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# STUDIES ON THE FORMATION AND MIGRATION OF THE PRONEPHRIC RUDIMENT IN THE MEXICAN AXOLOTL

by

Laura Gillespie

A thesis
presented to the University of Ottawa
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in
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The University of Ottawa requires the signatures of all persons using or photocopying this thesis. Please sign below, and give address and date.

To Mom and Dad

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## ABSTRACT

This study was undertaken to examine the origin of the pronephric rudiment in the mexican axolotl and to investigate the properties of the guidance mechanism that directs the migrating tip of the rudiment to the cloaca.

The formation of the presumptive pronephros and duct was studied by scanning electron microscopy and in histological sections. Unlike avian embryos where the nephric system is derived from the intermediate mesoderm (Balfour, 1876), our observations indicate that the presumptive pronephros in the axolotl is derived from the posterio-lateral region of somite 2 and the lateral region of somites 3 and 4. In fact, a clearly defined intermediate mesoderm does not exist in the axolotl. The duct, on the other hand, appears to originate from the lateral mesoderm, as suggested by early studies in other urodelan species (Mollier, 1890; Field, 1891; O'Connor, 1938).

In order to obtain information about the guidance mechanism for duct migration, the role played by the tissues of the duct path, namely the lateral mesoderm, the somitic mesoderm and the epidermis, was examined.

The role of the mesoderm in duct migration was investigated by experimental and morphological studies. Transplantations of secondary ducts to various positions posterior to the primary duct tip have demonstrated that only a small area of the lateral mesoderm, the active

region, is competent to support duct migration at any one time. This **active** region is about 2 somites in width and moves craniocaudally through the mesoderm in register with duct migration and somite segmentation. The anterior boundary of this region, the **post-migration** wave, corresponds to the wave of guidance information previously described by Poole and Steinberg (1982). The posterior boundary, is located immediately posterior to the last segmentation furrow and has, therefore, been named the **segmental** wave.

The presence of a third wave, the **pre-segmental** wave, that travels craniocaudally through the mesoderm was brought into evidence by briefly exposing the mesoderm to trypsin. This treatment was found to disrupt somite segmentation in a localized region and the pronephric duct was unable to migrate through this region. The affected area, consisting of 3-5 somites, moves in synchrony with, and about 3.5 somite widths ahead of segmentation, thus placing it approximately 3.5 somite widths ahead of the **segmental** wave. The trypsin sensitivity of the duct path was found to be calcium-dependent whereas that of the somitic mesoderm was not.

Examination of the lateral mesodermal cells, both histologically and in the scanning electron microscope, at several different developmental stages revealed a series of morphological changes that takes place in cells at the same anterio-posterior (A-P) level as the duct tip. These

morphological changes were found to correspond spatio-temporally to the post-migration wave.

By transplanting the epidermis in reverse A-P, D-V or A-P and D-V orientation, it was shown that this tissue is not directly involved in guiding the duct, but can affect its ability to migrate. The effects of the transplanted epidermis depended on the embryonic stage of donor and host, suggesting that spatio-temporal dependent changes are also taking place in the epidermis.

During duct migration, the subepidermal space was found to contain a thick layer of extracellular matrix (ECM) that ended abruptly at the anterior end of the last formed somite. Thus, the spatio-temporal distribution of this material parallels that of somite segmentation and pronephric duct migration. The posterior limit of ECM did not, however, coincide with either post-migration or segmental waves.

The importance of cell surface proteins for duct migration was shown by treating embryos with trypsin and tunicamycin. As mentioned above, pronephric duct migration was inhibited after treatment with trypsin. This inhibition was show to be calcium-dependent. Injection of tunicamycin during gastrulation also led to inhibition of duct migration. In this case, however, the duct deviated laterally from its normal path before migration was arrested.

It can be concluded from these studies that the duct

guidance information is contained within the lateral and somitic mesoderm, and not the overlying epidermis. The guidance information can be further localized to a narrow region of about two somite widths and this information moves craniocaudally in a wave-like fashion. In addition, duct migration depends on the developmentally regulated production of cell surface proteins; these proteins could be either glycoproteins of proteoglycans of the cell membrane or ECM.

## RESUME

Le but de cette étude était d'examiner la genèse de l'ébauche pronéphrique chez l'axolotl et de faire une enquête des facteurs qui dirigent la migration de l'uretère primaire.

L'étude de la formation de l'ébauche pronéphrique a été faite en utilisant le microscope électronique à balayage ainsi que des coupes histologiques. Contrairement à ce que l'on trouve chez le poulet, le mésoderme intermédiaire n'existe pas chez l'axolotl. Plutôt, le pronéphros provient de la région latérale du mésoderme somitique et l'uretère primaire tire son origine du mésoderme latéral. Cette observation est d'accord avec les conclusions tirées d'études antérieures (Mollier, 1890; Field, 1891; O'Connor, 1938).

Le rôle joué par chacun des tissus associés avec la voie suivie par l'uretère primaire a été étudié. En ce qui concerne le mésoderme, des études expérimentales et morphologiques ont été faites. Des uretères supplémentaires ont été greffés à divers positions postérieures au primaire. Les résultats de cette expérience ont démontré que seulement une région très particulière, la région active, peut entretenir la migration des cellules de l'uretère. Cette région active a la largeur d'environ deux somites et se déplace dans la direction caudale en correspondence avec la migration de l'uretère et la segmentation des somites. La

limite antérieure de cette région, la vague **post-migration**, correspond a la vague décrite par Poole et Steinberg (1982). La limite postérieure est située tout auprès du dernier sillion de segmentation, donc le nom vague **segmentale**.

Il y a aussi une troisième vague, la vague **pre-segmentale**, qui a été démontrée par un traitement de trypsine. Ce traitement désorganisa la segmentation des somites dans une région particulière et l'uretère ne pouva pas traverser cette région. La région affectée comprend 3 à 5 somites et se déplace en advance de la segmentation. Cette vague **pre-segmentale** se trouve à 3.5 largeurs de somite postérieure à la vague **segmentale**. L'effet sur le mésoderme somitique était indépendent de la presence de calcium tandis que la migration de l'uretère fut affectée seulement en absence de calcium.

La morphologie des cellules du mésoderme latéral a été examinée en utilisant le microscope électronique à balayage ainsi que des coupes histologiques, à divers stades développementals. Les cellules situées près de l'extrémité de l'uretère subient des changements morphologiques qui correspondent avec la vague **post-migration**.

Il a été démontré par des greffes d'épiderme que ce tissu n'est pas impliqué dans le mécanisme par laquelle l'uretère se dirige vers sa destination, mais peut empêcher la migration.

La distribution du matériel extracellulaire (MEC) a été examinée à différents stades embryonnaires. L'espace sous

l'épiderme est remplie de MEC qui abouti toujours à la limite antérieure de la dernière somite. Donc, la production de ce matériel suit la segmentation des somites ainsi que la migration de l'uretère.

Des traitements d'embryons avec la trypsine et le tunicamycin ont démontré l'importance des protéines à la surface des cellules pour la migration de l'uretère. L'effet du tunicamycin est différent de celui du trypsine car l'uretère s'écarte de la voie préférée avant de s'immobiliser.

En conclusion, le mésoderme latéral et somitique et non l'épiderme est responsible pour diriger la migration de l'uretère. Seulement une région mesurant la largeur de 2 somites peut supporter la migration; cette région se déplace en direction caudale. Cette étude a aussi démontré l'importance des protéines qui se trouvent soit dans la membrane cellulaire ou dans le MEC.

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## ABBREVIATIONS

| | |
|---|---|
| A-P | anterio-posterior |
| $CaCl_2$ | calcium chloride |
| CIg | cold insoluble globulin |
| CPC | cetyl pyridinium chloride |
| CSP | cell surface protein |
| DMSO | dimethyl sulfoxide |
| D-V | dorso-ventral |
| ECM | extracellular matrix |
| FN | fibronectin |
| GAG | glycosaminoglycan |
| GlcNAc | N-acetylglucosamine |
| HA | hyaluronic acid |
| HCG | human chorionic gonadotropin |
| KCl | potassium chloride |
| LDCF-F | lymphocyte-derived chemotactic factor for fibroblasts |
| LETS | large external transformation sensitive |
| $MgSO_4$ | magnesium sulfate |
| NaCl | sodium chloride |
| $NaH(CO_3)_2$ | sodium bicarbonate |
| NaOH | sodium hydroxide |
| NGF | nerve growth factor |
| $OsO_4$ | osmium tetroxide |
| PAS | periodic acid-Schiff |
| PDGF | platelet-derived growth factor |

| | |
|---|---|
| RER | rough endoplasmic reticulum |
| RNA | ribonucleic acid |
| SEM | scanning electron microscope |
| UDP | uridine diphosphate |

# 1. INTRODUCTION

Embryonic development begins with the fertilization of the egg by a sperm. The egg then undergoes a series of rapid and synchronous divisions to produce, in amphibians, a hollow ball of cells, or, in fish, reptiles, birds and mammals, a two-dimensional disc of cells. This is followed by gastrulation, a process characterized by extensive cell migration and resulting in the formation of the three germ layers. Gastrulation is the first of a series of cell movements and rearrangements that give rise to the final shape of the organism.

Many of these morphogenetic processes involve changes in cell shape (e.g. neurulation) or cell migration (e.g. gastrulation) or both. Cells may migrate individually or in sheets and frequently cover considerable distances over what are sometimes circuitous paths, before reaching their final destination. The precision with which cells follow a particular pathway has led to the question of guidance mechanism.

Much effort has been concentrated on studies of the guidance mechanism(s) and more specifically on the nature of the guidance cues. These cues are environmental factors that direct the motile cell along a very specific pathway. They exist either as diffusible molecules or integral components of the cell membrane or extracellular matrix (ECM). The ECM is an intricate meshwork that surrounds most cells. Its

structural components consist of collagen fibers as well as other glycoproteins, glycosaminoglycans (GAGs) and proteoglycans.

## 1.1 Guidance mechanisms

There are several mechanisms by which cells can be guided during migration:

1) chemotaxis and mutual inhibition: involves directed cell movement along a concentration gradient of a diffusible substance emitted by a point source. Although chemotaxis can be positive or negative, in this context it has been equated with attraction, while repulsion has been termed mutual inhibition.

2) contact guidance: involves guidance by a physical structure in the environment.

3) haptotaxis: involves guidance of cells by transient complexing with specific molecules in the ECM or on cell surfaces.

4) galvanotaxis: involves directed cell movement along an electric field.

Both in vitro and in vivo studies have demonstrated that cells can be guided by a combination of cues. Popular subjects of study include gastrulating cells, fibroblasts, leukocytes, axons and neural crest cells.

### 1.1.1 Cell migration during gastrulation

As mentioned previously, cells first begin to migrate

at gastrulation. In amphibia, the presumptive mesodermal cells move anteriorly as a sheet along the inner surface of the blastocoelic roof. Nakatsuji et al. (1982) analysed the network of ECM fibrils that cover the inner surface of the ectoderm in **Ambystoma maculatum** gastrulae. They found that these fibrils were frequently aligned parallel to the axis of migration and cells preferentially attached to these fibrils. These authors postulated that ECM fibrils serve to direct cell migration during gastrulation by contact guidance. In a more recent report, Nakatsuji and Johnson (1984) have provided convincing evidence for the role of contact guidance in orienting migrating cells during gastrulation. They found that the orientation of ECM fibrils could be altered by mechanical tension. Furthermore, mesodermal cells migrated along the newly aligned fibrils even when these fibrils were aligned along an axis perpendicular to the normal axis.

The role of fibronectin (FN) in gastrulation was examined by Boucaut et al. (1984). Fibronectin is a ubiquitous glycoprotein of the ECM (Akiyama et al., 1981) and has been shown to mediate cell adhesion to collagen (Hauschka and White, 1972; Klebe,1974). It exists in two major forms: 1) plasma FN, originally called cold insoluble globulin (CIg; Morrison et al., 1948; Edsall et al., 1955), exists as a soluble dimer in the blood, 2) cellular FN, also known as cell surface protein (CSP; Yamada and Weston, 1974) and large external transformation-sensitive (LETS; Hynes,

1973), exists as an insoluble multimer on the cell surface. The two forms have similar domains for binding actin, fibrin, heparin, transglutaminase, **Staphlococcus aureus** and a cell surface receptor but differ in their specific activities. They are thought to be products of different genes or differently spliced messenger RNA's.

Boucaut et al. (1984) reported that injection of monovalent antibodies to FN into late blastula or early gastrula of **Pleurodeles waltlii** completely inhibited gastrulation. These authors concluded that fibronectin is required for cell migration during gastrulation.

### 1.1.2. Fibroblast migration

Fibroblasts are mesodermal in origin and located in the connective tissue of the adult organism. Using the Boyden technique, Postlethwaite et al. (1976) examined the chemotactic response of human fibroblasts to "lymphocyte-derived chemotactic factor for fibroblasts" (LDCF-F) produced by stimulated human lymphocytes. The Boyden technique (Boyden, 1962) involves placing cells and presumptive chemoattractant in different compartments of a chamber. The compartments are separated by a millipore filter with pores large enough to allow the cells to pass through. Analysis of the distribution of the cell population after a set period of time shows whether directed migration has occured.

