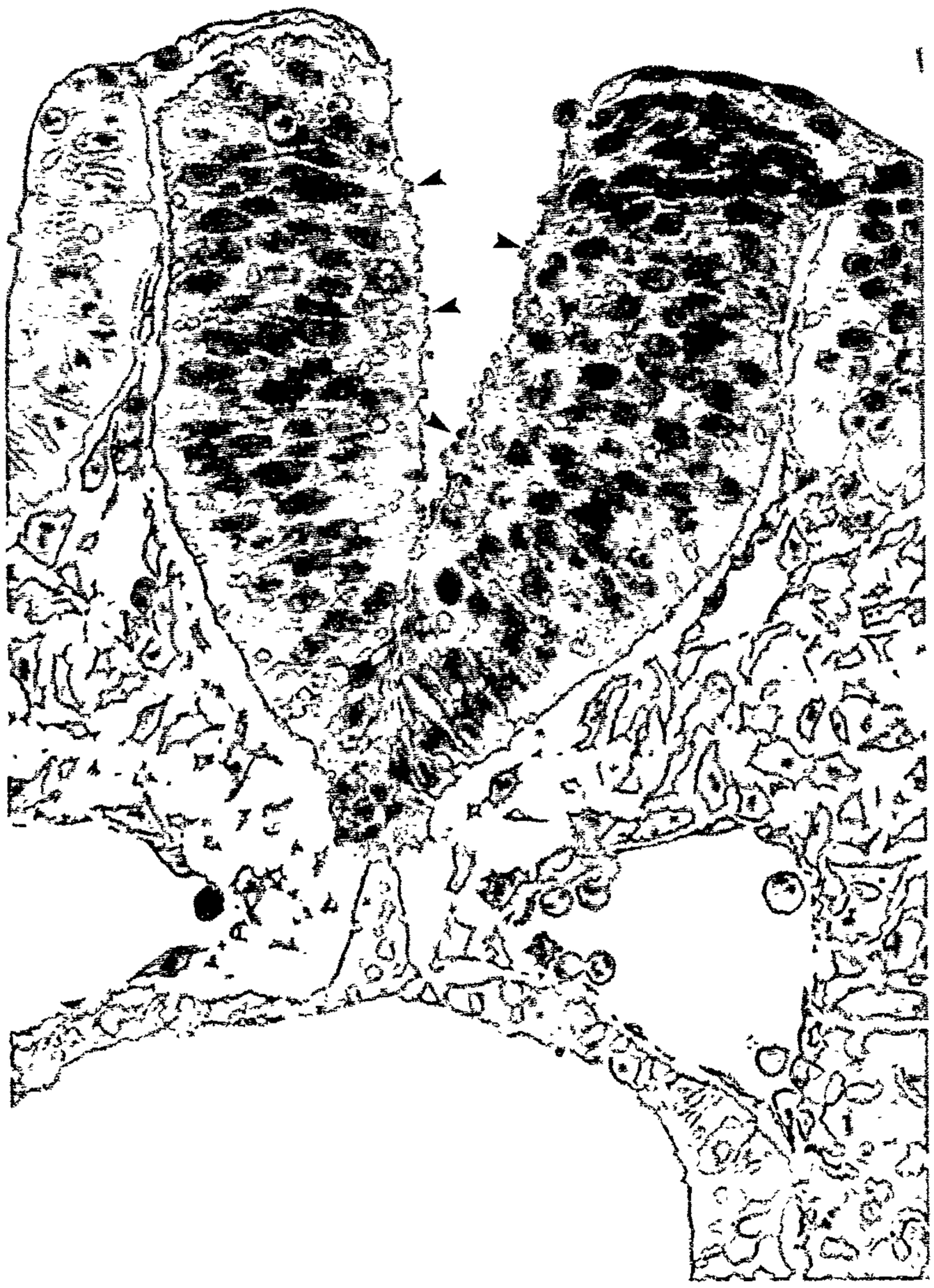


FIGURE 5.6C: Neural folds undergoing bending to achieve apposition. Unlike the cranial neural folds there is no dorso-lateral hinge point formation and the lumen of the neural tube almost becomes obliterated during the fusion process. There is a high level of proliferation within the neurectoderm. The characteristic blebbing of the lumen surface is shown (arrows) [430X].



The use of PC10 antibody to PCNA was trialed in order to gain a better understanding of the level of cell proliferation. Unfortunately, the technique was not successful on many specimens due to the method of fixation that had been used prior to embedding. While the negative and positive small intestine control tissue provided good results (see Figures 5.7A and 5.7B) indicating that correct procedural steps were being taken, the results on the embryonic tissue were somewhat variable and the information gained was limited.

However, Figure 5.8 shows a section of neural tube at a stage of apposition between Figures 5.6B and 5.6C that has been labelled with PC10. It confirms the extensive proliferation of the neurectodermal cells during the process of bending and fusion of the neural folds as seen by the multiple labelled cells within all areas of the neural folds. However, with only a small sample of specimens that displayed reliable labelling with PC10 it is difficult to make any conclusions about the role of proliferation in fusion of the neural folds.

Figure 5.6A shows the newly fused tube with intact surface ectoderm. Another example of newly fused neural folds can be seen in Figure 5.9. This Haematoxylin and Eosin stained paraffin section shows a bridge of cells one to two layers thick joining the neural folds in the midline. The surface ectoderm is already intact at this stage.

Four neural crest cells labelled with small arrows can be seen beginning to migrate through the cell-free zone. The cell labelled with a large arrow appears to be losing adhesion from the adjacent neurectodermal cells and may represent a presumptive neural crest cell.

Figures 5.10A - 5.10B show two Haematoxylin and Eosin stained paraffin sections that illustrate the finding of cells hanging from the neural folds during apposition and fusion. The number of sections in the study showing cells hanging into the lumen of the neural tube is summarised in Table 5.2.

FIGURES 5.7A AND 5.7B: 4 μm paraffin sections of mature rat intestine in cross-section used as controls during immunohistochemical staining with PC10 antibody to Proliferating Cell Nuclear Antigen (PCNA). This process labels proliferative cells. Figure 5.7B is shown on the following page.

FIGURE 5.7A: Negative rat intestine control showing the absence of any reactivity to PC10 in the highly proliferative basal cells [215X].

FIGURE 5.7B: Positive rat intestine control showing the abundant PC10-labelled nuclei of the highly proliferative basal cells and sporadic labelling of the cells at the base of the villi. The tips of the villi are free of labelled cells (not shown) [215X].

FIGURE 5.8: Section of a paraffin-embedded 10.5 day embryo stained with PC10 antibody to Proliferating Cell Nuclear Antigen (PCNA), showing an apposed neural tube. Many neurectodermal cells are labelled indicating the high rate of proliferation throughout the neurectoderm during this stage of fusion [430X].

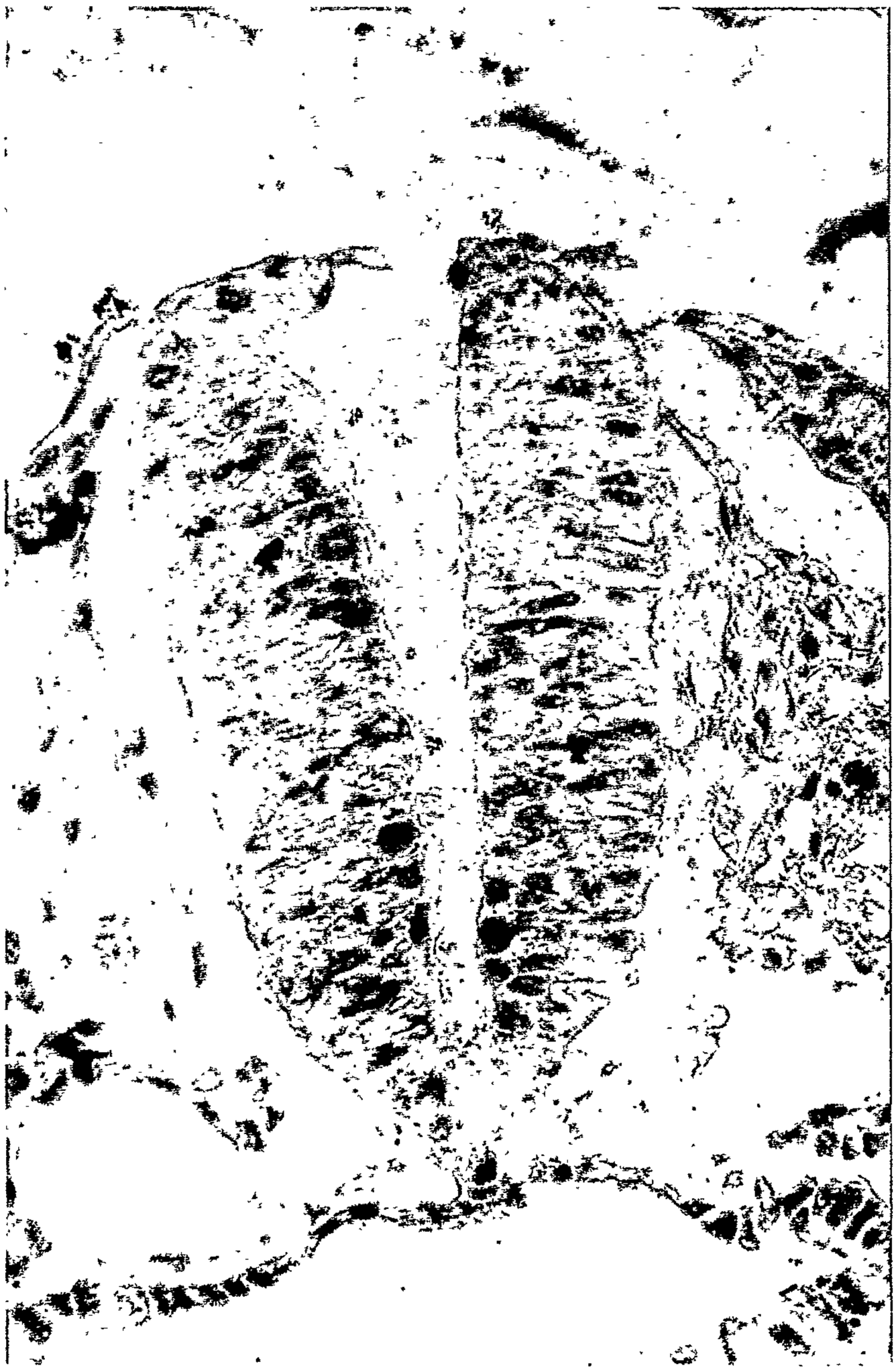
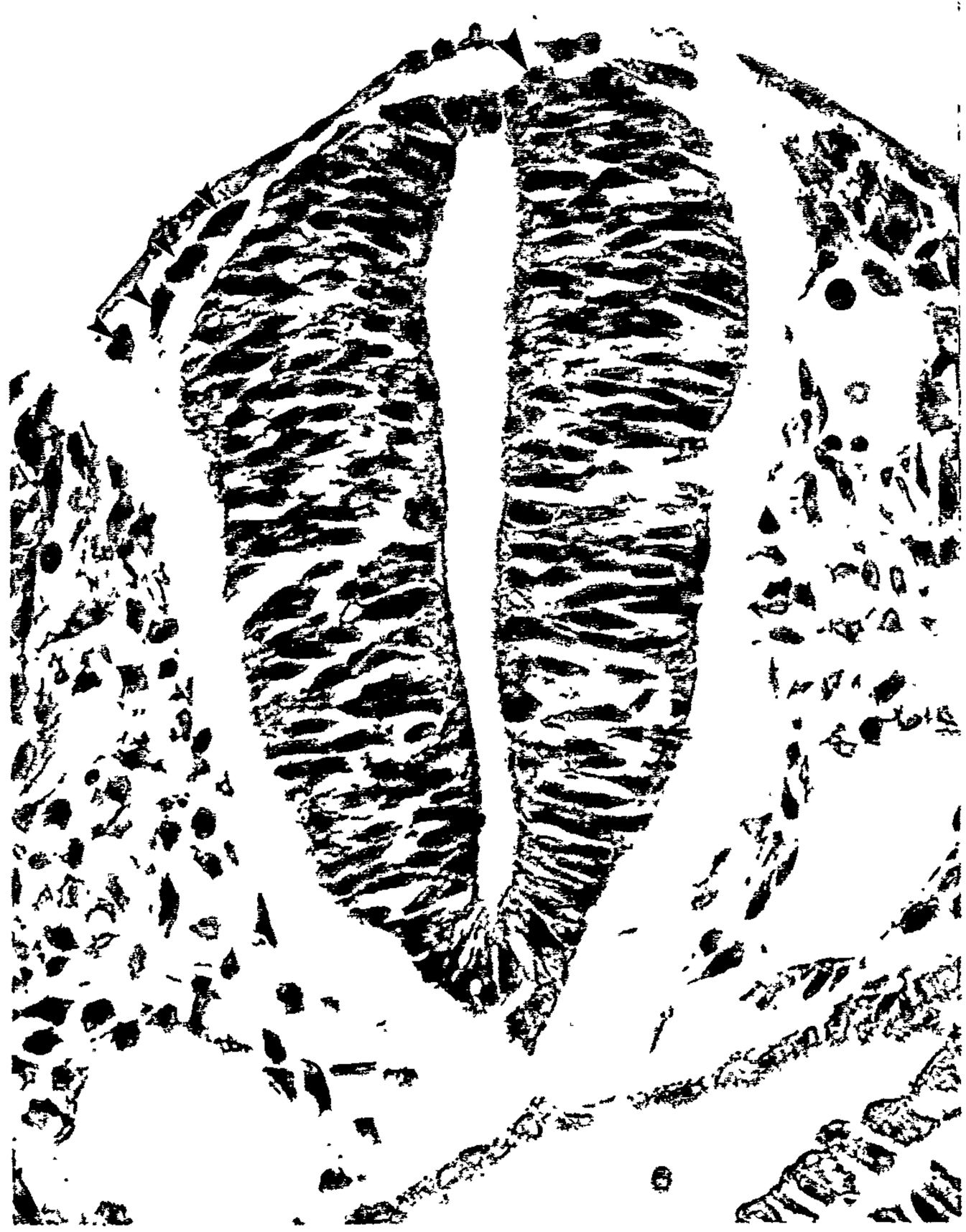