Fibroblasts were found to migrate between compartments

of a Boyden chamber only when a concentration gradient of LDCF-F existed across the filter. That this factor acts as a chemotactic stimulus rather than a stimulator of random migration was shown by comparing the rate of migration in the presence and absence of LDCF-F; the rate was the same in both cases. These authors speculated that this chemotactic response may be the mechanism by which fibroblasts are attracted to sites of inflammation in vivo.

More recently, Senior et al. (1983) have demonstrated the chemotactic activity of three platelet alpha granule proteins: platelet factor 4, PDGF and beta-thromboglobulin in fibroblast migration.

The role of various ECM components in fibroblast migration has been studied both in vitro and in vivo (Armstrong and Armstrong, 1980; Toole and Trelstad, 1971). Using radioactive isotopes, Toole and Trelstad examined the distribution of hyaluronic acid (HA) during avian corneal fibroblast migration. Hyaluronic acid is an polysaccharide composed of repeating glucuronic acid and N-acetylglucosamine (GlcNAc) units. They found that HA was present in the migration path in high concentration during fibroblast movement but declined rapidly after cessation of migration. Armstrong and Armstrong reported that fibroblast motility in aggregates was stimulated when these aggregates were treated with collagenase and restricted when the collagen content was increased by treatment with ascorbic acid.

Electric fields have been shown to influence fibroblast migration *in vitro* (Erickson and Nuccitelli, 1984). Quail somite fibroblasts respond to an electric field by migrating towards the cathode. Since electric fields have been detected in many *in vivo* systems (Borgens el al., 1977; Jaffe and Stern, 1979; Robinson and Stump, 1984), Erickson and Nuccitelli have postulated that during embryonic development, migratory cells might be guided by electric fields.

### 1.1.3. Leukocyte migration

Studies of leukocyte migration have concentrated on identification of chemoattractants as well as metabolic events of the chemotactic response. During the inflammation response, circulating leukocytes become attached to the endothelial lining of a blood vessel near the site of inflammation. They then move along the endothelium, passing through the vessel wall at the site of inflammation. Although it is not known what the natural attractant is, several compounds have been shown to be chemoattractants *in vitro*. Among these are N-formylmethionyl peptides (Schiffman et al., 1975), leukocyte factors (Zigmond and Hirsch, 1973) and complement components (Ward et al., 1968).

Leukocytes have also been shown to display a galvanotactic response (Fukushima et al., 1953; Monguio, 1933). These cells will migrate towards the anode or the cathode, depending on the strength of the electric field.

### 1.1.4. Axonal migration

During embryonic development, the first neuron of a nerve bundle to project an axon, the pioneer axon, traces the stereotyped route to be followed by all other neurones of the same bundle. The axons of these late arising neurones are thought to follow the pioneer by contact guidance.

The pioneer axons have been shown to navigate by several different mechanisms. Gunderson and Barrett (1979) demonstrated the importance of chemotaxis in directing the migrating growth cones of dorsal root sensory neurones. The axons of these neurones turned towards a pipette releasing nerve growth factor (NGF). That the same can occur in vivo was shown by Menesini Chen et al. (1978). They found that sympathetic nerve fibers would grow into regions of the cerebrum that been injected with NGF.

Chemotaxis is not the only means by which pioneer axons can navigate. In the grasshopper, these axons bind to specific non-adjacent cells, named guidepost cells, along their route from the limb bud to the CNS (Bate, 1976). The importance of these cells in directing axonal migration was demonstrated by selectively destroying these cells. Bentley and Caudy (1983) reported that in such cases, the pioneer axon was no longer able to navigate along its normal route and consequently failed to reach the CNS.

Neurite growth can also be guided by electric fields (Hinkle et al., 1981; Freeman et al., 1981). In such cases,

growth was oriented towards the cathode.

### 1.1.5. Neural crest cell migration

Neural crest cell migration is probably the most intensively studied system, both *in vitro* and *in vivo*. Neural crest cells originate in the neural folds. They form a mass of mesenchymal cells which, prior to migration, are located on the dorsal side of the neural tube, just beneath the epidermis. In the head region, neural crests migrate laterally and form most of the cranial cartilage. In the trunk region, migration can take place along two pathways: 1) the dorsal pathway: migration proceeds laterally between the epidermis and mesoderm; cells migrating along this pathway will differentiate into pigment cells; 2) the ventral pathway: migration proceeds between the neural tube and somites; cells that migrate along this pathway will differentiate into the spinal ganglia, ganglia of the sympathetic nervous system, adrenal medulla and Schwann cells.

*In vivo* studies have involved transplantation of a second neural tube to the flank of the host (Twitty, 1966). In such cases, the transplanted neural crest cells migrated radially from the graft. Thus, on one side of the graft, crest cells migrated dorsally along the host flank, a direction opposite to the normal migration path of the host's crest cells. These results demonstrate that the neural crest pathway contains no polarity.

The neural crest migration pathways are acellular, ECM-rich spaces. It has, therefore, been postulated that the ECM is important in guiding neural crest cells either by contact guidance or providing specific recognition molecules. Much effort has been devoted to determining the role of ECM components in neural crest cell migration.

Lofberg and Ahlfors (1978) examined the organization of the ECM in the dorsal region of **Ambystoma mexicanum** and found that the fibrils along the neural tube were aligned dorso-ventrally. Based on this observation, the authors proposed that these fibrils serve to orient the neural crest cells by contact guidance. In contrast, Tosney (1978) reported that in the chick embryo the ECM fibrils have no particular orientation.

Analysis of GAG components of the ECM in the chick embryo revealed that hyaluronic acid was the major component (Pratt et al., 1975). Hydration of HA would increase the volume of the ECM (Laurent, 1970), thus permitting invasion by migratory cells (Pratt et al., 1975). Pratt et al. proposed that controlled synthesis and removal of hyaluronic acid could mediate neural crest cell migration.

Greenburg et al. (1981) reported that fibronectin was chemotactic for avian neural crest cells. However, these authors did not distinguish between increased adhesion, chemokinesis and chemotaxis. The role of fibronectin was further studied by Rovasio et al. (1983). They have demonstrated that avian neural crest cells adhere to a glass

substrate or collagen only in the presence of fibronectin; adhesion was inhibited by antibodies to the cell binding fragment of fibronectin. They also found that migration on fibronectin was random and concluded that fibronectin was important for adhesion but not for directing migration.

Bronner-Fraser (1982) proposed that fibronectin may act as a mechanism by which neural crest cells discriminate between the dorsal and ventral pathways. She studied the movement of uncoated, BSA and fibronectin coated latex beads injected onto the ventral pathway in chick embryos. Uncoated and BSA coated beads were translocated along the ventral pathway whereas fibronectin coated beads remained near the site of implantation. This not only demonstrates that active migration is not required for movement along the ventral pathway, but that the presence of fibronectin on the surface inhibits this movement. However, since neural crest cells lack cell surface fibronectin (Newgreen and Thiery, 1980), the significance of these findings remains unclear.

These examples serve to illustrate the many different mechanisms by which migrating cells are guided: chemotaxis, contact guidance, galvanotaxis and haptotaxis. They also serve to demonstrate that cells often navigate by more than one type of cue.

## 1.2 Systems for studying guidance mechanisms

By far, most of the studies on guidance mechanisms for

cell migration have centered on in vitro sytems. Although in vitro systems offer some obvious advantages over in vivo systems, such as being readily observed and manipulated, one major problem is that the results are often not easily extrapolated to embryonic processes and, more importantly, are not necessarily indicative of what is occuring in the embryo.

For example, Greenburg and Hay (1982) have shown that the cell morphology and behaviour are at least in part a function of the cell's environment. Epithelial cells which normally form a flat, tightly cohesive sheet, express the morphological and behavioural characteristics of migrating mesenchymal cells when cultured on a collagen matrix. In addition, Sugrue and Hay (1982) have found that stress fibers, adhesion plaques and ruffles, all morphological characteristics of fibroblasts in culture, do not develop when these cells are grown on ECM. Thus, in studies designed to examine guidance cues for cell migration during embryonic development, in vivo systems would be the best choice.

### 1.2.1 The pronephric duct as a model system

As mentioned previously, neural crest cell migration is one of the most intensively studied systems. One less well studied system is that of pronephric duct cell migration. Like neural crest cells, the duct is located just beneath the epidermis, thus allowing easy access for experimental manipulation. One advantage that the pronephric duct offers

over neural crest cells is that its migration pathway is very specific and can be readily identified, even before the cells begin to migrate. Therefore, one can not only perform experimental studies on the duct cells but on the pathway itself.

In vertebrates, the pronephric rudiment first appears as a thickening of the mesoderm at the ventrolateral border of the somites in the anterior region of the trunk (Field, 1891). The pronephros, the larval kidney, develops from the anterior portion of the rudiment while the duct develops in the adjacent posterior region by caudal growth, as in the chick embryo (Overton, 1959), by recruitment of cells in situ, as in Xenopus (Poole and Steinberg, 1977) or by cell migration, as in the axolotl (Poole and Steinberg, 1981).

That the duct cells in the axolotl do indeed migrate was shown by Poole and Steinberg (1981). They placed vital dye marks over the duct cells as well as the adjacent somitic and lateral mesoderm. Several hours later, the position of the dye mark on the duct had moved posteriorly while those in the somitic and lateral mesoderm remained stationary.

#### 1.2.1.1 Guidance information

During its migration to the cloaca, the pronephric duct follows a very specific pathway: the juncture between the somites and lateral mesoderm. The duct cells make contacts with both somitic and lateral mesodermal cells (Poole and

Steinberg, 1981). Very little is known about the cues that guide the duct. Holtfreter (1944) was one of the first to manipulate this system experimentally and observe the effects on duct migration. In the first set of experiments, a transverse incision was made through the somitic mesoderm and the duct path, prior to duct migration. Holtfreter found that the advancing duct would attempt to deviate ventrally around the wound and in successful cases, the duct always returned dorsally to the duct path.

In the second set of experiments, Holtfreter bisected early stage embryos and allowed the anterior half to heal to the posterior half in reverse dorso-ventral (D-V) orientation. In many cases the duct would reach the cloaca, doing so by following the normal but discontinuous duct path. In other words, once the duct had migrated into the posterior half, it would change direction and migrate dorsally with respect to its new environment until it reached the normal duct path. It would then migrate caudally to the cloaca. These experiments demonstrate the tendency of the duct to follow its normal path.

More recently, Poole and Steinberg (1982) have performed a series of experiments to study the properties of the guidance information. In order to determine the source of the guidance cues, they examined the effect of removal of the surrounding tissues on migration of a second duct transplanted onto flank. By amputating the posterior quarter of an embryo, the cloaca and caudal somitic mesoderm were

removed. They found that both the primary and secondary ducts continued to migrate even in the absence of these tissues. From this, the authors concluded that the guidance information is not produced by a distant source, such as the cloaca, but must be contained within the lateral mesoderm.

In order to determine the possible influence of the primary duct on secondary duct migration, primary duct migration was blocked by making a dorsal incision. They found that secondary ducts migrated dorsally to the duct path and in all cases turned caudally to migrate along the virgin duct path. From these experiments, the authors concluded that the migration path taken by the secondary duct is not influenced by the primary duct. In addition, the guidance information on the primary duct path must be directionally polarized. According to Poole and Steinberg this would argue against simple contact guidance.

In order to further study the polarity of the guidance information, Poole and Steinberg (1982) grafted secondary ducts in reversed D-V or A-P orientation. Reversing the D-V axis of the graft with respect to the host had no effect on subsequent migration of the secondary duct. Reversal of the A-P axis, on the other hand, resulted in complete inhibition of migration. The authors concluded from this that the duct could only migrate caudally over the lateral mesoderm.

Poole and Steinberg examined the spatio-temporal localization of the guidance information by transplanting secondary ducts in normal orientation at various A-P levels

along the lateral mesoderm. They found that secondary ducts were unable to migrate in anterior regions of the flank; migration was observed only when the secondary duct was placed at the same A-P level as the primary duct.

To briefly summarize these results, Poole and Steinberg have shown that the duct guidance information is contained within the lateral mesoderm and travels craniocaudally as a wave.

#### 1.2.1.2 Relationship of duct migration to other morphogenetic processes

Poole and Steinberg (1982) noted that pronephric duct migration proceeds caudally in synchrony with somite segmentation, an observation that led them to suggest a causal relationship between the two morphogenetic processes.

In vertebrates, somite segmentation begins just behind the head and this process moves caudally through the embryo in a wave-like fashion. A detailed account of the process of somite segmentation in the chick embryo has been provided by the work of Bellairs et al. (1978, 1980) and Bellairs (1979). The mesenchymal cells of the unsegmented mesoderm undergo an increase in adhesiveness to one another, causing them to condense. These cells become spindle shaped, thus allowing for an increased area of contact between the cells. In the resulting rosette shape of the individual somite, each cell has one end anchored via desmosomes at the center of the somite (Lipton and Jacobson, 1974) and the other

anchored via collagen fibrils to the adjacent epithelium. Beloussov and Naumidi (1983) have examined ECM distribution during somitogenesis in the chick embryo and found that ECM accumulation begins several hours prior to somite segmentation. Though the molecular details of somitogenesis have not been examined in the axolotl, the general process of segmentation is quite similar to that in avian embryos (Youn and Malacinski, 1981).

In addition to the craniocaudal wave of visible segmentation, a prior wave of cellular change precedes somite segmentation (Elsdale et al., 1976). This wave was made visible by its sensitivity to heat shock. Elsdale et al. (1976) discovered that short exposure of **Xenopus** embryos to heat shock resulted in a region of abnormality several somites ahead of the last formed somite at the time of treatment.

## 1.3 The thesis project

My overall objective was to obtain information about the guidance mechanism of pronephric duct cell migration by

1) examining the role played by the surrounding tissues, namely the lateral mesoderm, the somitic mesoderm and the epidermis, in directing the migrating duct; and

2) examining the role of cell surface proteins in duct migration.

### 1.3.1 Formation of the pronephros and pronephric duct rudiment

During my investigation, it became apparent that the pronephric rudiment in the axolotl has a dual origin; this is contrary to what has been reported for other vertebrates (reviewed in Fraser, 1950). Therefore, I began my investigation by an examination of the formation of the pronephros and pronephric duct rudiment in histological sections as well as in scanning electron micrographs.

### 1.3.2 Studies of the lateral and somitic mesoderm

Poole and Steinberg (1982) have provided evidence that the guidance information is contained within the lateral mesoderm and travels craniocaudally in a wave-like fashion. Due to the synchrony between migration and somite segmentation, these authors have suggested a causal relationship between the two. I felt that the first step should be to continue the work of Poole and Steinberg by further examining the properties of this wave of guidance information and by determining whether a causal relationship exists between somite segmentation and duct migration.

Poole and Steinberg's wave represents a boundary to the region of the lateral mesoderm that cannot support duct migration. One question that remains to be answered is: are all lateral mesodermal cells posterior to this boundary capable of supporting duct migration at any one time, or is this property limited to those cells in the immediate

vicinty of the primary duct tip? This question was answered by transplanting ducts to A-P levels posterior to the primary duct. In some cases, these grafts were also placed at a level posterior to the last formed somite, thus providing information about the relationship between somite segmentation and duct migration.

One of the advantages of using the pronephric duct to study guidance mechanisms is that the path that the cells will follow is readily identifiable long before migration begins. However, no one has examined the duct path cells for any morphological changes that might provide clues as to the guidance mechanism. Therefore, the next step in my investigation was to examine these cells histologically as well as in the SEM, at different stages during duct migration.

### 1.3.3 Role of the epidermis in duct migration

During migration, the duct cells are not only in contact with the lateral and somitic mesoderm, but also with the overlying epidermis. The question of a possible role for the epidermis in duct migration was never addressed by previous studies on this system, yet the epidermis has been shown to be important for neural crest cell migration (Bogomolova and Korochkin, 1973; Keller et al., 1982).

In the white (d/d) axolotl, melanoblasts are unable to migrate along the dorsal pathway and therefore remain close to the dorsal midline (Dalton, 1950). By performing

reciprocal epidermis transplants between normal (D/-) and white embryos, Dalton demonstrated that the defect lay in the ability of the environment to support migration, rather than in the ability of the melanoblasts to migrate. By performing reciprocal epidermal transplants between white embryos at different stages, Bogomolova and Korochkin (1973) concluded that the problem resulted from delayed "maturation" of the epidermis. Spieth and Keller (1984) reported that the structure of the ECM in the subepidermal space is abnormal; this led them to propose that the absence of an ECM component could be responsible for the abnormal appearance of the matrix as well as for inhibited neural crest cell migration.

The role of the epidermis in duct migration was examined by transplanting the epidermis in reverse A-P, D-V or A-P and D-V orientation.

### 1.3.3 Distribution of ECM during duct migration

Most of the recent studies on cell migration have concentrated on the role of the ECM in directing migration either by contact guidance along aligned fibrils (Nakatsuji and Johnson, 1984) or by selective adhesion to specific ECM components (Boucaut et al., 1984).

The ECM is composed of an intricate network of collagen fibrils as well as other glycoproteins, glycosaminoglycans (mucopolysaccharides) and proteoglycans (mucoproteins). Collagen is a fibrous glycoprotein that is characterized by

its triple stranded helical structure and its high hydroxyproline content. Two of the major non-collagen glycoproteins of the matrix are fibronectin and laminin. Fibronectin has been shown to promote adhesion in mesenchymal cells while laminin is important for epithelial cell adhesion (for review, see Kleinman et al. 1981).

Glycosaminoglycans are long unbranched chains of repeating disaccharide units. Most are covalently linked to serine residues of a polypeptide chain to form proteoglycans. The one exception is hyaluronic acid. Hyaluronic acid is composed of repeating N-acetylglucosamine-glucuronic acid units and is a major constituent of the ECM in regions where cells are migrating (Pratt et al., 1975; Markwald et al., 1978; Orkin and Toole, 1978; Weston et al., 1978). Some of the common proteoglycans include heparin, keratan sulfate and chrondroitin-sulfate, a major component of cartilage.

Before attempting a detailed study of the role of the ECM and its components in duct migration, one must determine whether ECM is present during the duct migration stages and, if so, one must examine the spatio-temporal distribution. The ECM was identified in sectioned material by the periodic acid-Schiff method (McManus, 1946), a stain specific for polysaccharides. The spatio-temporal distribution was determined by examining embryos histologically and in the SEM at different stages.

### 1.3.4 Role of cell surface proteins

Since many developmental processes are thought to be mediated by cell surface proteins, I decided to examine the role of such proteins in duct migration by

1) interfering with glycoprotein production with tunicamycin
2) enzymatic digestion with trypsin

#### 1.3.4.1 Glycoprotein structure and synthesis

Most secreted and cell membrane proteins are glycoproteins: complexes consisting of a protein core to which various oligosaccharide chains are attached. Such proteins are synthesized on the rough endoplasmic reticulum (RER) (Sabatini and Blobel, 1970). The nascent polypeptide chain is inserted into the lumen of the RER by interaction of specific receptors in the membrane with the signal peptide, a short sequence at the amino-terminal (Blobel, 1977).

The carbohydrate moiety is usually attached to the amino group on the side chain of asparagine (N-linked oligosaccharide) or, less frequently, to the hydroxyl group on the side chain of serine, threonine or hydroxylysine (O-linked). My work is restricted to study of the former and, therefore, the remaining discussion will be concerned with the synthesis of N-linked oligosaccharides.

Addition of the carbohydrate moiety occurs in two steps. The core region, consisting of two N-acetylglucosamine and three mannose residues, is added in

the RER; subsequent modification takes place in the golgi complex. The core oligosaccharide is synthesized by step-wise addition of activated sugars from a UDP-sugar intermediate to dolichol, a lipid located in the membrane of the RER. The completed core region is subsequently transferred to an asparagine residue by glycosyl transferase. During passage through the golgi, subsequent addition of monosaccharides results in the generation of a mature glycoprotein.

1.3.4.2 Tunicamycin

Tunicamycin is an antibiotic produced by **Streptomyces lysosuperificus** (Takatsuki et al., 1971) that specifically inhibits the transfer of activated GlcNAc from UDP to dolichol (Takatsuki et al., 1975), thus interfering with the synthesis of N-linked glycoproteins. Due to its specificity, tunicamycin has been very useful for studying the role of this class of glycoprotein in embryonic development. When applied at early cleavage stages, no effect was observed until gastrulation (Schneider et al., 1978; Romanovsky and Nosek, 1980; Sanchez and Barbieri, 1983). In **Arbacia, Bufo** and **Xenopus**, the embryos either failed to gastrulate or exogastrulated. This demonstrates the importance of N-linked glycoproteins for morphogenetic movements.

My study involved injection of tunicamycin into late blastula or early gastrula to determine the role of N-linked glycoproteins in pronephric duct migration.

### 1.3.4.3 Trypsin

Trypsin is a pancreatic proteolytic enzyme, first isolated from bovine pancreas. It belongs to a group of homologous enzymes, the serine proteases. Trypsin catalyses the hydrolysis of ester and peptide bonds involving the carboxyl groups of arginine and lysine.

Trypsin has been widely used to identify cell-cell adhesion molecules which are thought to be important for intercellular recognition during embryonic development (Yoshida and Takeichi, 1982). A variety of different cell types have been shown to possess two distinct adhesion mechanisms (Takeichi, 1977; Urushihara et al., 1977; Atsumi and Uno, 1979; Takeichi et al., 1979). One is sensitive to trypsin only in the absence of calcium while the second is cleaved independently of calcium.

The present study was designed to study the importance of trypsin sensitive proteins for duct migration.

# 2. MATERIALS AND METHODS

## 2.1 ANIMALS

### 2.1.1 Maintenance

The majority of the animals in our colony have been bred and raised in our laboratory and are descendants from a small group of animals obtained from L. Delanney, Ithaca College; H.C. Dalton, Pennsylvania State University and R.R. Humphrey, Indiana University. Animals are maintained in plastic cages containing two liters of 50% Holtfreter's solution (1.7 gm NaCl, 100 mg $MgSO_4$, 100 mg $NaH(CO_3)_2$, 50 mg $CaCl_2$, 25 mg KCl per liter of dechlorinated tap water). The cages are cleaned and fresh water added, three times a week. Newly hatched larvae are feed brine shrimp on a daily basis. At about two months of age, the animals are switched to a diet of beef heart. Young animals are fed 8-10 appropriate sized strips of beef heart on a daily basis, while adults are fed three times a week.

### 2.1.2 Spawning

The breeding procedure involves the use of Human Chorionic Gonadotropin (HCG) to induce ovulation in the female axolotl. Prior to spawning, the female is injected with 250 or 500 I.U. of HCG; the smaller dose is usually sufficient. The male may also be injected with 250 I.U. of HCG; this was found to facilitate spawning in less

responsive males.

After injection, the female was placed in a plastic mating pan (35 x 30 x 15 cm) containing 5 liters of 50% Holtfreter's solution at room temperature. The bottom of the pan is lined with gravel.

The best results are obtained if the male is placed with the female 8-12 h after injection. If the male is added too soon after injection, the female does not usually display any interest; if the spawning is initiated too late, the female frequently begins to lay eggs before picking up any sperm. The time interval between injection and spawning can be lengthened by incubating the injected animals at a temperature several degrees below room temperature.

Courting normally begins within 15 min after the male is placed with the female and the latter usually begins to lay eggs two to four hours later.

### 2.1.3 Embryos

#### 2.1.3.1. Maintenance

Eggs are collected in finger bowls containing 25% Holtfreter's solution and maintained either at room temperature ($20^{o}C$) or in a low temperature incubator ($5-15^{o}C$). Thus, embryonic development can be slowed down sufficiently to allow time to perform several experiments with eggs from a single spawning. Incubation of pre-neurula

stage embryos at 5-10°C frequently give rise to developmental abnormalities (personal observations) and therefore, early stage embryos are always incubated at temperatures above 10°C.

2.1.3.2. Staging

Embryos were usually staged by external morphology, according to the comprehensive set of diagrams prepared by Bordzilovskaya and Dettlaff (1979; appendix 1). However, it was frequently observed that the number of somites did not correspond to the external appearance of the embryo. For example, a stage 25 embryo would have anywhere form 8 to 11 somites, instead of 8 as described by Bordzilovskaya and Dettlaff. Therefore, in all experiments were exact staging was critical the embryos were staged according to the number of somites.

In the tunicamycin experiments, gastrula were staged according to thee shape and size to the blastopore. Some of these stages were arbitrarily redefined to facilitate classification in the various stages. Stages 10 1/2 and 10 3/4 were defined as described by the staging table. Stage 11 was redefined as the stage at which the ventral lip has begun to involute; thus, the blastopore forms a full circle. Stage 11 1/2 was redefined as the stage at which the blastopore is reduced to about on half its original size. This stage corresponded to Bordzilovskaya and Dettlaff's

stage 11 1/2 or 12. It was difficult to distinguish between these two stages due to the variation in the size of the blastopore.

## 2.2.MICROSURGERY

### 2.2.1.Instruments

Most of the instruments required for microsurgery are not available commercially and therefore, were custom made. The following is a list of instuments used and instructions for their manufacture.

Tungsten needles: this type of needle is used in preference to glass needles because of the ease with which the tip can be re-sharpened. These needles are used for cutting tissues and consist of a 3 cm piece of tungsten wire (0.1 mm diameter) inserted into a pasteur pipet; the latter serves as a holder. The tip of the wire is bent to a $45^{o}$ angle with a pair of watchmaker's forceps. The bent tip is sharpened by electrolysis in a 1N NaOH solution. The straight end of the wire is inserted into the tapered end of a pasteur pipet that has been pulled to a suitable diameter and broken off at an appropriate length. The junction is flamed in order to seal the wire in place. The tip can be re-sharpened by electrolysis many times before the needle must be replaced.

Hair loops: these loops are used for manoeuvering tissues or

whole embryos without damaging them. Both ends of a 3 cm piece of hair (preferably blond since excessive pigment causes kinking in the loop) are inserted into a pasteur pipet holder, prepared in the same manner as for tungsten needles. It was found that one end of the hair would tend to slip out when the second end was inserted. This could be avoided by first filling the tapered end of the holder with a liquid. Ethanol is best for this purpose since it will evaporate quickly afterwards. Once adjusted to the appropriate size, the hair loop is held in place by sealing the end with melted paraffin. After the paraffin has solidified, any excess wax adhering to the loop is gently scraped off.