FIGURE 5.9: Haematoxylin and Eosin stained cross-section of a recently fused neural tube in a 10.5 day paraffin-embedded rat embryo. The surface ectoderm is intact lying above the neural tube. The area of fusion shows a bridge of cells between the neural folds that is 1-2 cell layers thick. Four cells seen in the cell-free zone (small arrows) appear to be migratory neural crest cells. A cell adjacent to the area of fusion appears to be detaching from the adjacent neurectodermal cells (large arrow). This cell is a presumptive neural crest cell [430X].



FIGURES 5.10A AND 5.10B: Haematoxylin and Eosin stained cross-sections of two different rat neural tubes embedded in paraffin showing cells hanging from the fusion area into the lumen. Figure 5.10B appears on the following page.

FIGURE 5.10A: Section showing apposed neural folds. The arrow indicates a cell that has lost adhesion from the adjacent neurectodermal cells and is within the lumen of the forming neural tube [430X].

FIGURE 5.10B: Section showing a recently closed neural tube. There are two cells found hanging from the area of fusion into the lumen of the neural tube (large arrows). Several cells within the fusion area show the features of apoptosis (small arrows) [430X].

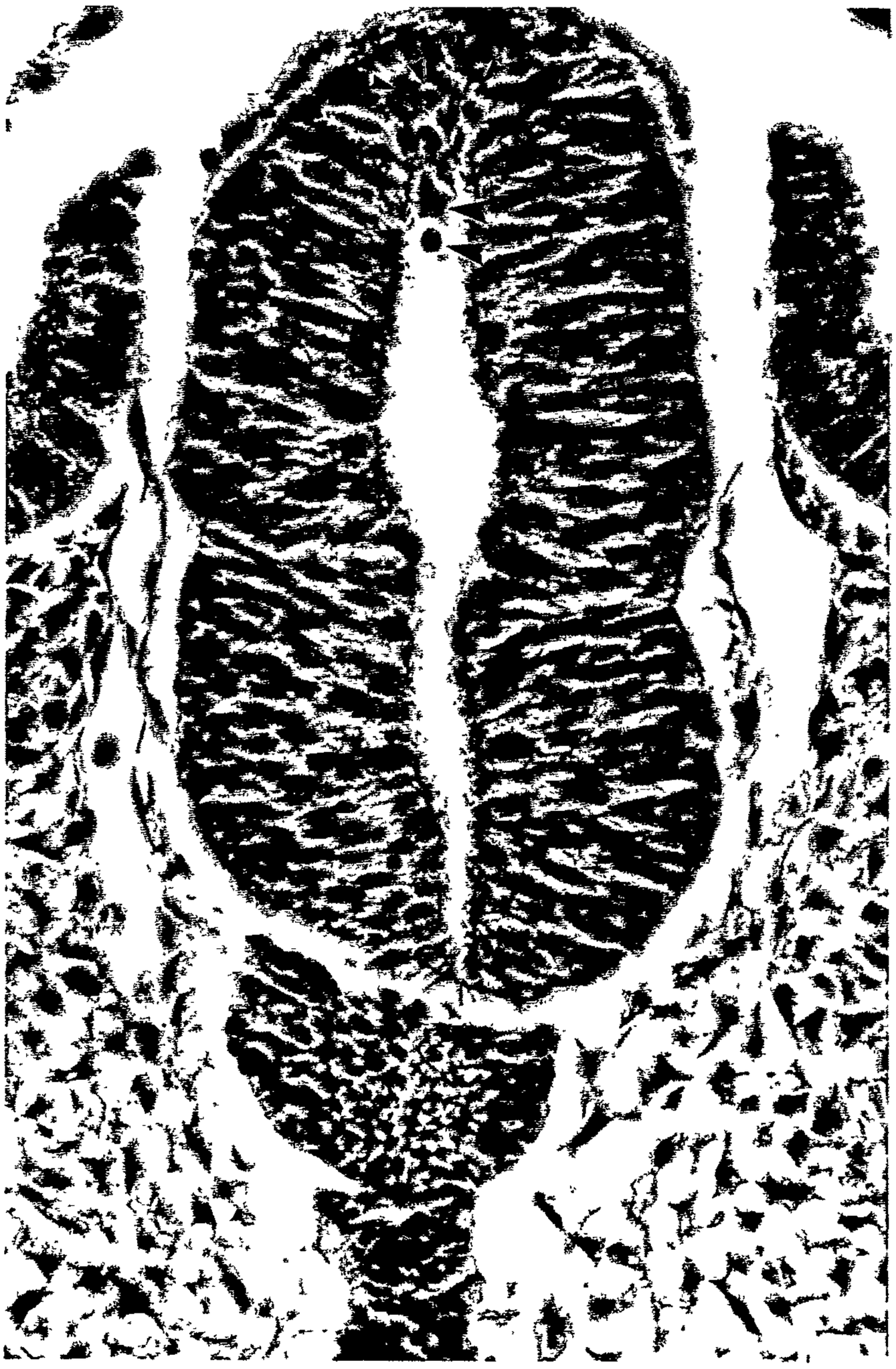


TABLE 5.2: A number of sections studied with closed neural tubes displayed cells hanging from the fusion line into the lumen. This table shows the number and percentage of sections and embryos displaying hanging cells.

	NUMBER OF SECTIONS	NUMBER OF EMBRYOS
CLOSED TUBE	20	12
HANGING CELL	4	3
PERCENTAGE	20%	25%

5.3.2 *IN-SITU* END-LABELLING

Figures 5.11A and 5.11B show negative and positive rat intestine controls for the *in-situ* end-labelling process. The negative controls showed a total absence of labelling. Positive controls were successful when labelling was present at the tips of the villi but the basal cells remain unlabelled.

A preliminary study of 9.5 day paraffin-embedded embryos was carried out in order to gain an understanding of the general level of apoptosis found during early embryonic development. Thirteen 9.5 day embryos were harvested, embedded in paraffin within their embryonic sacs and sectioned. Only one of these specimens was adequately orientated and well enough preserved for the application of the *in-situ* end-labelling technique.

A Haematoxylin and Eosin stained section of this specimen is shown in Figure 5.12A. It shows a head-fold stage embryo that has not yet undergone entypy and folded into the amniotic cavity. At this early stage, there is no evidence of a defined neural plate or craniofacial structures, but the embryo has attained axial and dorso-ventral patterning. The sections shown in Figure 5.12B - 5.12D are low-power (110X) and higher-power (215X and 430X) views of an adjacent serial section to the one in Figure 5.12A. The section has been processed for *in-situ* end-labelling. There is a low level of apoptosis seen throughout the embryo at this stage. The labelled cells appear to be at random distribution with no areas of higher concentration.

Of the seventy-five 10.5 day paraffin-embedded embryos, twelve were considered to have yielded serial sections of adequate quality for the use of the *in-situ* end-labelling technique. These specimens were both of good orientation and at appropriate stages of fusion. The results of the use of this technique in examining specimens at different stages of neural tube closure are summarised in Table 5.3. Only those specimens with successful positive and negative controls have been included in this data.

FIGURES 5.11A AND 5.11B: Sections of paraffin-embedded rat intestine used as negative and positive controls during the *in-situ* end-labelling technique. Figure 5.11B appears on the following page.

FIGURE 5.11A: Cross-section through the villi of the intestine in a negative control for the *in-situ* end-labelling technique. There is no evidence of labelling of the cells being shed from the tips of the villi [215X].

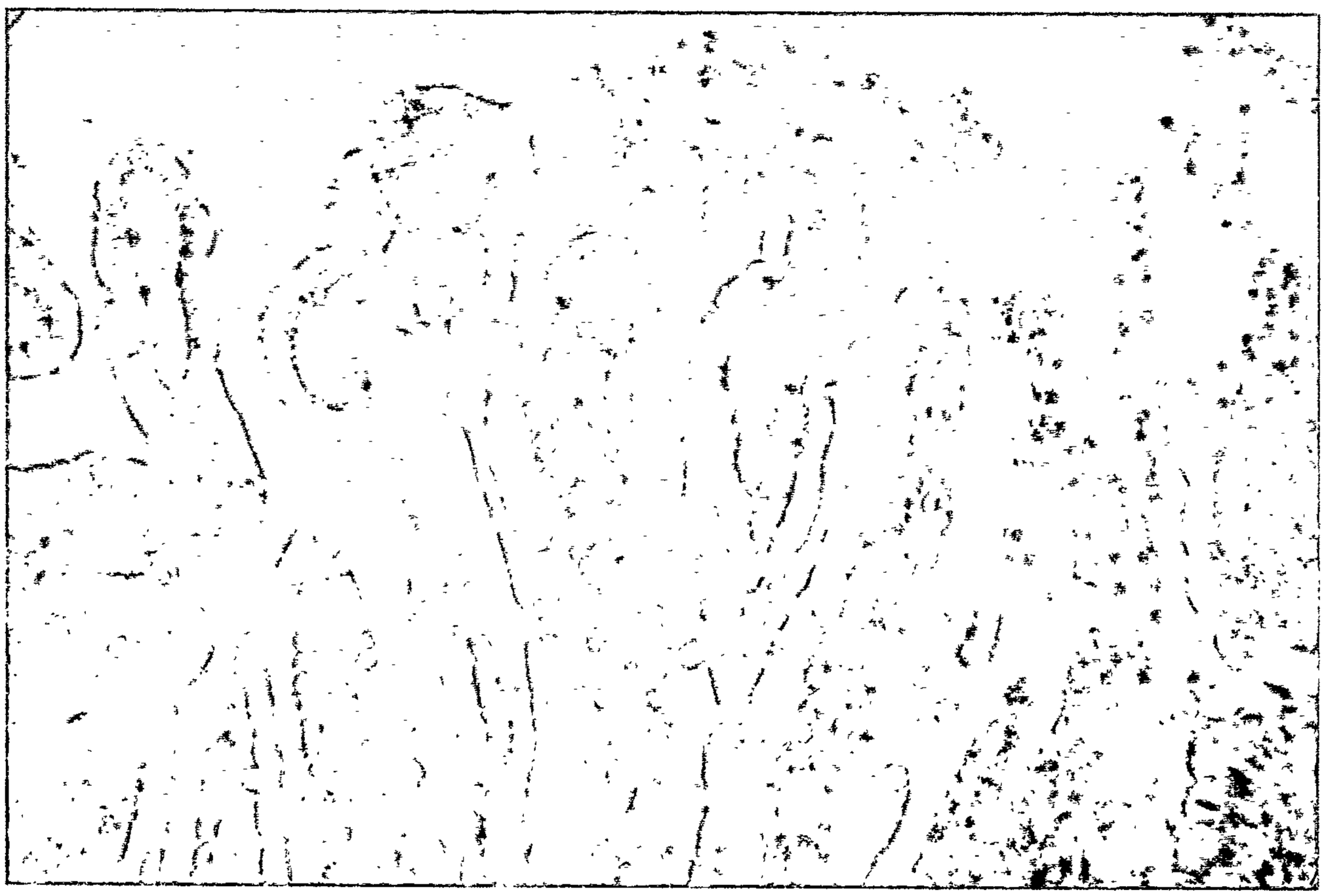
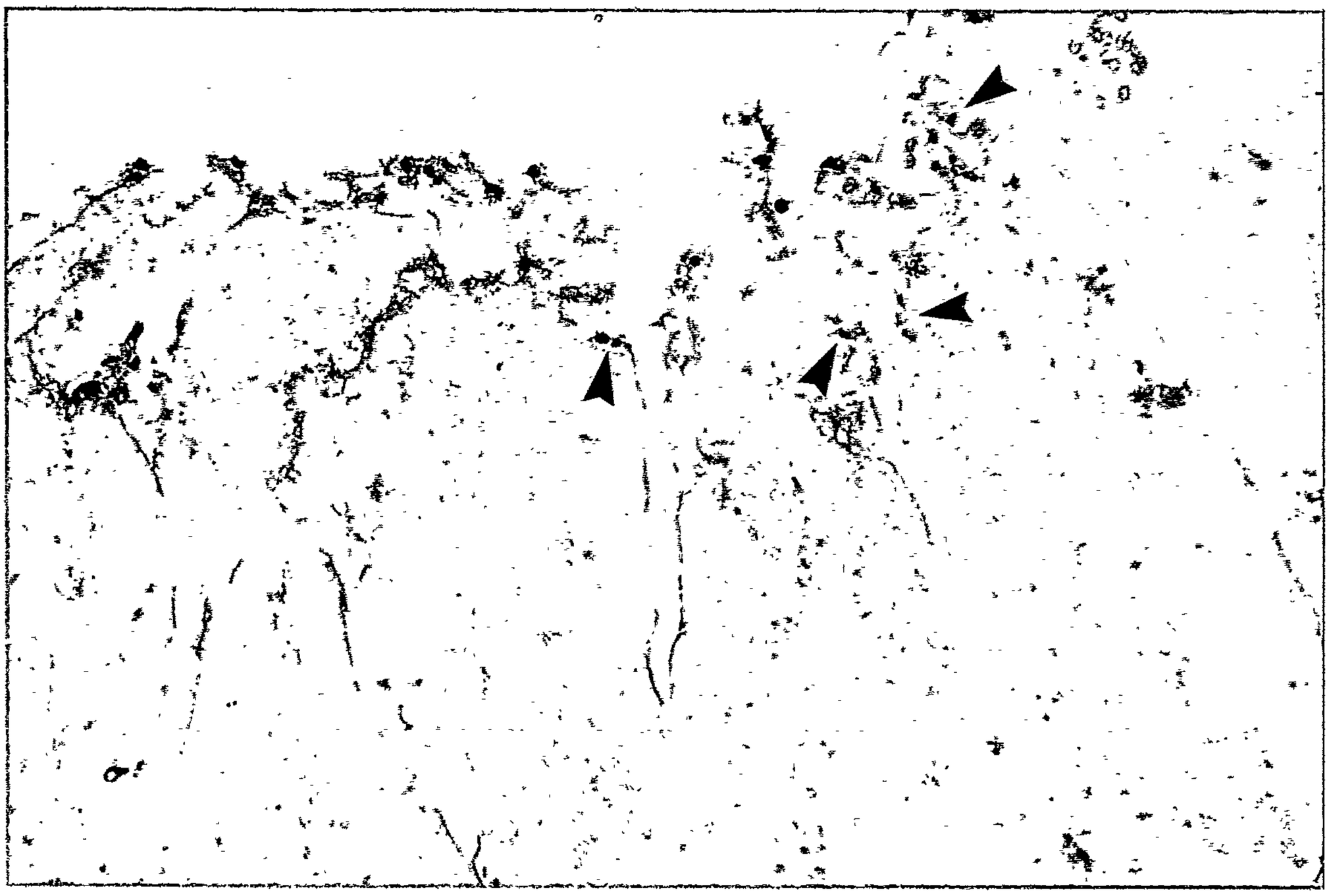


FIGURE 5.11B: Cross-section through the villi of the intestine in a positive control for the *in-situ* end-labelling technique. There are numerous labelled cells at the tips of the villi (arrows). Further down the lateral borders of the villi and in the basal region (not shown), there is no labelling of cells [215X].



FIGURES 5.12A - 5.12D: Cross-sections of a 9.5 day paraffin-embedded rat embryo. This stage of development is the head-fold stage. The embryo has not yet undergone entypy, folding into the amniotic cavity. Figures 5.12B - 5.12D appear on subsequent pages.

FIGURE 5.12A: A Haematoxylin and Eosin stained section showing the neural fold (N) the ectoderm (E), the amniotic cavity (A) and the extra-embryonic coelom (EC) [110X].