Ball tip rods: these are useful for making depressions in the permoplast-paraplast lining of the operating dish. The embryo is placed in this depression and the lining is mounded around the embryo in order to immobilize it during the experiment. The narrow end of the pasteur pipet is broken off leaving 3 cm of taper. The broken end is flamed, causing the glass to retract. Twirling of the end during flaming ensures that a relatively spherical ball is formed.

Glass Bridges: these are used to hold grafts in place until healing has taken place. Glass coverslips (#1 thickness) are scored with a diamond pencil into 1 x 2 mm rectangles and a gentle pressure applied to break along the score lines. The

edges of each bridge are smoothed by quick flaming.

Operating dishes: Corning spot depression plates were used for this purpose. The bottom is lined with a soft pliable material so that depressions for holding the embryos can be made easily. An 80:20 permoplast (American Clay Co.): paraplast (Lancer) mixture has proven best for this. The mixture is heated to melting (approximately 55°C) and poured into the wells. Once the lining has cooled, it is spread evenly with the bottom of an appropriate sized test tube.

Watchmaker's forceps: no. 5 forceps were purchased from Fine Science Tools. Forceps are used for dejelling embryos and manipulating glass bridges. For easy manipulation, it is imperative that the two tips of the forceps match perfectly. The forceps are, therefore, sharpened with an oilstone under the dissecting microscope.

### 2.2.2 Operations

Embryos kept at 5°C - 15°C were allowed to warm up to room temperature for 0.5-1.0 h before the beginning of the experiment. Except for the trypsin experiments, all operations were carried out in calcium-free Steinberg's solution (3.4 gm NaCl, 0.05 gm KCl, 0.21 gm $MgSO_4$, 0.56 gm Tris base per liter of distilled water, pH 7.7; 100% contains 0.05 gm $CaCl_2$ in addition to the above). Embryos were washed three times in calcium-free solution prior to

being transfered to depressions made in the operating dish. The embryos were held in place by mounding the permoplast-paraplast over the embryo's head and around its sides with a ball tip rod.

#### 2.2.2.1 Duct transplants

The presumptive pronephros and duct, as well as the overlying ectoderm, were cut out with a tungsten needle and transfered to a slit made in the flank of a host embryo. The graft was held in place by a glass bridge until healing had taken place. Embryos were transfered to 100% Steinberg's and left to incubate for 6-24 h.

#### 2.2.2.2 Epidermis transplants

The epidermis on one side of the embryo was slit with a tungsten needle laterally behind the gill bulge, dorsally along the neural tube and ventrally along the mid-ventral line. To reverse the A-P axis, the epidermis was transplanted from the left side of one embryo to the right side of another. To reverse the D-V axis, the epidermis from the left side of one embryo was transplanted in an inverted position onto the right side of a second embryo. To reverse both axes, the epidermis was replaced in an inverted position on the same side. Controls consisted of reciprocal epidermis transplants in the proper A-P and D-V orientation.

## 2.3 MEASUREMENTS OF CELL HEIGHT

Measurements were made from frontal and cross sections of embryos staged according to the number of somites present. Cells were measured with an ocular micrometer that had been calibrated with a hemocytometer grid.

The height of the duct path cells in the region of the duct tip cell was measured along the mediolateral axis. In order to compare the height of different cells, measurements were made at the same point in each cell; heights were determined from sections passing through the nucleus.

Three or four cases from each of four different stages were examined. In each case, 7-9 contiguous duct path cells were measured, beginning with third or fourth cell anterior to the duct tip cell. For each stage, the heights of cells in the same A-P position were averaged. The A-P position of each cell was determined by first matching the tallest cell of each case; all other cells were matched according to their A-P position with respect to the tallest cell.

## 2.4 EXAMINATION OF THE ECM

Various stage embryos were fixed for 1 h in 2.0% glutaraldehyde made up in 0.1M cacodylate buffer, pH 7.7. A short fixation time was important in order to preserve the ECM; very little ECM was visible when embryos were fixed for 6 h or longer. Typical preservatives, such as cetyl pyridinium chloride (CPC) or ruthenium red, added to the fixative, did not improve the preservation of the ECM. In

fact, glutaraldehyde with CPC was less effective than glutaraldehyde alone. Therefore, in this study, embryos were fixed in glutaraldehyde alone. The fixative was removed with two washes in 0.1 M cacodylate buffer and the embryos were stored at 4°C. The samples were prepared for light or electron microscopy as described in sections 2.6 and 2.7.

The ECM was stained in thick sections by the PAS method. The first step consisted of an aldehyde block with chlorous acid (80 mls of 0.03% sodium chlorite; 20 mls of 5N acetic acid). The slides were incubated in this solution for 48 h followed by a 10 min rinse in tap water. The slides were then treated with 1% periodic acid for 10 min, washed for 5 min in distilled water and stained with Schiff reagent (BDH chemicals) for 20 min. The unreacted dye was removed with two 2 min washes in sulfurous acid (5 mls of 1N HCl; 5 mls of 10% sodium metabisulfite; 100 mls distilled water). After a final 5 min wash in distilled water, the slides were mounted in permount with a glass coverslip (#1 thickness).

In order to view the ECM in the scanning electron microscope, the epidermis on one side was removed after the embryo had been dried, using pieces of double sided sticky tape, as described for the lateral mesoderm fractures in section 2.7.6.

## 2.5. ENZYME AND DRUG TREATMENT

### 2.5.1. Trypsin

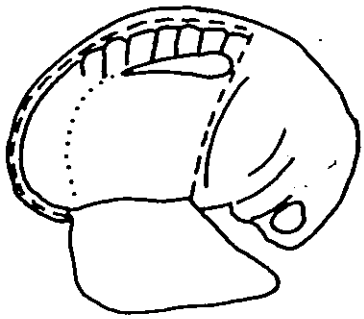
These experiments were carried out in either calcium-free or 100% Steinberg's. The epidermis on one side of the embryo was slit with a tungsten needle laterally, behind the gill bulge, and dorsally, along the neural tube (Fig. 1). The epidermis was gently pulled off with out disturbing the underlying mesoderm and a 0.01% trypsin (made up in Steinberg's) solution was added. After three minutes, the trypsin solution was washed out with 7 changes of calcium-free Steinberg's. The epidermis was gently eased back over the mesoderm with a hair loop and held in place with a glass bridge. After healing was complete (approximately one half hour), the embryos were transfered to 100% Steinberg's and left to incubate for 24-36 h.

### 2.5.2. Tunicamycin

The injection needles were made from capillary tubes pulled on a model 700C vertical pipet puller (David Kopf Instruments). The injection needle was manoeuvered into position with a MM33 Brinkman micromanipulator. Gastrula were carefully staged according to the Bordzilovskaya and Dettlaff staging tables and placed into depressions made in lined operating dishes containing 100% Steinberg's solutions. Each embryo was injected with 200 nl of a solution containing 10 or 25 ng of tunicamycin in a modified

Figure 1

Diagram illustrating temporary removal of the epidermis. The dashed line indicates where the cuts were made; the dotted line represents the duct path.



Steinberg's solution (calcium-free Steinberg's plus 0.005% calcium chloride plus 5% dimethylsulfoxide (DMSO)), pH 8.0. Control embryos were injected with the same volume of modified Steinberg's solution. After injection, embryos were transfered to 25% Steinberg's solution and left to incubate for 48 h.

## 2.6 PREPARATION FOR LIGHT MICROSCOPY

### 2.6.1 Fixation

Appropriate stage embryos were fixed overnight at 4°C in 2.5% glutaraldehyde made up in 0.1 M cacodylate buffer, pH 7.7. The fixative was removed with two 15 min washes with cacodylate buffer.

### 2.6.2 Dehydration

The embryos were dehydrated at room temperature in the following ethanol series: 30%, 50%, 70%, 80%, 90%; 15 min in each, followed by two half hour changes in 95%.

### 2.6.3 Infiltration and Embedding

Embryos were placed in glycol methacrylate infiltrating medium (Sorvall) for 48 h at 4°C, with one change with fresh infiltrating medium after 24 h. The infiltrating medium was replaced with embedding medium (Sorvall) and the embryos were pipetted into rectangular molds. The molds were placed in a vacuum dessicator and the blocks allowed to polymerize

and dessicate overnight.

### 2.6.4 Sectioning

Four micron sections were cut with a glass knife on a Sorvall JB-4 microtome. Individual sections were removed from the knife edge with watchmaker's forceps and dropped into a beaker of water to maximize spreading. Serial sections were picked up with a clean glass slide and dried overnight on a 60°C slide warmer.

### 2.6.5 Staining

Sections were stained with Lee's basic fuschin-methylene blue (1.2 mls of 0.1% basic fuschin, made up in distilled water; 1.2 mls of 0.1% methylene blue; 1.5 mls of 95% ethanol; 2.1 mls of phosphate buffer, pH 7.2) for 1.5 min, rinsed in distilled water, dipped in 95% ethanol to remove the excess stain from the background, and mounted in permount with a 24 x 60 mm glass coverslip (#1 thickness).

Frequently, the sectioned material would not stain properly with basic fuschin-methylene blue. Such sections would show virtually no staining of nuclei, while the cytoplasm stained normally. A new staining method was developed for these sections. Sections were stained for 1 min in 1% acid fuschin, made up in distilled water, rinsed briefly in water and dipped in 95% ethanol. Sections were then stained for 15 min in 0.1% toluidene blue, made up in a 0.02M solution of benzoic acid, pH 4.4. After rinsing

briefly in water and dipping in 95% ethanol, the sections were examined under the microscope. If the nuclei were not stained enough, the sections were stained in toluidene blue for an additional 5-15 min. The slides were washed briefly in distilled water and mounted as described above.

## 2.7 PREPARATION FOR SCANNING ELECTRON MICROSCOPY

### 2.7.1 Fixation

Embryos were fixed overnight at $4^{o}C$ in 2.5% glutaraldehyde made up in 0.1M cacodylate buffer, pH 7.7. The fixative was removed with two 15 min washes with cacodylate buffer. Using insect pins, the embryos were immobilized in an operating dish and the epidermis on the operated side was removed with a tungsten needle. At this point, the embryos could be stored in buffer at $4^{o}C$ for several weeks. The embryos were post-fixed at room temperature for 1 h in 1% $OsO_4$ made up in 0.1M cacodylate buffer.

### 2.7.2 Dehydration

Dehydration was performed at room temperature in the following ethanol series: 15 min in each of 30%, 50%, 70%, 80%, 90% and 95%, followed by two half hour washes in 100%.

### 2.7.3 Critical point drying

Critical point drying was performed under $CO_2$ in a

Polaron critical point dryer. Special containers for holding the embryos during this process were made from BEEM capsules (JB EM supplies; size 00). The capsules were perforated over the entire surface and labelled with a hot needle. The length of the capsule was then adjusted by cutting off the tapered end and capping it with the lid from a second BEEM capsule. In this way, 15 separate containers can be fitted into the critical point dryer instead of the usual three. Embryos were pipetted into the capsules and these were in turn placed into the critical point drying boat. Both transfers are done under 100% ethanol. The metal screen was fitted on the boat and the latter placed inside the dryer.

The ethanol was gradually replaced with liquid $CO_2$ by flushing the dryer several times. The temperature was maintained at 10°C during the flushing. The first was a 10 min flush, followed by three 3 min flushes. After each of the first three flushes, the system was allowed to equilibrate for 3 min; the fourth was followed by a half hour equilibration period. The system was then flushed for a final 3 min after which the temperature was gradually increased to ~36°C. As the temperature increased, some $CO_2$ was released so that the internal pressure did not exceed 1200 pounds per square inch. When the internal temperature reached ~36°C, the pressure was gradually released over a 10 min period. After cooling the apparatus to room temperature, the capsules were removed and stored in a dessicator.

### 2.7.4 Mounting

Silver paste was used to mount embryos on aluminum stubs. Paste was preferred to carbon or silver paint because its thicker consistency allowed for greater flexibility in the orientation of the specimens. A wooden applicator stick that had one end whittled to a point was used to apply a streak of paste onto a stub. A small amount of paste on the tip of the applicator stick was used to transfer the specimen onto the stub. The embryo was gently manoeuvered into the proper orientation with a clean applicator stick.

### 2.7.5 Coating

Once the paste had dried, the stubs were placed in a Polaron sputter coater and coated for 3 min with gold or a 60:40 gold:palladium mixture. Specimens were examined in either a JEOL JSM 35 or a Philips 505 scanning electron microscope.

### 2.7.6. Fracturing

The lateral mesoderm was fractured after the dried specimens had been mounted on aluminum stubs. Appropriate sized pieces (1 x 3 mm) of double sided sticky tape were cut with a pair of fine dissection scissors. The tape was gently lowered onto the lateral mesoderm and pulled off again. This process was repeated until all the desired cells were removed.

## 2.8.PHOTOGRAPHY

### 2.8.1. Negatives

Scanning electron micrographs were taken with Polaroid type 55 positive-negative film. The negatives were processed immediately, by dipping into an 18% solution of sodium sulfite. Once the processing film had peeled away (about 1 min), the negative was washed for on half hour in a 16°C water bath and hung up to dry.