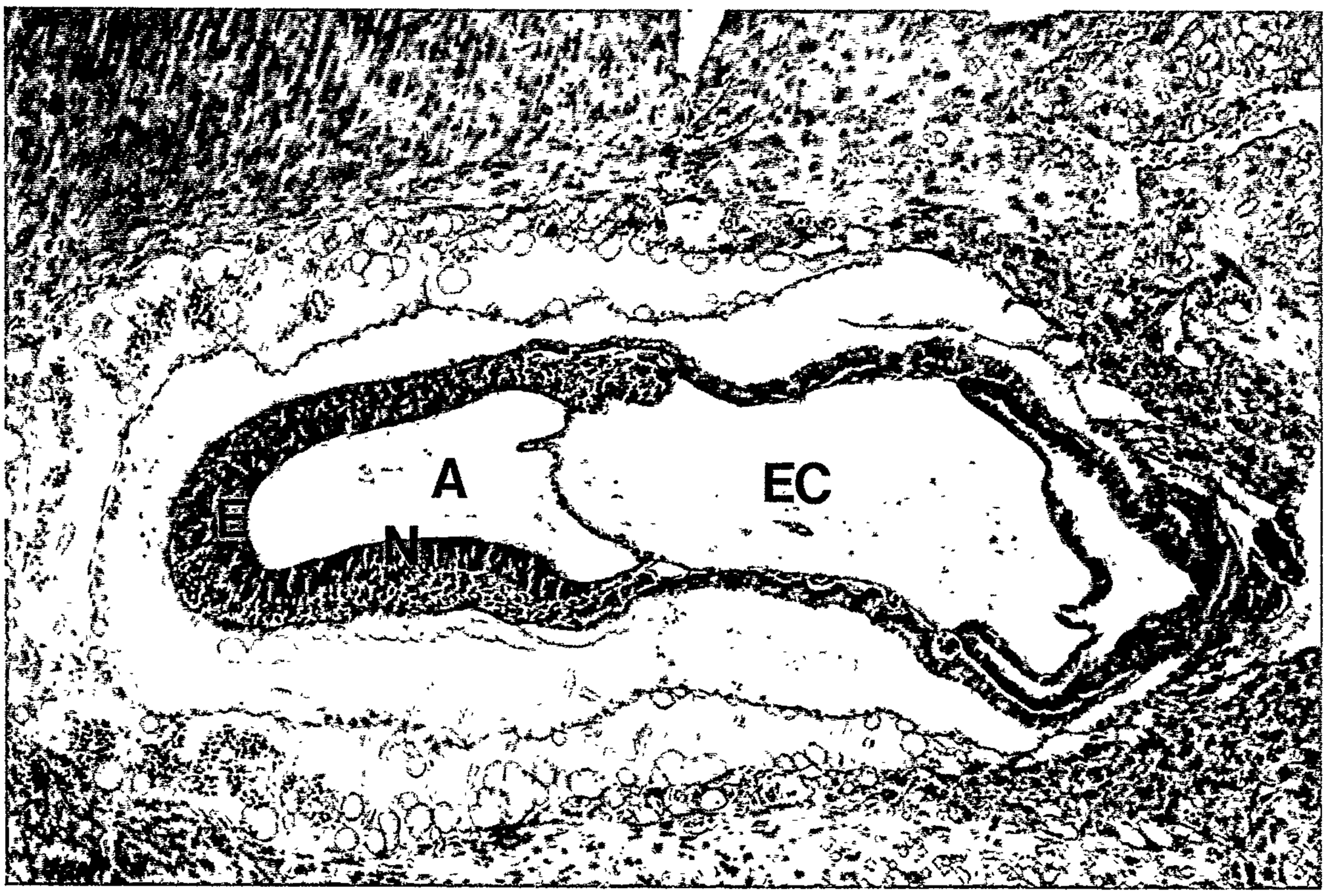


FIGURE 5.12B: A serial section adjacent to the one shown in Figure 5.12A. This section has been labelled by the *in-situ* end-labelling technique to show apoptotic cells. There is a very low level of cell death apparent in the embryo at this stage [110X].

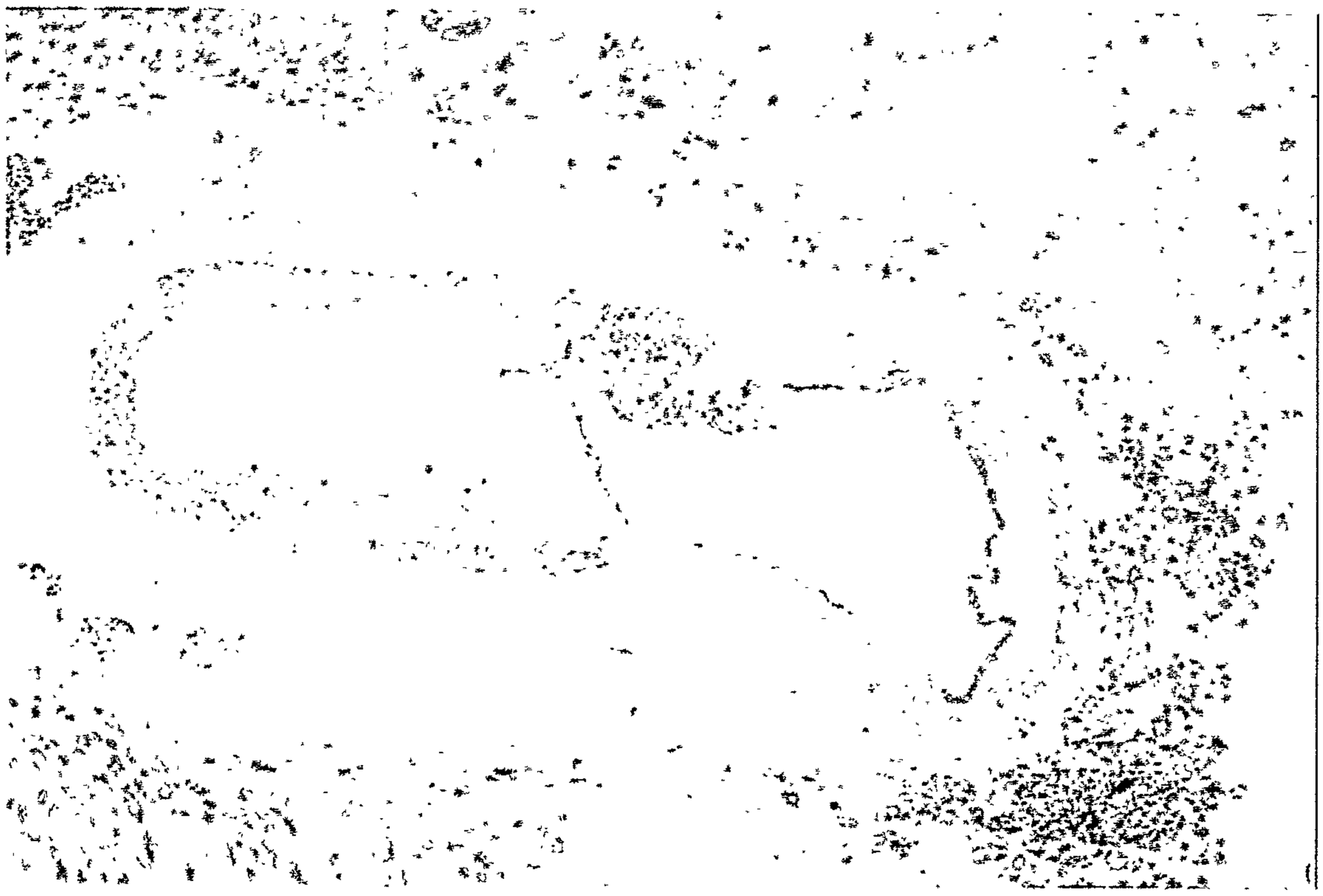


FIGURE 5.12C: The same section as shown in Figure 5.12B. At this magnification it can be seen that there are five labelled cells in the embryonic tissue [215X].

FIGURE 5.12D: At high magnification the discrete nature of the labelled cells within the neural fold area can be seen [430X].

TABLE 5.3: Summary of results of *in-situ* end-labelling, showing total number of sections and stage of closure. Some sections displayed a differential background staining, where the level of staining in the dorsal one-third of the neural folds was higher than in the remainder of the neurectoderm. The number of sections showing this phenomenon can be found in this table.

NUMBER OF SECTIONS	STAGE OF CLOSURE			NUMBER OF SECTIONS SHOWING DIFFERENTIAL BACKGROUND STAINING
	OPEN	APPOSED	CLOSED	
34	11	3	20	10

The bar graphs in Figures 5.13 and 5.14 show the number of *in-situ* end-labelled cells found in the sections studied. The results of the *in-situ* end-labelling technique showed a very low number of labelled cells within the neurectoderm during the stages of fusion of the neural tube (see Figure 5.15B). Most specimens did not show any labelled cells in the neural folds. Of the specimens that did show some labelling, most showed only one or two cells undergoing apoptosis (see Figure 5.15C).

Figure 5.16 shows the section with the highest number of *in-situ* end-labelled cells. There is a low level of background staining present. This section highlights the degree of organisation found within the bridge region of a recently closed neural tube.

A common morphological feature of recently fused neural tubes was the presence of a solitary cell hanging from the fusion line into the lumen of the tube (see Figures 5.10A and 5.10B). Although, prior to the use of the *in-situ* end-labelling technique, it was hypothesised that these cells were undergoing apoptosis, there was not consistent labelling of such cells (see Figure 5.15C and 5.16). Again, from morphological features alone, these cells were suspected to be neural crest cells that had undertaken an inappropriate migratory pathway and had become trapped within the lumen of the recently fused tube.

Throughout the study an interesting staining phenomenon occurred. There was often a low to moderate amount of background staining found throughout the specimen (see Figures 5.17B). This was also found in some of the rat intestine positive controls specimens (see Figure 5.17A), where cells in the basal regions showed moderate staining but were completely distinguishable from the true positive labelling seen in the apoptotic cells at the tips of the villi (see Figure 5.11B). However, Table 5.3 shows that a number of sections studied displayed a differential background staining. In these sections there was an increased level of background staining found in the dorsal one-third of the neural folds compared with the remainder of the neurectoderm. This increased level of background staining did not equate to true positive labelling as seen in apoptotic cells.

FIGURE 5.13: The bar graph below shows the number of closed, apposed and open sections showing labelled cells by the *in-situ* end-labelling technique. It can be seen that the majority of sections did not contain any labelled cells (22/34), a number of sections displayed one apoptotic cell (7/34) and the remaining sections contained two or more labelled cells.