Sections were photographed with Kodak Panatomic-X (32 ASA) film on a Leitz microscope. Negatives were developed for 12 min at 20°C in Kodak Microdol X (diluted 1:3 in water immediately before use). The negatives were then rinsed for 1 min in tap water an fixed for 8 min with Kodak Fixer. The negatives were dipped in a 1:200 dilution of Photoflo and hung up to dry.

### 2.8.2. Prints

Negatives were printed on Kodabrome (resin coated) paper. The prints were developed for 60 sec in a 1:2 dilution of Kodak Dektol stock solution (made up as directed on the package). The prints were rinsed in tap water for 30 sec and fixed for 3 min in Kodak Rapid Fix. After rinsing in tap water for 5 min, the prints were hung up to dry.

# 3. RESULTS

## 3.1 THE FORMATION OF THE PRONEPHROS AND PRONEPHRIC DUCT RUDIMENT

Traditionally, the vertebrate excretory system has been described as originating entirely from the intermediate mesoderm or cell-mass (Balfour, 1876; for review, see Fraser, 1950). The latter has been defined by Balfour as the cell-mass formed by a partial fusion of the somatic and splanchnic layers of the lateral mesoderm, at the outer border of the somites.

A re-examination of some of the original literature reveals a different origin for the urodelan pronephros and duct. According to Field (1891), the pronephros of **Ambystoma punctatum** arises as a continuous thickening of the somatic layer of the lateral mesoderm beneath somites 3 and 4, while the duct develops beneath somites 5-7. These observations were consistent with those of Mollier (1890) in **Triton alpestris** and were later confirmed by O'Connor's (1938) studies on **Ambystoma punctatum** and **Triton taeniatus.** This discrepancy could be simply due to a difference in terminology or to a genuine difference in the origin of the urodelan pronephros and duct.

The present study was undertaken in order to examine the question of the origin of the pronephric rudiment in **Ambystoma mexicanum** by use of the high resolving power of

the scanning electron microscope. My observations indicate that a clearly defined intermediate mesoderm does not exist during the stages of pronephros and duct formation; instead, the pronephros appears to be derived from the lateral region of somites 2, 3 and 4. The duct, on the other hand, appears to originate from the lateral mesoderm, as suggested by Mollier (1890), Field (1891) and O'Connor (1938) in other urodelan species.

### 3.1.1 Results

#### 3.1.1.1 Pronephros formation

The presumptive pronephros begins to form at the 4 somite stage, while segregation of the duct rudiment takes place at the 7-8 somite stage. During the initial stages of formation, the pronephros can be seen as a slight bulge beneath somites 2, 3 and 4 (Fig. 2 a-d). During subsequent stages, this bulge becomes more predominant and compacted into the typical ovoid shape of the pronephros (Fig. 2e).

The presumptive pronephros is initially continuous with the posterior-lateral region of somite 2 and the lateral region of somites and 4, as seen in scanning electron micrographs and light microscope sections (Fig. 3a-c), but gradually separates from them (Fig 2f, 3d). Examination of the somitic and pronephric cells at higher magnification reveals that at the 4-5 somite stage, the two cell types have similar morphology and cell processes (Fig. 4), such

Figure 2

Development of the pronephric rudiment. A total of 30 embryos were examined. The epidermis has been removed in order to expose the mesoderm. The anterior end of the embryo is on the right hand side in each micrograph. (a) a 3 somite embryo; the rudiment has not begun to develop. (b) a 4.5 somite embryo; the pronephric rudiment has begun to form. Note the dorsolateral fissure in somite 2 (arrow). This fissure develops as the posterio-lateral portion of somite 2 segregates to form part of the pronephric rudiment. (c and d) a 6 and a 7 somite embryo; the bulging pronephros is more apparent. Note the continuity with somites 2, 3 and 4. (e) an 8 somite embryo; the developing pronephros has formed a very compact ovoid tissue. It is still continuous with somite 3, but not with somite 2 or 4. (f) a 10 somite embryo; the pronephric rudiment (PN) is now completely separated from somite 3. (a, x56; b, x90; c-e, x73; f, x210)

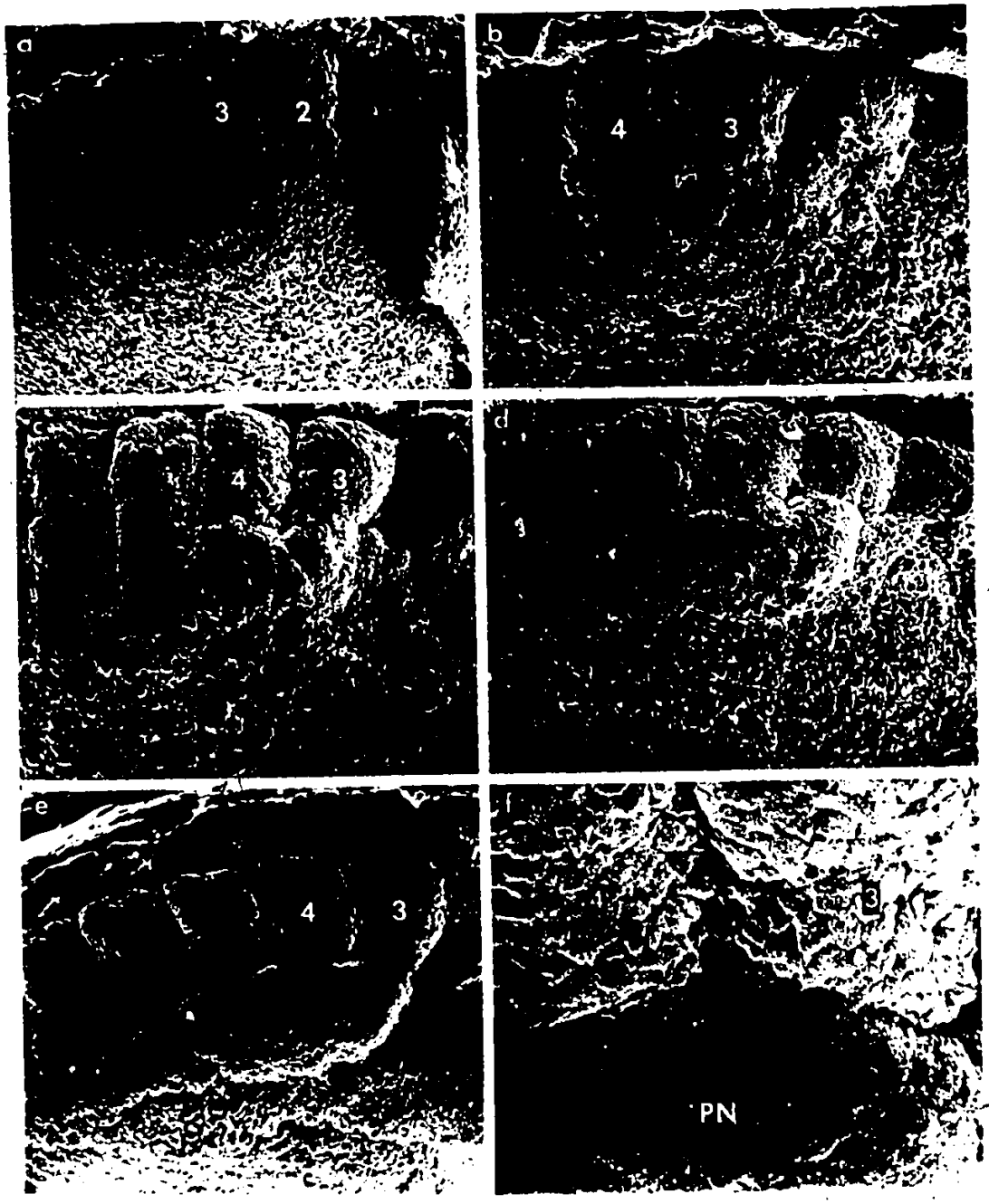

a
3
2
b
4
3
2
c
4
3
d
3
e
4
3
f
3
PN

Figure 3

Continuity between the somites and the pronephric rudiment. (a) Cross-section through a 5 somite embryo at the level of somite 3. Note the continuity between the somitic (S) and pronephric (PN) tissue. The same continuity was observed at the level of somites 2 and 4. (b) Frontal section through a 6 somite embryo. (c) Cross-section through a 7 somite embryo at the level of somite 4. (d) Frontal section through a 9 somite embryo. The pronephric rudiment has almost completely segregated from the adjacent somites. NT=neural tube, N=notochord. (x90)

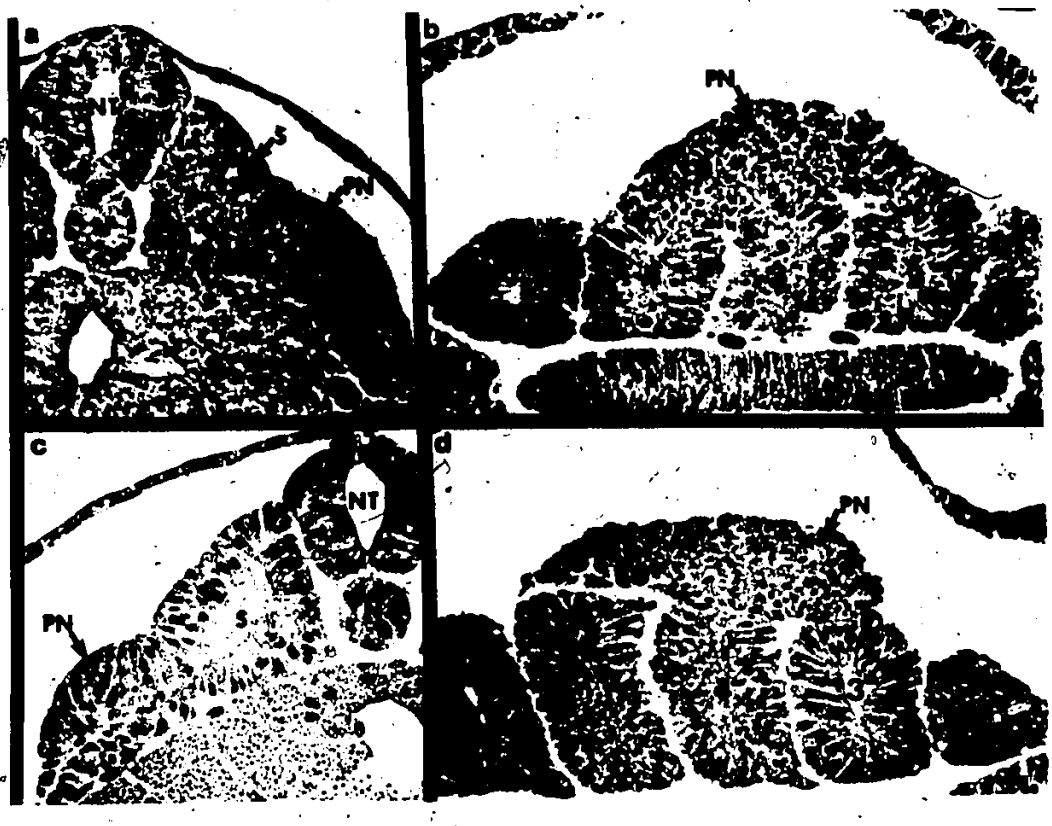

a
NT
S
PN
b
PN
c
NT
PN
S
d
PN

Figure 4

Comparison of cell morphologies in the somitic mesoderm and presumptive pronephros at the 5 somite stage. Cells of somite 2 (a), 3 (b), 4 (c) and of the presumptive pronephros (d) show no difference in morphology. (x925)

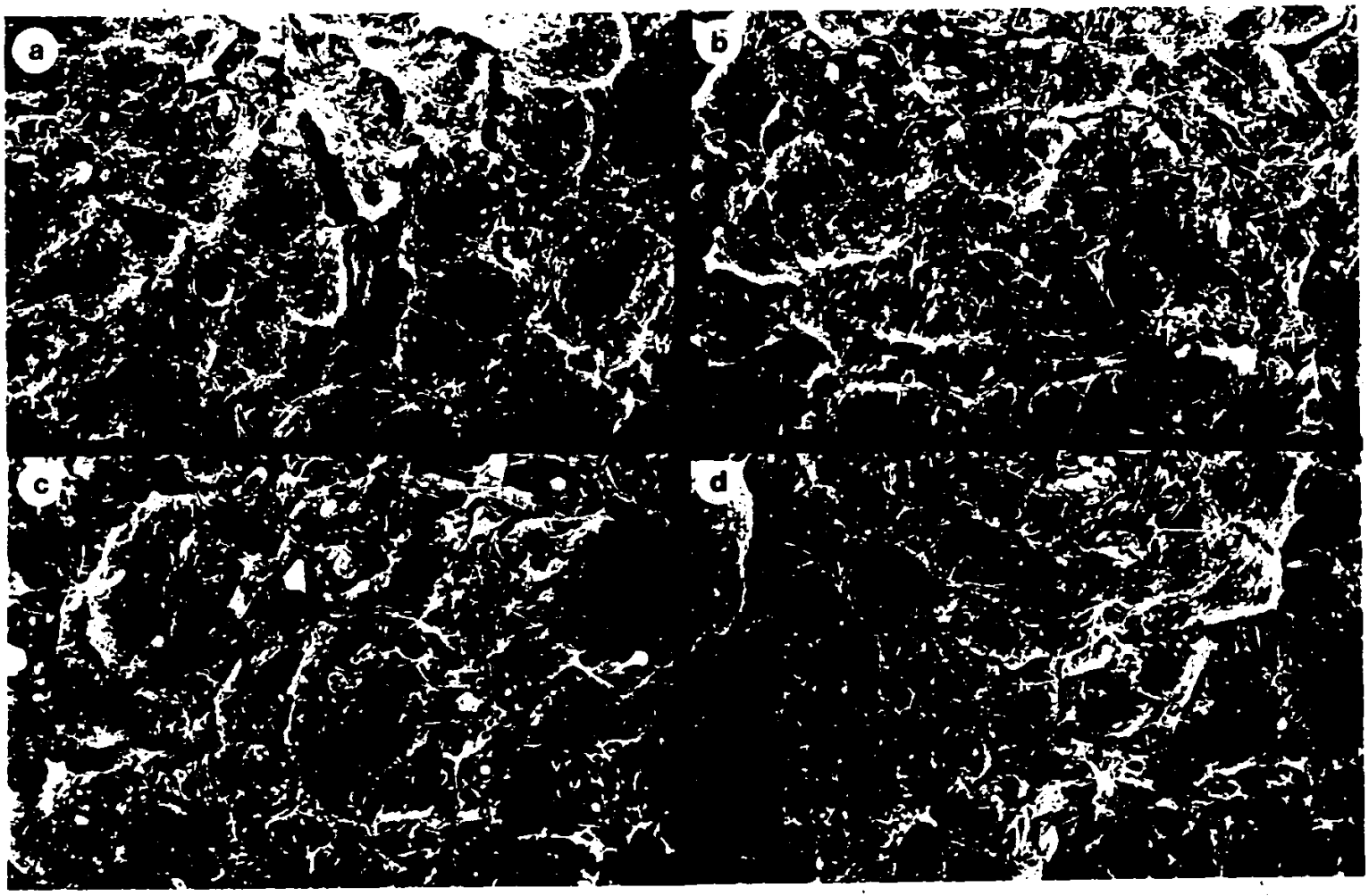
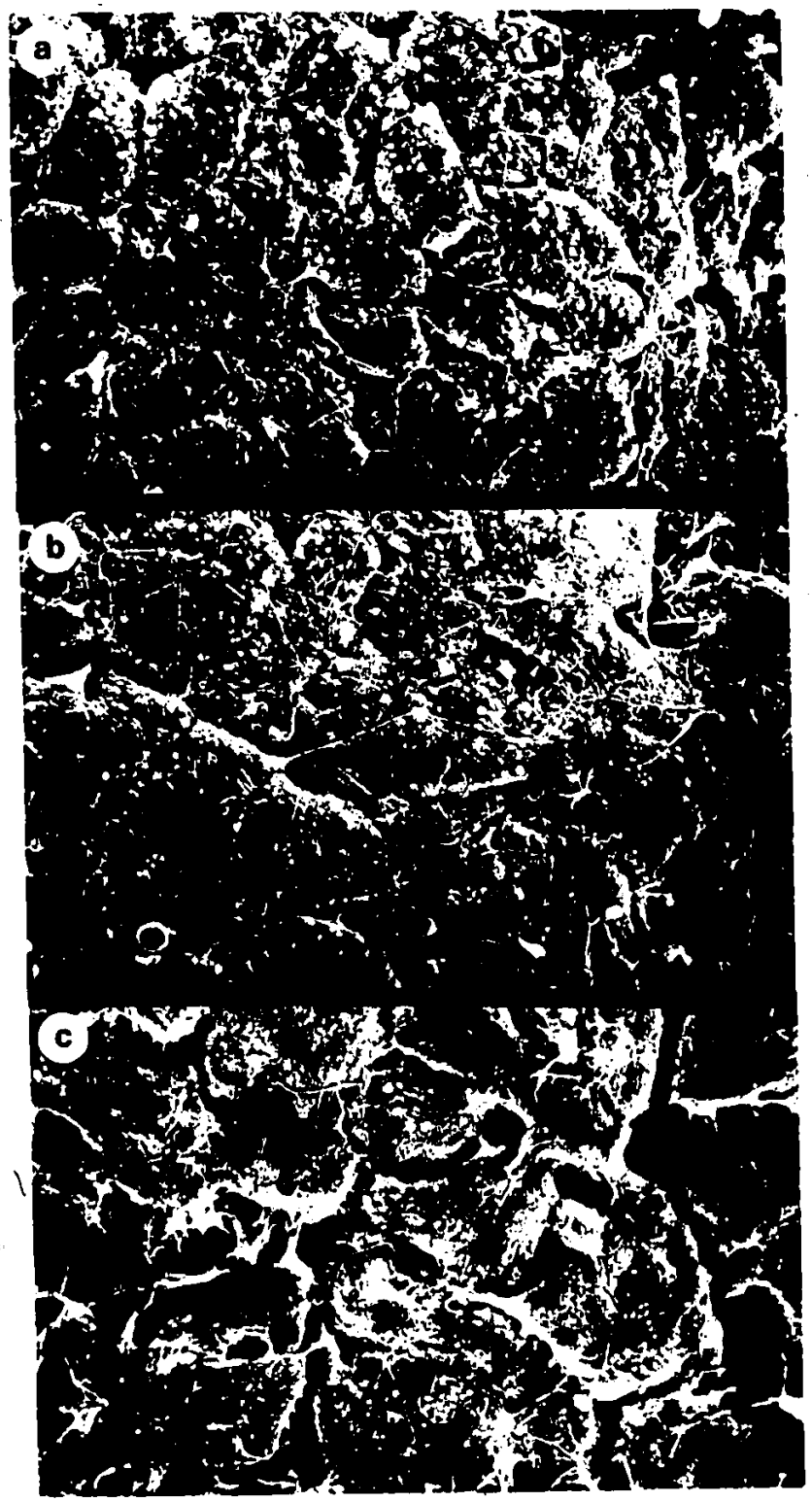

a
b
c
d

Figure 5

Comparison of cell morphologies in the somitic mesoderm, presumptive pronephros and lateral mesoderm at the 7 somite stage. Cells of the presumptive pronephros (b) differ in morphology from either somitic (a) or lateral mesodermal (c) cells. (x925)


a
b
c

that it is difficult to distinguish between them. By the 7 somite stage, however, the morphology of the pronephric cells are quite different from that of the somitic cells. The former have become flattened superficially and it is difficult to discern cell boundaries while the somitic cells remain well separated from one another (Fig. 5). Gradually, the pronephric cells form an extremely compact tissue. Thus, the cells of the pronephros are originally similar to somitic cells in morphology but gradually change. This, along with the continuity between the somites and pronephric rudiment, suggest that the pronephros is derived, at least in part, from somites 2, 3 and 4.

3.1.1.2 Pronephric duct formation

The pronephric duct rudiment forms during the 7-8 somite stages. In scanning electron micrographs, one can see a mass of loosely connected cells immediately below the pronephros at the 7 somite stage (Fig. 6a). At progressively later stages, this mass of cells can be seen in more dorsal positions until they become located on the duct path (8 somite stage (Fig. 6b-d). These cells are aligned along the dorso-ventral axis of the embryo (Fig. 6c), suggesting that migration occurs along this axis. This mass of cells reaches the duct path below somites 5, 6 and 7. As the cells begin to migrate caudally along the duct path (9 somite stage), they become aligned along the anterio-posterior (A-P) axis, as shown previously by Poole and Steinberg (1981).

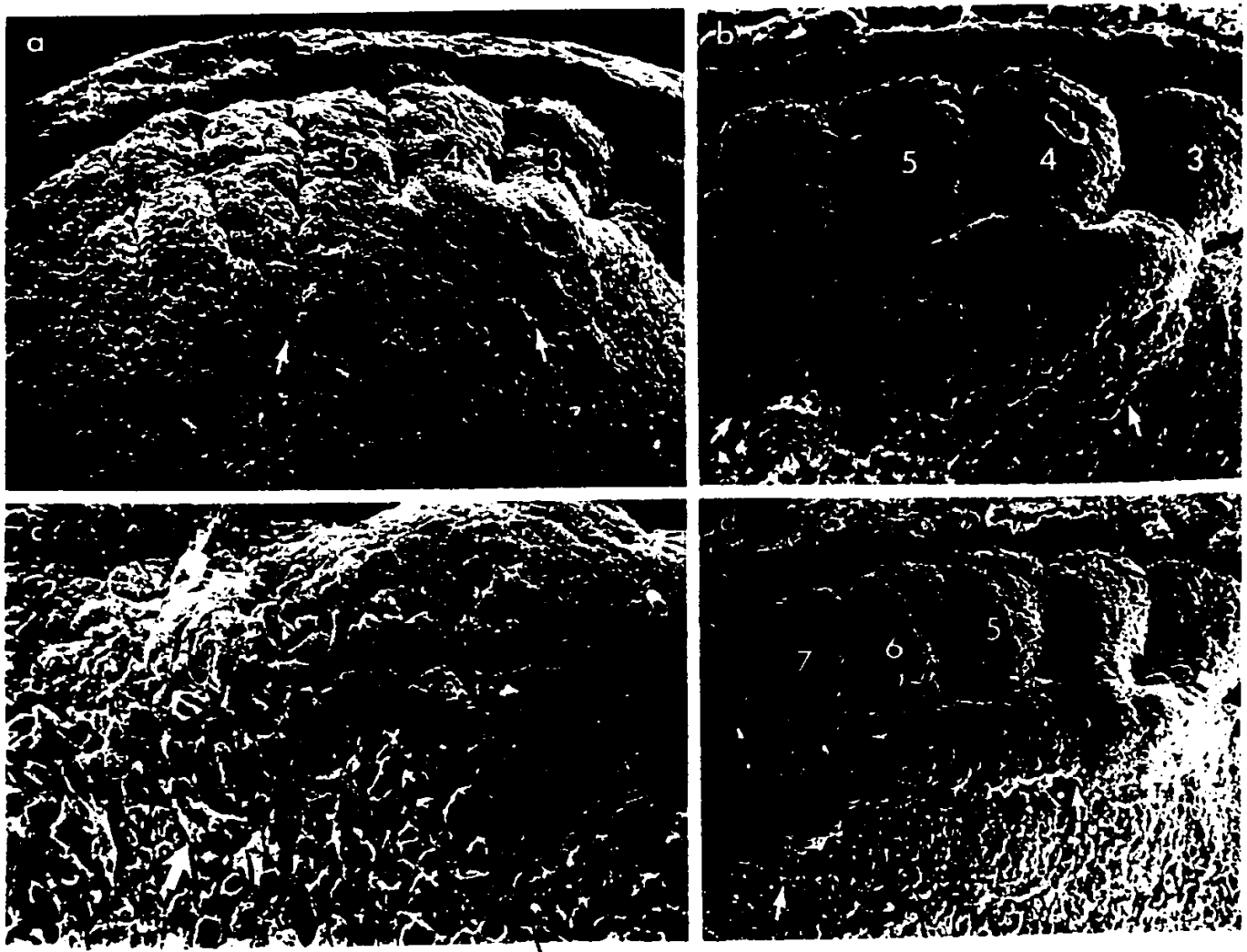

a
5
4
3
b
5
4
3
c
d
7
6
5

Figure 6

Formation of the pronephric duct. A total of 30 embryos were examined. The anterior end of the embryo is on the right hand side in each micrograph. (a) A mass of cells (between arrows) begins to segregate from the surrounding lateral mesodermal cells at the 7 somite stage. (b) The mass of loosely connected pronephric duct cells (between arrows) can be seen at the lateral edge of the presumptive pronephros at the 7.5 somite stage. (c) The dorso-lateral alignment of the long axis of pronephric duct cells (arrow) suggests that they are migrating in this direction. (d) 9 somite embryo; the duct cells have reached the duct path and the tip (arrow) has begun to migrate posteriorly. (a-b, x100; c, x200; d, x93)

These observations suggest that the cells of the duct rudiment arise from the lateral mesoderm below the pronephros and migrate dorsally to the duct path before beginning their caudal migration to the cloaca.

### 3.1.2 Discussion

This study demonstrates that, like all other vertebrates, the pronephric rudiment in the axolotl develops in the region between the somites and the lateral mesoderm. However, my observations fail to provide evidence for the existence of intermediate mesoderm, as is seen in the chick embryo (Balfour, 1876). Instead, the pronephric rudiment appears to be derived from both somitic and lateral mesoderm.

O'Connor (1938) states that the pronephros is derived from somatic mesoderm. However, this conclusion, as well as those reached by Mollier (1890) and Field (1891), was based on observations of external appearance and examination of cross-sections. It is obvious from our cross-sections (Fig. 3) that a distinction between a somatic and a somitic origin would be extremely difficult to make. In fact, it was only through examination of scanning electron micrographs that the contribution of the somites to the pronephros became apparent. Therefore, it is conceivable that the difference between my findings and those of earlier workers simply reflects the limitations of the methodology they employed.