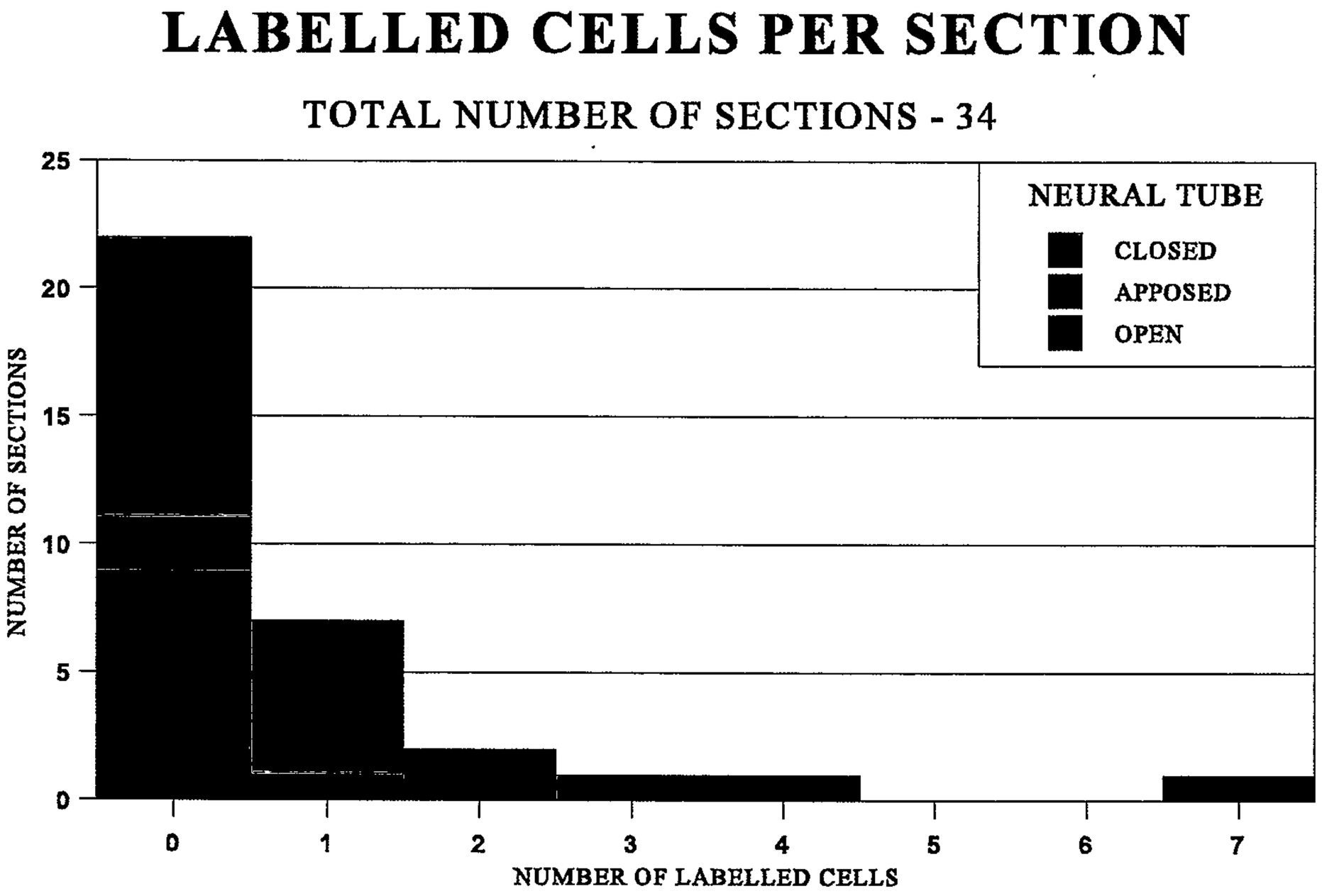
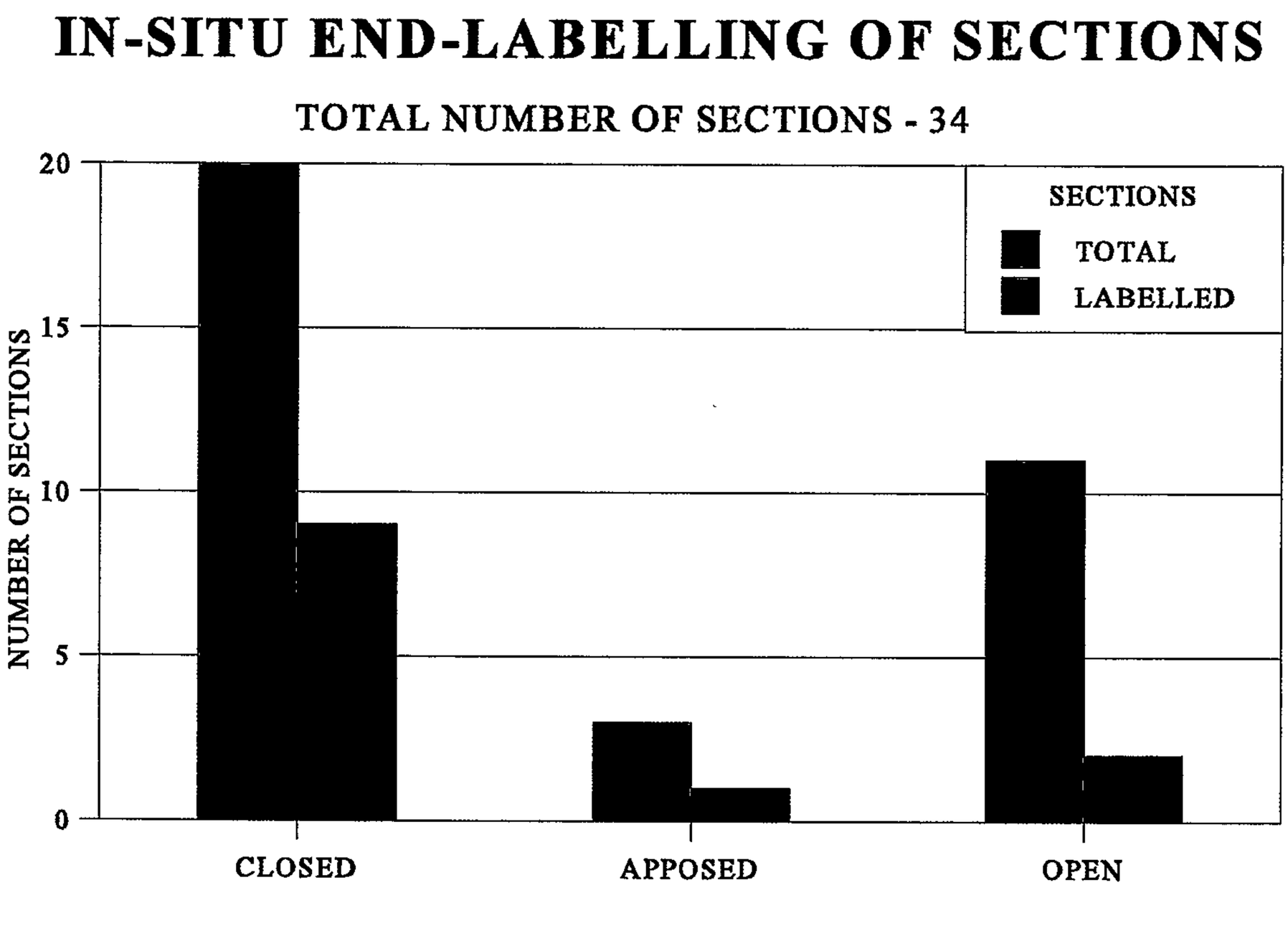


FIGURE 5.14 The bar graph below shows the proportion of closed, apposed and open sections labelled with the *in-situ* end-labelling technique.



FIGURES 5.15A - 5.15C: The *in-situ* end-labelling technique was used to label cells undergoing apoptosis. The following photomicrographs show sections of paraffin embedded 10.5 day rat embryos. Figures 5.15B and 5.15C appear on subsequent pages.

FIGURE 5.15A: This specimen served as a negative control during *in-situ* end-labelling. There is no evidence of labelling of any cells [430X].

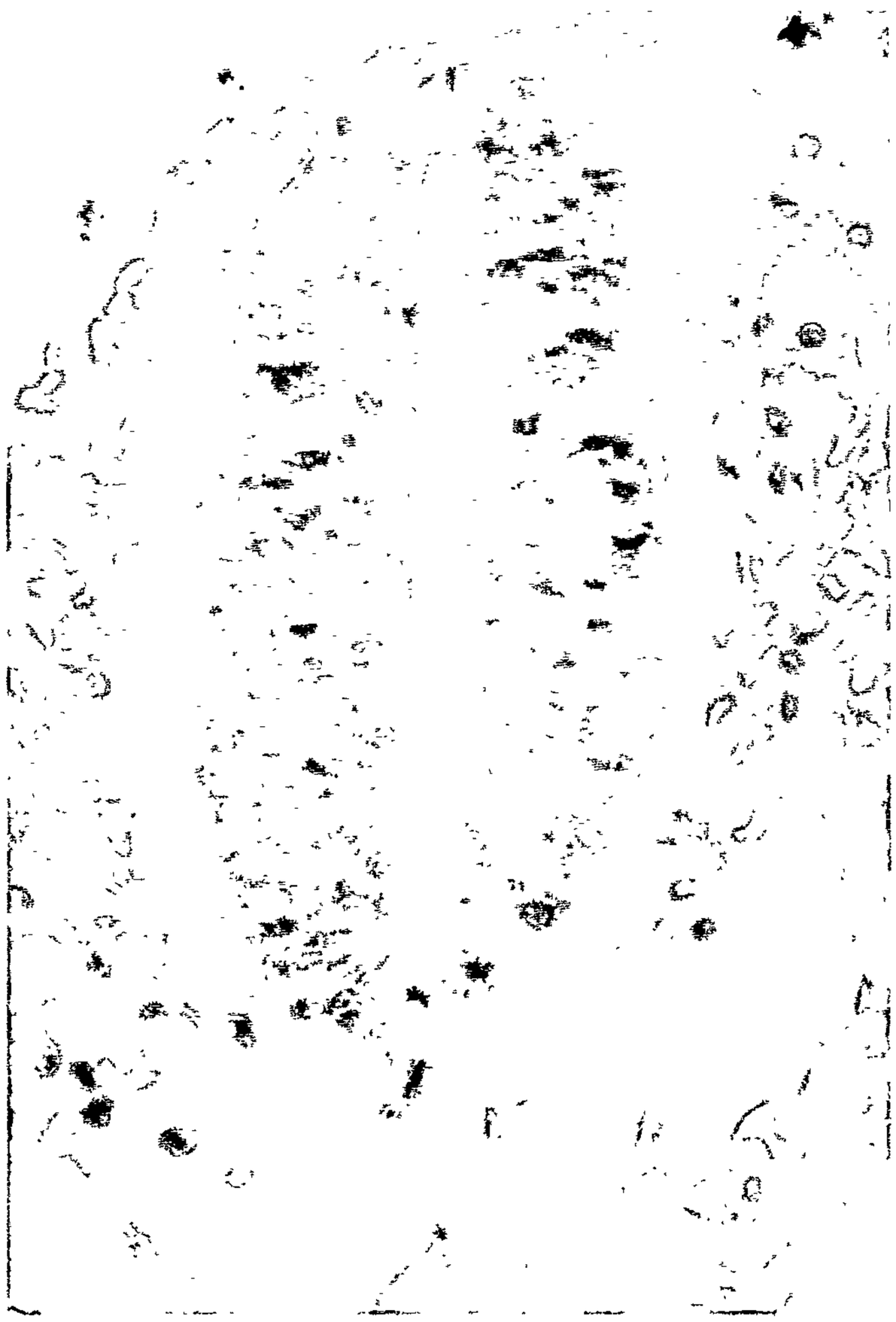


FIGURE 5.15B: An *in-situ* end-labelled section of a paraffin-embedded 10.5 day embryo showing the absence of labelled cells in any other area of the embryo apart from the fusion area of the neural tube [215X].

FIGURE 5.15C: This section is a high-power view of the one shown in Figure 5.15B. Three labelled cells in the area of fusion are indicated by small arrows. The cell hanging from the fusion area into the lumen of the neural tube has not been labelled (large arrow) [430X].