Evidence in support of this view comes from the fact that my observations are entirely consistent with the vital staining experiments performed by O'Connor (1938). He found that when the region below somites 3 and 4 were stained, the stain was subsequently located in the pronephros only. Likewise, stain applied to the region below somites 5-7 was distributed throughout the duct but not in the pronephros. This is exactly what one would expect to find, based on my observations. O'Connor (1938) concluded from his results that the pronephros and duct are distinct from one another, i.e. derived from different segments.

## 3.2 SPATIO-TEMPORAL DEPENDENT PROPERTIES OF THE MESODERM AND EPIDERMIS

Many morphogenetic processes proceed in a craniocaudal sequence; for example, neural crest cell migration, somite segmentation and, of course, pronephric duct migration. In this section, I will examine the tissues surrounding the duct cells for changes that proceed in the same craniocaudal sequence and, thus, may play a role in guiding the duct.

### 3.2.1 ABILITY TO SUPPORT DUCT MIGRATION

#### 3.2.1.1 Lateral and somitic mesoderm

In studies on pronephric duct migration, Poole and Steinberg (1982) demonstrated the presence of a wave that passes craniocaudally through the lateral mesoderm in synchrony with duct migration. This **post-migration** wave results in a change such that lateral mesodermal cells are no longer capable of supporting duct migration. In these studies, however, only the lateral mesoderm anterior to and immediately posterior to the wavefront was examined. Thus, one question that remains to be answered is: do all lateral mesodermal cells posterior to this wave contain guidance information at any one stage, or is this property also acquired in a craniocaudal sequence? This question can be answered by transplanting secondary ducts at various A-P

levels within the posterior lateral mesoderm.

In some of the transplants, the secondary duct was placed posterior to the last formed somite, therefore, the results of these experiments will also test the hypothesis that somite segmentation is causally related to duct migration.

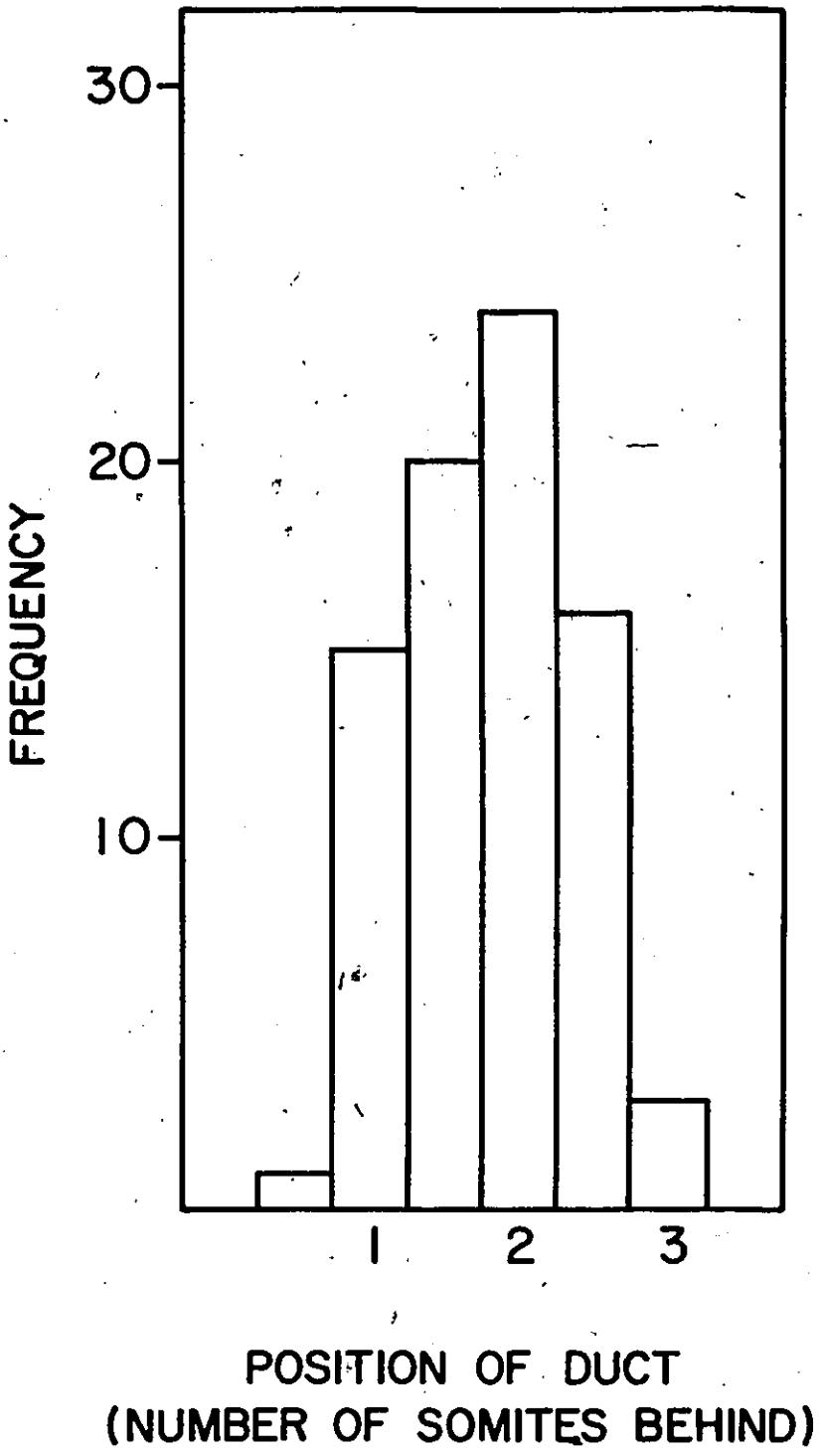
In this section, I will show that only a small area of the lateral mesoderm, the active region, is competent to support duct migration at any one time. In addition, I will show that duct migration can proceed in advance of somite segmentation.

3.2.1.1.1 Results and Discussion

Poole and Steinberg (1982) previously noted that the duct tip was always located about 2 somites behind the last formed somite. However, in order to evaluate the effect of any experiment on duct migration a more precise measurement must be made. I examined the position of the duct tip with respect to the last formed somite in 79 normal untreated embryos. The position of the duct tip with respect to the last formed somite did not vary significantly with developmental age ($0.5 < P < 0.9$). This indicates that somite segmentation and pronephric duct migration proceed at the same rate. The values for all stages (8-19 somites) were, therefore, pooled (Fig. 7). I found that the duct tip was never located ahead of the last formed somite and occupied a very narrow range of positions behind it (0.5-3.0

Figure 7

Location of the duct tip in normal, untreated embryos. The duct's position was recorded in terms of number of somites behind the last formed somite. The data were collected and pooled from all stages during duct migration since there was no significant difference in duct position as determined by a t-test. The total number of cases was 79; $\bar{x} = 1.80 \pm 0.58$


30
20
10
FREQUENCY
1
2
3
POSITION OF DUCT
(NUMBER OF SOMITES BEHIND)

somites); the mean was 1.80 ± 0.58.

Secondary ducts were transplanted such that the anterior end of the rudiment was placed 0-4.5 somite widths posterior to the anterior end of the primary pronephric rudiment. In 6 cases (2 at stage 22; 4 at stage 25) where the duct was not shifted posteriorly, no duct migration was observed (Table 1). When the duct was shifted posteriorly by one somite width (1 case), migration was observed but the secondary duct failed to reach the duct path. In 11 cases (5 at stage 22; 6 at stage 25) where the secondary duct was shifted posteriorly by more than one somite width, the secondary duct reached the duct path before the primary duct and was located significantly ahead of the normal duct position (Table 1; Fig. 8b-d); in some cases, the secondary duct was located ahead of the last formed somite. The primary duct failed to continue migration beyond the point where the grafted duct joined the duct path (Fig. 8).

The precise location of the post-migration wave was not determined by Poole and Steinberg (1982). It was described as being located in the vicinity of the duct tip. The results of the duct transplants presented here show that secondary ducts transplanted at exactly the same A-P level do not migrate. In order to explain why the primary duct can migrate but a secondary duct placed immediately below it cannot, one must assume that the wavefront is not parallel to the D-V axis but at a slight angle to it. Thus, secondary ducts transplanted at the same A-P level as the primary

* The duct tip position was determined with respect to the last formed somite; a positive value indicates that the duct tip was located posterior to the last formed somite, while a negative value indicates a position anterior to the last formed somite.

Table 1. Migration of secondary ducts transplanted onto posterior regions of the lateral mesoderm

| Stage of Transplant | Posterior Shift | Migration | Duct Tip Position* |
|---|---|---|---|
| 22 | 0.0 | - | - |
| 22 | 0.0 | - | - |
| 25 | 0.0 | - | - |
| 25 | 0.0 | - | - |
| 25 | 0.0 | - | - |
| 25 | 0.0 | - | - |
| 22 | 1.0 | + | - |
| 22 | 2.0 | + | +1.0 |
| 22 | 2.5 | + | +0.5 |
| 22 | 3.0 | + | +0.5 |
| 22 | 3.0 | + | +0.5 |
| 22 | 3.5 | + | +1.5 |
| 22 | 4.5 | + | +0.5 |
| 25 | 1.5 | + | 0.0 |
| 25 | 2.5 | + | +0.5 |
| 25 | 2.5 | + | -0.5 |
| 25 | 3.0 | + | +0.5 |
| 25 | 3.0 | + | -0.5 |
| 25 | 3.5 | + | -0.5 |

Figure 8

Secondary duct transplants. (a) The transplant was performed at stage 25 and the embryo was fixed 6 hours later. The primary and secondary duct tips are indicated by the curved and straight arrows, respectively. (b) The secondary duct was transplanted at stage 22 and the embryo was fixed 9.5 hours later. (c) The transplant was performed at stage 22 and the embryo was fixed 15 hours later. (d) The duct was transplanted at stage 25 and the embryo fixed 24 hours later. (a, x46; b, x38; c, x41; d, x34)


a
b
c

would not be located in the active region and could not migrate (Table 1).

One can obtain information about the angle of the wavefront by examining the angle at which secondary ducts migrate over the lateral mesoderm. First, one must assume that the secondary ducts were migrating along a line perpendicular to the wavefront. This assumption is based on the fact that a migration path perpendicular to the wavefront would constitute the shortest distance to migrate in order to remain within the active region. In all cases examined, the secondary duct migrated dorsocaudally at a fairly constant angle to the D-V axis. The data from Table 1 was pooled with 12 cases obtained from Poole and Steinberg (1982) since the average angle from the two sets of data were not significantly different. The average angle was $49^{o} \pm 6^{o}$ From this, one can calculate that the angle between the wavefront and the D-V axis is $41^{o} \pm 6^{o}$.

In the one case where the duct was shifted posteriorly by one somite width, limited migration was observed. The fact that this duct failed to reach the duct path suggests that the tip was just slightly behind the wavefront. Thus, the migrating tip was overtaken by the advance of the post-migration wave.

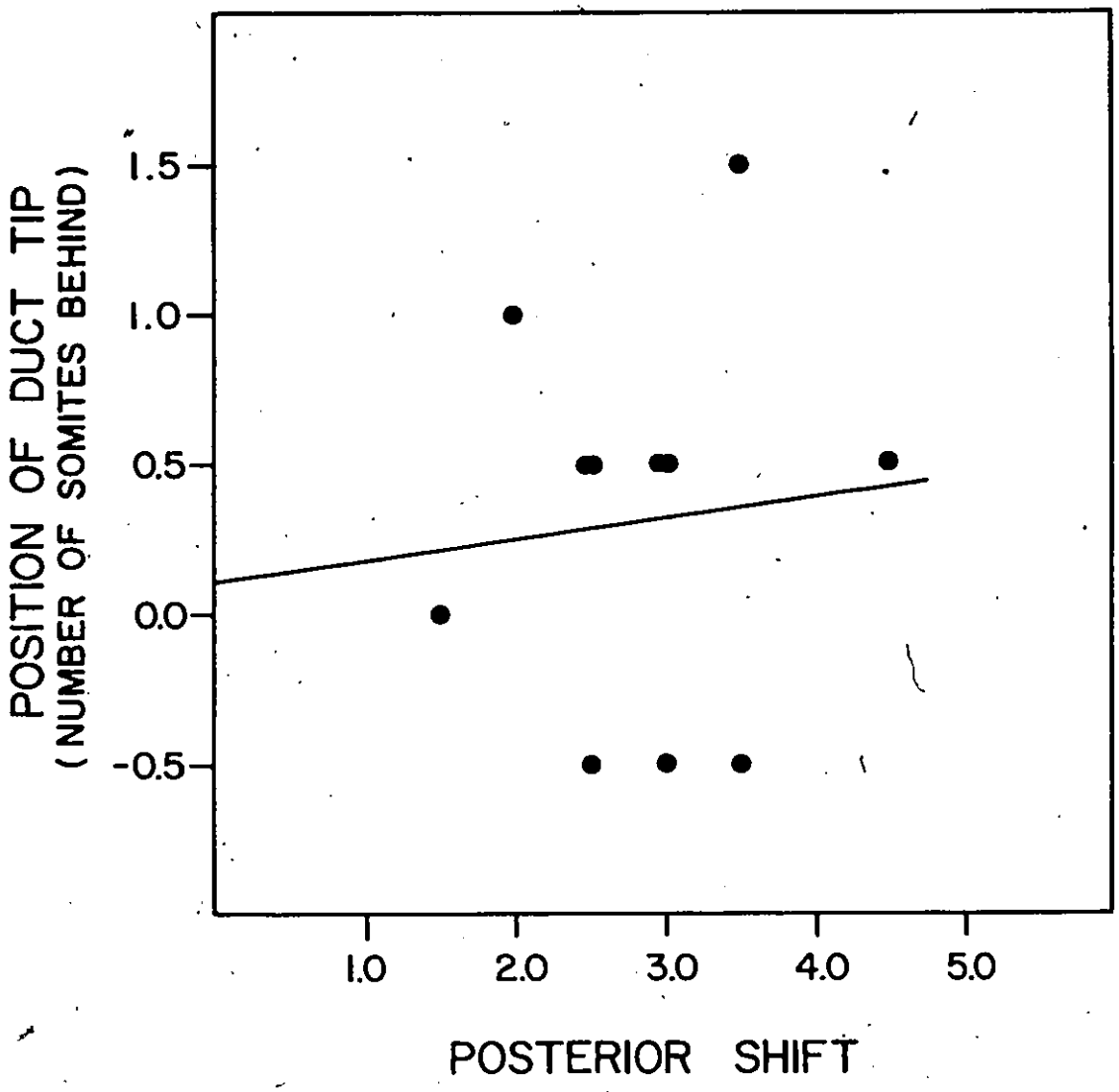
The fact that in all other cases the secondary duct reached the duct path ahead of the primary duct indicates that the lateral mesoderm in the region posterior to the primary duct tip is competent to support duct migration.