FIGURE 5.16: This photomicrograph shows a section of the same embryo seen in Figure 5.10B. The section has been *in-situ* end-labelled and shows several labelled cells within the fusion area of the recently closed neural tube. This was the maximum number of labelled cells found during the study. Of particular interest are the two cells within the lumen of the neural tube (arrow) which have not been labelled despite the morphological features suggestive of apoptosis [430X].

FIGURE 5.17A AND 5.17B: Sections illustrating a level of background staining found in some series of *in-situ* end-labelled sections. It occurred in both the basal area of the mature rat gut and embryonic tissues but was easily distinguished from positively labelled cells. Figure 5.17B appears on the following page.

FIGURE 5.17A: This section of rat intestine shows a level of background staining of the basal cells using *in-situ* end-labelling. This mild background staining can be easily distinguished from the strongly labelled cells at the tips of the villi seen in Figure 5.11B [430X].

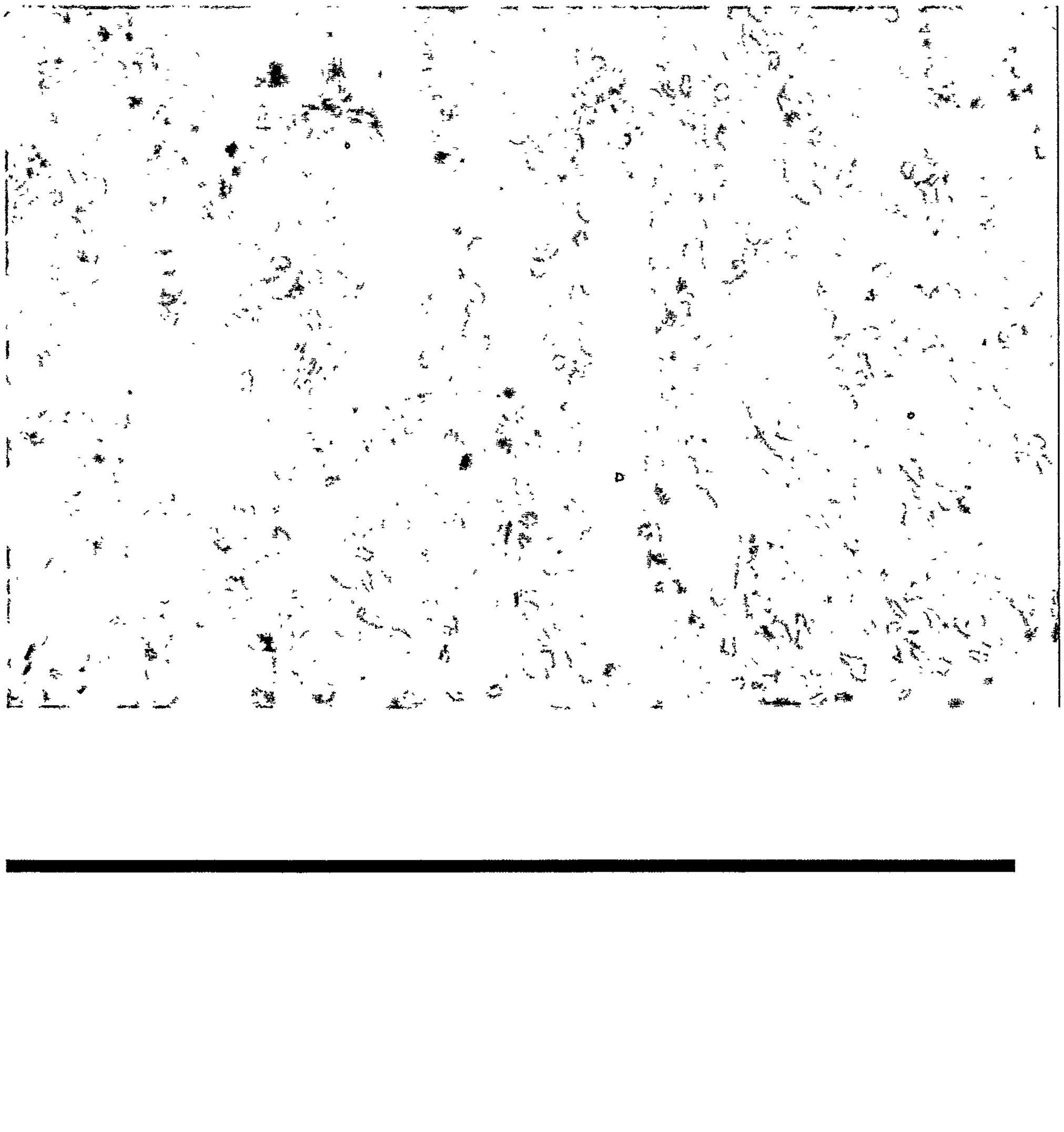
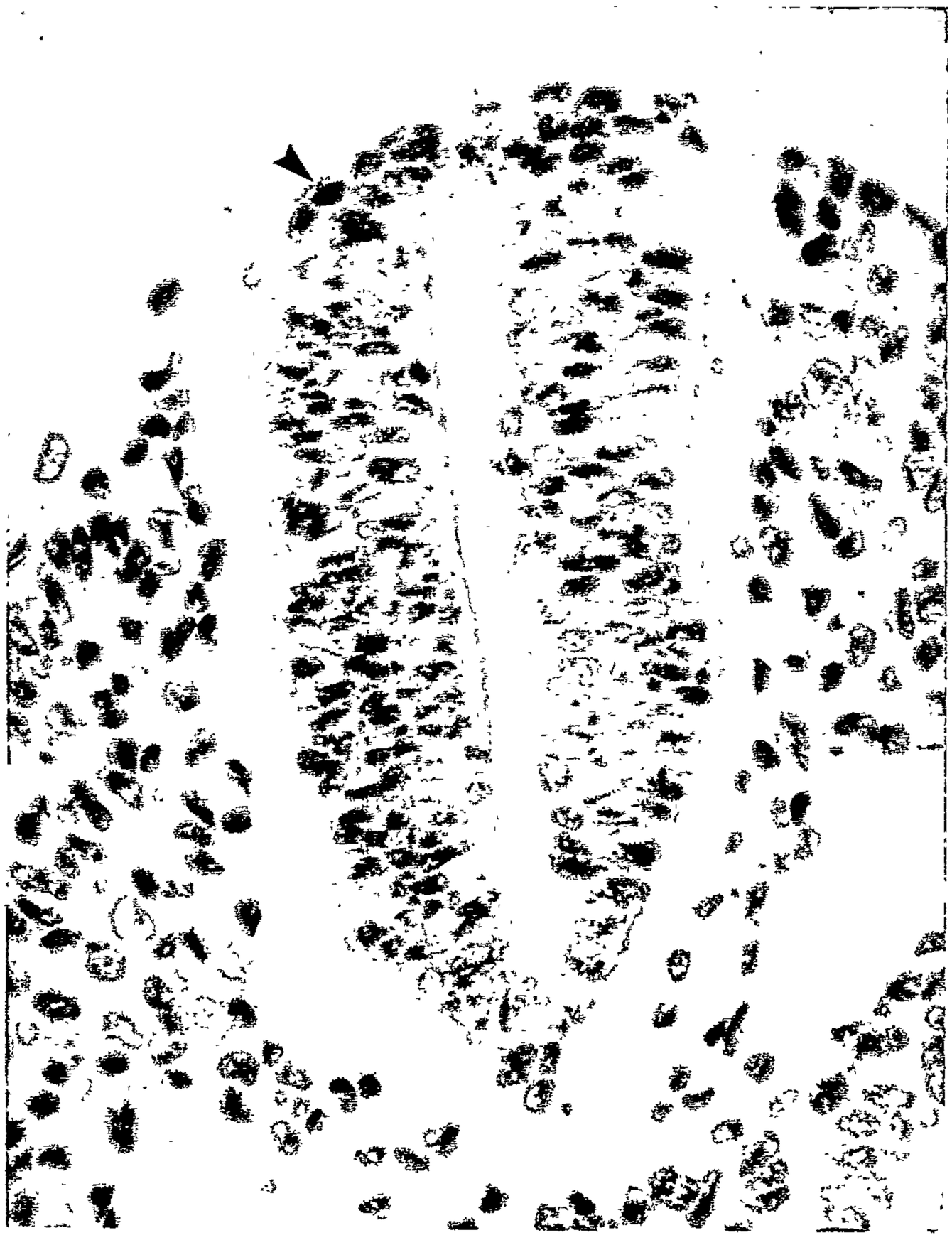


FIGURE 5.17B: This section of neural tube from a 10.5 day embryo was labelled using the *in-situ* end-labelling technique. Although there is a degree of background staining within the neurectoderm and surrounding mesenchyme one positively labelled cell can be differentiated from those exhibiting the low background level of staining (arrow) [430X].



There did not appear to be a correlation between the area of differential background staining found with *in-situ* end-labelling and that found on the PC10 sections. If the differential background staining was due to the labelling of the Okazaki fragments produced during DNA replication, it would be expected that the dorsal one-third of the neural folds would give a more positive PC10 reaction than the rest of the neurectoderm. Although the sample of specimens labelled with PC10 was very small, making it difficult to reach any conclusions, it appears that the differential background staining found in the *in-situ* end-labelling technique was not a phenomenon of highly proliferative cells.

5.3.3 PHENOTYPIC MARKERS

The use of monoclonal antibodies to S_{100}, Keratin 8 and Vimentin were trialed. It was hoped that these immunohistochemical markers would provide valuable information regarding the phenotype of cells that were intimately involved with the fusion process. It is known that cells of the neural crest begin their migration during the time in which the neural folds appose and commence the fusion process. Without the aid of a phenotypic marker, it is impossible to distinguish between cells committed to becoming neural crest cells and those that will remain within the neurectoderm.

The trials with the phenotypic markers were equivocal giving no strong differentiation between cells of ectodermal, mesenchymal and neural crest origin (see Figures 5.18A to 5.18E). As it was felt that these markers would not be beneficial in the study of the fusion process, the use of phenotypic markers was not extended beyond an initial trial.

FIGURES 5.18A - 5.18E: These photomicrographs show the trials using immunohistochemical markers to differentiate between cells of ectodermal, mesenchymal and neural crest phenotype. The sections used show an open neural tube from a paraffin-embedded 10.5 day embryo. Figures 5.18B - 5.18E appear on subsequent pages.

FIGURE 5.18A: This specimen has a routine Haematoxylin and Eosin stain used as a reference point for the following figures [430X].

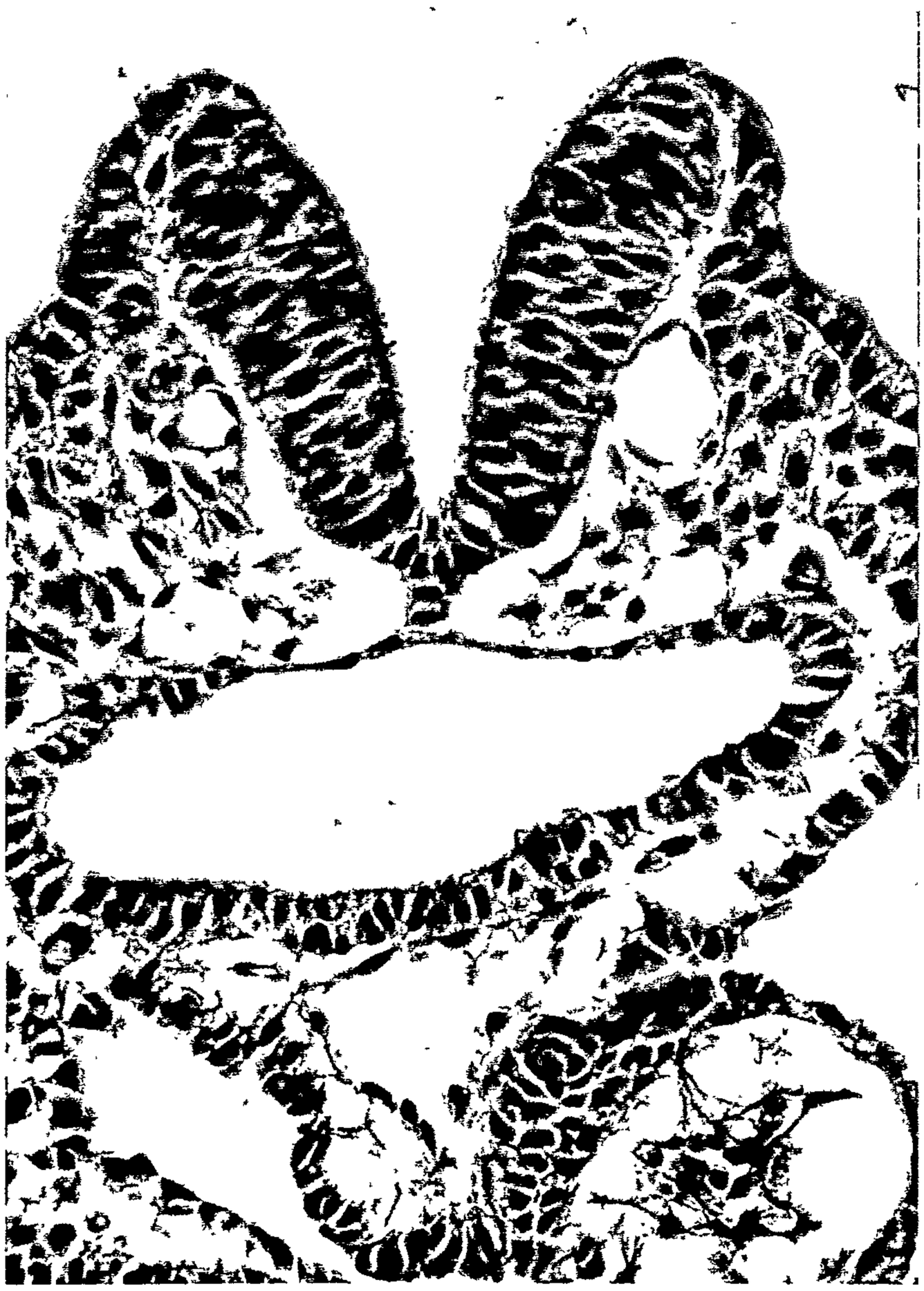
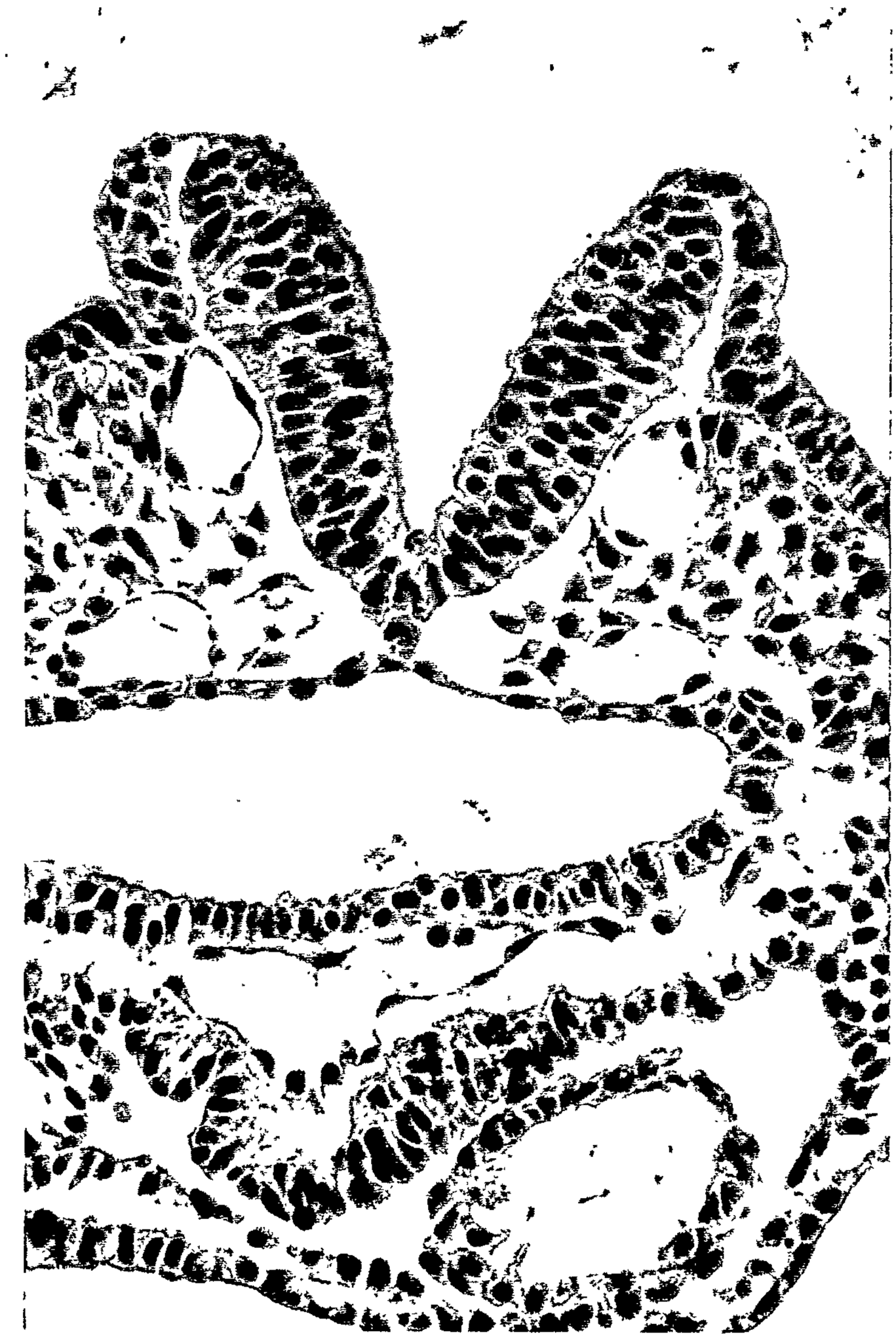


FIGURE 5.18B: This section of neural fold served as a negative control for the sections undergoing immunohistochemical marking for cells of ectodermal, mesenchymal and neural crest origin. An isotype-matched irrelevant monoclonal antibody was substituted for the primary antibody [430X].

FIGURE 5.18C: A section stained with the immunohistochemical marker for cells of ectodermal origin (Monoclonal antibody to Keratin 8). The arrows show the positive cell marking of the ectodermal and neurectodermal cells. However, the mesenchymal cells also appear to show some staining [430X].

FIGURE 5.18D: This section has been labelled with the immunohistochemical marker for cells of mesenchymal origin (Monoclonal antibody to Vimentin). This labelling seems to be non-specific with most cells throughout the section displaying marking compared with the negative control in 5.18B [430X].

FIGURE 5.18E: This specimen has been labelled with the immunohistochemical marker for cells of neural crest origin (Monoclonal antibody to S_{100}). There is no distinct marking of any cells in this section compared with the negative control seen in Figure 5.18B [430X].



5.4 DISCUSSION

The morphological findings in this study in relation to fusion of the neural folds have not been previously described. Although there has been a considerable amount of research into the process of neurulation, to date the work has focused on the bending and shaping of the neural folds and the final adherence and fusion has received comparatively little attention. Due to the difficulties in the preparation of embryos and the application of immunohistochemical and *in-situ* end-labelling techniques, the amount of data available for examination in this study is limited. Despite these difficulties it has been possible to make observations of the fusion process at a cellular level and arrive at a more comprehensive description of the process.

During folding of the neural plate there is a high level of cell proliferation within the neurectoderm as seen from the use of PC10 labelling (see Figure 5.8). This cell proliferation appears to occur throughout the neurectoderm, including the tips of the neural folds. Following apposition of the neural folds, a bridge of cells one to two cell layers thick forms. It is not known how these cells achieve initial adhesion, although it has been suggested that it involves the synthesis of cell surface glycoconjugates (Takahashi and Howes, 1986; Takahashi 1988, 1992).

During the process of initial fusion, neural crest cells were found in the cell-free zone. Other cells appeared to be losing adhesion to the surrounding neurectoderm, suggestive of pre-migratory neural crest cells. There is no complete understanding of the role of this population in the fusion of the neural folds. However, there is an intimate relation of these cells with the neurectoderm during the fusion process.

The level of cell death in the neural folds during fusion was very low. A significant finding of this study was the absence of cell death in any other areas of the neurectoderm apart from the tips of the fusing neural folds. However, due to the difficulties in using immunohistochemical markers, it was not possible to identify the phenotype of those cells undergoing apoptosis.

Without the use of such phenotypic markers it is impossible to know if the labelled cells were of neural crest or neurectodermal origin. However, from observation of the morphological characteristics of the labelled cells it seems likely that these cells are most often neural crest cells.

It has been reported that cranial neural crest cells from rhombomeres r3 and r5 undergo apoptosis leading to the unique patterning of neural crest cell migration from these regions (Jeffs *et al.*, 1992; Graham *et al.*, 1993). However, throughout the caudal neural tube there is no such patterning and neural crest cells emerge uniformly from the neural folds at all axial levels. Any cell death seen in areas of the caudal neural tube may, therefore, represent a regulation process whereby neural crest cells are eliminated if they become trapped within the fusing neural folds, are surplus or embark on an inappropriate migratory pathway.

A frequent observation found in recently fused neural folds, was the presence of one or two cells hanging from the fusion line into the lumen of the neural tube. Although, it was expected that these cells would be undergoing apoptosis, the use of the *in-situ* end-labelling technique did not give consistent labelling of these cells. As it is not necessary for cells of the neurectoderm to undergo cell death in order to achieve fusion, it is hypothesised that the cells found hanging from the fusion line represented neural crest cells trapped within the fusing neural folds.

The *in-situ* end-labelling technique is based on the detection of DNA strand breaks which are abundantly present in apoptotic cells. The Klenow fragment of DNA polymerase adds nucleotides to 3'-hydroxyl ends of a DNA strand in the presence of a template, extending the strand in the 5' to 3' direction. Although, the exact type of cleavage in the internucleosomal linker DNA of apoptotic cells remains unknown, it is believed to involve the presence of recessed 3'-hydroxyl termini thus allowing for the detection of apoptosis by the *in-situ* end-labelling technique. The detection of other types of DNA breaks by this technique, for example, occurring during necrosis and DNA replication, although theoretically possible does not seem to interfere with the identification of individual cells undergoing apoptosis. During necrosis, tracts of contiguous cells are affected, making this process morphologically distinguishable from apoptosis.

The hypothesis that non-linked DNA fragments (Okazaki fragments) present during DNA replication might be labelled by the end-labelling technique has also been considered. However, Gold *et al.* (1993) investigated this by combining cell proliferation studies using 5-bromo-2-deoxyuridine (BrDU) immunocytochemistry and an end-labelling technique on a myeloma cell line. The results showed clearly that although the entire cell population entered S-phase at least once within the 24 hour period as seen by BrDU immunocytochemistry, the cells remained completely negative using the end-labelling technique. Thus replicating cells should not be detected by the *in-situ* end-labelling technique.

There is presently no explanation for the observed differential background staining found in this study. Wijsman *et al.* (1993) discussed a phenomenon of cytoplasmic staining of apoptotic cells following *in-situ* end-labelling of rat and human tissues. It was unclear whether this was a true representation of DNA fragments leaking out of the nucleus or an artefact resulting from tissue processing as it only occurred in apoptotic cells and therefore appeared to be unrelated to the background staining observed in this study.

The findings of this study are significant in the context of gaining a better understanding of the processes that lead to fusion of the neural folds. While it has not been possible to address all aspects of this complex developmental mechanism, some points are now clear. The level of cell death within the neurectoderm and in fact throughout the embryo is very low during the time of closure of the neural tube. Conversely, all areas of the neurectoderm display a high level of proliferation during the same stage. Initial fusion of the neural folds is achieved by the formation of a bridge of cells, one to two cell layers thick. During apposition and fusion of the neural folds, neural crest cells can be observed losing their adhesion to cells of the neurectoderm and beginning a migratory pathway through the cell-free zone. The surface ectoderm from each neural fold joins in the midline after it is drawn into apposition by the neural folds. Confluence of this single-layer occurs simultaneously with fusion of the neural folds.

Many details of the fusion process remain unexplained at this time. The adhesion of cells of both the neurectoderm and surface ectoderm of the fusing neural folds is as yet undefined. The interaction between the pre-migratory neural crest cells and the neurectodermal cells during this time is not understood. It seems likely that this population of specialised cells has an influence on the events leading to fusion, due to the intimate spatial and temporal relation between neural crest cell migration and neural tube closure. However, whether or not these cells undergo apoptosis if trapped within the fusing neural folds and whether or not failure of apoptosis of such cells is implicated in neural tube defects is not known.

Closure of the neural tube represents the first example of fusion in embryology. The comparison of this process to other subsequent fusion processes may or may not be applicable. While an understanding of the mechanisms of cell adhesion in fusion of the neural folds may lead to a similar understanding in other fusion processes, for example palatal fusion, other aspects of fusion may be quite unique to each individual process.

The fusion of the palatal shelves can only be accomplished following the disappearance of the medial edge epithelium, so it is reasonable to believe this may, at least in part, be brought about by apoptosis. On the other hand, while the neural folds do not have a defined cellular layer or basal lamina interposed between the presumptive fusion areas that needs to be disrupted prior to fusion, the consequences of having neural crest cells in this area is not understood. It may be necessary for those neural crest cells trapped within the fusion line to undergo apoptosis in order to achieve confluence of the neurectoderm, but whether or not these cells often become trapped, making it a programmed event or whether it represents the ability of the embryo to regulate the process as required is not known.

6

GENERAL DISCUSSION

Although the study of embryological fusion processes began over a century ago, there is still much to be learned. Despite the power of the molecular biological techniques available in recent times, there are limitations to the information that can be gained from studies, *in vitro*. Results from studies such a this must be critically analysed, keeping in mind that all conclusions are drawn from the observation of an delicate embryo that has undergone extensive manipulation and been exposed to harsh histological techniques. Further, the closure of the neural tube has been studied spatially not temporally, that is, the closure process has been investigated by studying a section of neural tube that is open in one area and closed in another. While it is hoped that this method of study leads to an accurate description, visualisation of the process, *in vivo*, would provide the only true answer. If the advances in technology during the past decade are an indication of things to come, it is not unreasonable to believe that such a study will be possible.

Despite the limitations of the techniques used in this morphological study there are several important findings. A very low level of apoptotic cell death was found throughout the embryo over the period of development studied. As expected, during a time of such immense growth and development, the rate of cell proliferation must exceed the rate of cell death. Whether or not the limited apoptosis found in the 9.5 day embryo is truly programmed, as in *C. elegans*, was unable to be determined because of the small sample studied. In the mammalian model, it seems unlikely that the same cells in every embryo would undergo apoptosis at the same time in development. A more likely explanation of the observed apoptosis would be that it represents the suicidal death of cells in surplus of the requirements at any given spatial and temporal position.

In the same way, the sparse apoptosis found in the fusion area of the neural tube of the 10.5 day embryos did not seem to be programmed in nature. Instead, it appeared to occur as part of a regulation process allowing organisation of the original bridge of cells into a consolidated area of neurectoderm. The role of the presumptive neural crest cells during the process of neural tube closure remains to be understood.

The similarities and differences between this original fusion process and that of a subsequent embryological fusion process such a palatogenesis also remain to be detailed. A major similarity between these two events is that both are frequently perturbed resulting in severe malformations while the other craniofacial processes consistently obtain fusion and confluence. Other similarities may include the forces required for bending of the processes into a position of apposition and the molecular structure of the initial adhesion of the apposed processes. An obvious difference between the two events is that the neural folds consist of neurectodermal tissue only, while the palatal processes are formed by two different tissues, epithelium and mesenchyme. It is therefore mandatory in the palate for the medial edge epithelium to disintegrate to allow mesenchymal confluence.

Why these two events are frequently perturbed while others are spared is an interesting question. Perhaps the answer lies in the regulation of apoptosis. It is known that this process of cell death has a role to play in many embryological events including the breakdown of cells in the interdigital spaces during digit formation. This is another event in development that is frequently perturbed leading to syndactyly, a soft tissue fusion of the digits. So it appears that events requiring the apoptotic breakdown of cells are often perturbed during development. In this context, the progenitor neural crest cells in the area of neural tube fusion could act in a similar manner to cells of the medial edge epithelium of the palate and the cells in the interdigital spaces. That is neural tube closure could depend on selected apoptosis of neural crest precursors concentrated in the zone of fusion.

Further evidence of the role of the neural crest progenitors in closure of the neural tube can only result once there is a reliable marker for these cells. While S_{100} is expressed in cells of neural crest origin, like pigment cells, it apparently is not expressed in the presumptive neural crest cells that lie within the neurectoderm. If the neural crest cells within the neurectoderm could be reliably marked and observed during fusion of the neural folds it may become evident that these cells do, in fact, act in a similar way to the cells of the medial edge epithelium and that a failure of apoptosis at the correct time and place results in neural tube defects.

The current study has utilised the caudal neural tube because of its ease of orientation. However, it is not known whether these observations correlate with the closure of the neural tube in cranial regions. In particular, the hindbrain with its complex axial segmentation and areas of known apoptosis of neural crest cells in specific rhombomeres, may display a completely different pattern of events from those of the caudal neural tube.

Further answers to the questions will only be found by continuing the study and increasing the number of embryos examined. With the improved success rate in correct orientation by dissecting embryos from their embryonic sacs prior to embedding, a further 100 embryos should provide an adequate number specimens for analysis. Employing a computerised digitiser to plot the location of *in-situ* end-labelled cells within the neural tube may allow a better assessment of whether the labelled cells are always localised to a particular area within the fusing neural folds. Once there is a clear picture of the events of closure of the caudal neural tube, the cranial region should also be studied in order to document the similarities and differences along the rostrocaudal axis.

Bcl-2 protein functions as a death repressor molecule in the apoptotic death pathway. The use of specific antibodies to Bcl-2 to study the pattern of *bcl-2* expression in the cells of the fusing neural folds would provide additional information about the role of cell death during closure of the neural tube and allude to whether cell death plays an integral part of the fusion process or whether it is merely a mechanism of regulation.

Ultrastructural studies are also expected to provide information about the fusion process. Using the transmission electron microscope the nature of the initial adhesion of cells of both the surface ectoderm and the neurectoderm may be found. In combination with immunohistochemical markers for products such as Bcl-2 and *in-situ* end-labelling techniques, a better understanding of the events of fusion at a cellular level, can be gained.

Confocal scanning microscopy, a relatively new optical microscopic technique, offers significant advantages over conventional microscopy (Shotton, 1989). Not only does it give improved resolution and enhanced contrast but most importantly, it allows the optical sectioning of living specimens obviating the necessity for chemical fixation. Thus it confers the ability to study temporal phenomena by repetitive observation during time-course studies. Future studies of the closure of the neural tube and other fusion events may be able to employ organ culture with vital dye staining, in combination with confocal scanning microscopy thus allowing a direct observation of the events in their correct spatial and temporal relationship.

The processes of neural tube closure and neural crest migration are fundamental to the study of craniofacial development. Perturbations of either of these events result in craniofacial malformations that range from mild clefting to severe anomalies, incompatible with life. The current study has investigated the events involved in fusion of the neural folds during normal neurulation in an attempt to gain a better understanding of this process. From the observations made it is postulated that the regulation of apoptosis in the population of neural crest progenitors within the fusing neural folds is a central event in normal neurulation and neural crest migration. Events leading to the disturbance of normal apoptosis in this area may therefore be responsible for the disruptions that result in craniofacial malformations.

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