However, this competent region appears not to include all of the lateral mesoderm posterior to the primary duct. If all the posterior lateral mesoderm could support migration at the time of the transplantation, one would expect that ducts shifted posteriorly by 4 somites would be located ahead of ducts shifted by 2 somites. This was not the case as shown in Table 1. In fact, all the duct tips ended up within a region that was two somites wide. Regression analysis of the degree of shift vs the duct tip position (Fig. 9) indicated no correlation between the two parameters. The slope of the line is not significantly different from zero ($0.5 < P < 0.9$).

There are two possible explanations for this lack of correlation between duct position and degree of shift. The first is that transplanted ducts migrated at different rates; those transplanted more posteriorly migrated more slowly. Alternatively, one could imagine that the ducts did not migrate immediately after transplantation but waited until some change took place in the lateral mesodermal cells in order to allow migration. This change would pass through the lateral mesoderm in advance of the **post-migration** wave and delineate a posterior boundary to the **active** mesoderm. Since this wave would pass through the embryo in a craniocaudal direction, ducts shifted posteriorly by four somites would have to wait longer than those shifted by two somites. Thus, the two would end up in the same A-P position.

Figure 9

Correlation between the degree of posterior shift and the final duct position. This graph was plotted using the data from Table 1. The slope (regression coefficient) of the line is not significantly different from zero, indicating no correlation between the two variables.


POSITION OF DUCT TIP
(NUMBER OF SOMITES BEHIND)
1.5
1.0
0.5
0.0
-0.5
1.0
2.0
3.0
4.0
5.0
POSTERIOR SHIFT

One can distinguish between these two possibilities by examining the secondary duct at various times after transplantation. If the duct waits, one should be able to observe this. Ducts were transplanted 3 somite widths posterior to the primary duct in stage 22 or stage 25 embryos and examined 6, 9.5 or 15 h later.

In all of the 16 cases, migration of the secondary duct was observed only after 9.5 h, at a time when somite segmentation had begun in the region where the tip was located (Fig. 8a). The primary duct, on the other hand, had migrated at least 4 somite widths in all cases. From these results, one can conclude that the transplanted duct was incapable of migrating until some change took place, thus providing evidence for a posterior boundary.

The cases in which the secondary duct tip was located ahead of the last formed somite demonstrate that actual segmentation is not required for duct migration. The average position of the secondary duct with respect to the last formed somite was $0.32 \pm 0.64$ somite widths ahead. This is significantly different from the average position of primary ducts in normal embryos ($P < 0.001$) determined above. Thus, the posterior boundary is temporally linked to an event that takes place immediately prior to the appearance of a segmentation furrow.

In summary, secondary duct's transplanted at an A-P level posterior to the primary duct end up, on average, 2.12 (1.80 + 0.32) somite widths ahead of the normal duct

position. From this, one can determine that the posterior boundary of **active** mesoderm would be located approximately 2 somite widths ahead of the primary duct as well as 2 somite widths ahead of the **post-migration** wave. This is true for both stage 22 and stage 25 embryos, indicating that the posterior boundary moves caudally with increased developmental age.

These duct transplants also provide information about the relationship between migration and the pre-segmental mesoderm. Ducts that were shifted more posteriorly were placed closer to the duct path (Fig. 8a). Thus, a duct shifted by four somites had its tip on the duct path in a region of unsegmented mesoderm. Since these ducts wait before migrating, neither the duct path nor the somitic mesoderm is competent to support duct migration at the time of transplantation. At some later time, these tissues acquire this competency. Thus, it appears that the posterior boundary extends from the ventral region of the lateral mesoderm all the way up through the somitic mesoderm.

So far, I have demonstrated that migration can be uncoupled from the actual segmentation process. Instead, migration appears to be linked to a change that takes place in the somitic and lateral mesoderm just posterior to the last formed somite. This change, the **segmental** wave, delineates the posterior boundary of active mesoderm and moves in a craniocaudal direction approximately two somites ahead of the anterior boundary described by Poole and

Steinberg (1982).

### 3.2.1.2 Epidermis

During migration, the pronephric duct is in contact with three tissues, the lateral mesoderm, the somitic mesoderm and the epidermis. Previous studies (Poole and Steinberg 1977, 1981, 1982) have concentrated on the role of the mesodermal tissues in duct migration. The epidermis has been shown to be important for neural crest cell migration in the axolotl (Dalton, 1950; Keller et al., 1982). Therefore, the present study was undertaken in order to examine the possible influence of the epidermis on pronephric duct migration.

If the epidermis actively guides the duct, then one would expect the orientation of the epidermis to be important. In order to investigate this, the epidermis was transplanted in abnormal orientations such that one or both axes were reversed (Fig. 10).

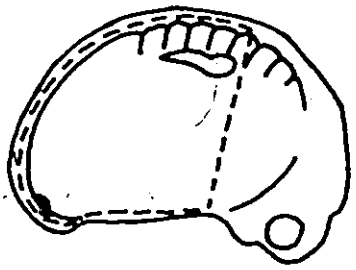
The results of these experiments demonstrate that the epidermis is not directly involved in guiding the duct but can affect its ability to migrate.

#### 3.2.1.2.1 Results

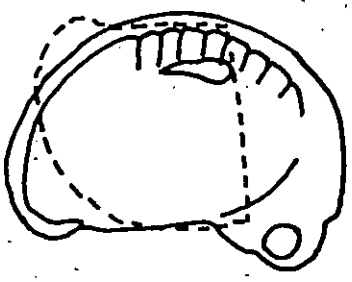
Transplants were done at two different stages in order to determine whether any observed effects is stage-dependent. In control cases, where the epidermis was reciprocally transplanted in normal orientation, no effect was observed

Figure 10

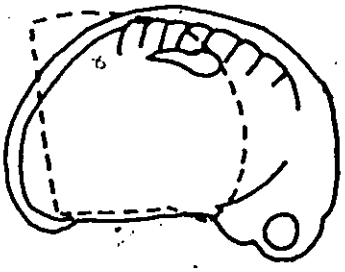
Diagram illustrating transplantation of the epidermis in different orientations. (a) Controls: the epidermis is transplanted in normal orientation. (b) D-V reversals: the epidermis from the left side of one embryo is inverted and then placed onto the right side of a second embryo. (c) A-P reversals: the epidermis from the left side of one embryo is transplanted onto the right side of a second embryo. (d) A-P and D-V reversals: the epidermis from the right side of one embryo is inverted and then placed onto the right side of a second embryo.



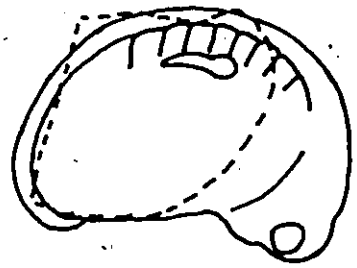
a



b



c



d

on duct migration (Table 2; Fig. 11a). The same was true of embryos in which the epidermis was transplanted in reverse D-V orientation (Table 2).

When the A-P axis was reversed in 11 somite stage embryos, no effect on duct migration was observed. However, when 7 somite embryos were used, the duct was always located at the posterior end of somite 14, regardless of how many somites were present at the time of fixation (Table 3; Fig. 11b). This suggests that the duct had stopped migrating.

When both axes were reversed, duct migration in both 7 and 11 somite embryos was affected (Table 2). However, the effect on migration was different for the two stages. When the transplants were performed at the 7 somite stage, the duct tip was later found behind its normal position (Table 4; Fig. 11c). Unlike the previous set of experiments, the final duct position was variable; the average position, measured in terms of somite number, was 14.3 $\pm$ 1.7. Due to this variation, I was unable to determine whether the duct had actually stopped migrating or merely slowed down.

When the transplants were performed at the 11 somite stage, the duct separated into two parts: an anterior portion which, in most cases, terminated beneath somites 6-8 and a posterior portion, the tip of which was located in the normal duct position (Table 4; Fig. 11e-f). There was always an empty portion of duct path separating the two parts of the duct.

In order to investigate the stage-dependence of the A-P

Table 2. Summary of Epidermis Transplant Experiments

| Type of transplant | Stage of transplant | No. of cases | No. with effect on duct migration |
|---|---|---|---|
| Control | 7S | 3 | 0 |
| Control | 11S | 4 | 0 |
| D-V reversal | 7S | 4 | 0 |
| D-V reversal | 11S | 8 | 0 |
| A-P reversal | 7S | 5 | 5 |
| A-P reversal | 11S | 6 | 0 |
| A-P and D-V | 7S | 7 | 6 |
| A-P and D-V | 8S | 4 | 4 |
| A-P and D-V | 11S | 8 | 7 |
| Young embryo + Old epidermis | - | 8 | 8 |
| Old embryo + Young epidermis | - | 8 | 0 |

Table 3. Effects of A-P reversed epidermis on duct migration in 7 somite embryos.

| No. of Somites at Fixation | Duct Position+ at Fixation | No. of Somites Behind++ |
|---|---|---|
| 17.5* | 14E | 4.0 |
| 18.0 | 14E | 7.0 |
| 18.5 | 14E | 5.0 |
| 19.0 | 14E | 4.5 |
| 21.0 | 14E | 3.5 |

+ the position of the duct tip was measured in terms of somite number; B, M and E stand for beginning (anterior end), middle and end (posterior end) of the somite, respectively

++ number of somites posterior to the duct tip

* a somite that had not completely segmented at the time of fixation was counted as a half somite

+ the position of the duct tip was measured in terms of somite number; B, M and E stand for beginning (anterior end), middle and end (posterior end) of the somite, respectively

++ number of somites posterior to the duct tip

* a somite that had not completely segmented at the time of fixation was counted as a half somite

** the first is the position of the anterior portion; the second is the position of the duct tip

Table 4. Effects of A-P and D-V reversed epidermis on duct migration

| No. of Somites at Transplantation | No. of Somites at Fixation | Duct Tip Position[+] | Somites Behind[++] |
|---|---|---|---|
| 7.0 | 18.0 | 12B | 7.0 |
| 7.0 | 19.0 | 13E | 6.0 |
| 7.0 | 19.0 | 14E | 5.0 |
| 7.0 | 19.5[*] | 15M | 5.0 |
| 7.0 | 20.0 | 12E | 8.0 |
| 7.0 | 20.0 | 16E | 4.0 |
| 8.0 | 17.5 | 10B | 8.5 |
| 8.0 | 19.0 | 12E | 7.0 |
| 8.0 | 20.0 | 11M | 9.5 |
| 8.0 | 22.0 | 16E | 6.0 |
| 11.0 | 16.0 | 6B, 15B[**] | 2.0 |
| 11.0 | 18.5 | 6E, 17E | 1.5 |
| 11.0 | 18.5 | 7M, 16E | 2.5 |
| 11.0 | 18.5 | 8E | - |
| 11.0 | 19.0 | 6M, 17E | 2.0 |
| 11.0 | 19.0 | 11M, 17E | 2.0 |
| 11.0 | 20.0 | 7B, 19E | 1.0 |

Table 5. Effect of an Old Epidermis on Duct Migration in Young Embryos

| No. of Somites at Fixation | Duct Position[+] at Fixation | No. of Somites Behind[++] |
|---|---|---|
| 16.0 | 12M | 4.5 |
| 17.5* | 13M | 5.0 |
| 18.0 | 13M | 5.5 |
| 18.0 | 14B | 5.0 |
| 18.5 | 15E | 3.5 |
| 19.5 | 14E | 5.5 |
| 20.0 | 12E | 8.0 |
| 21.0 | 12B | 10.0 |

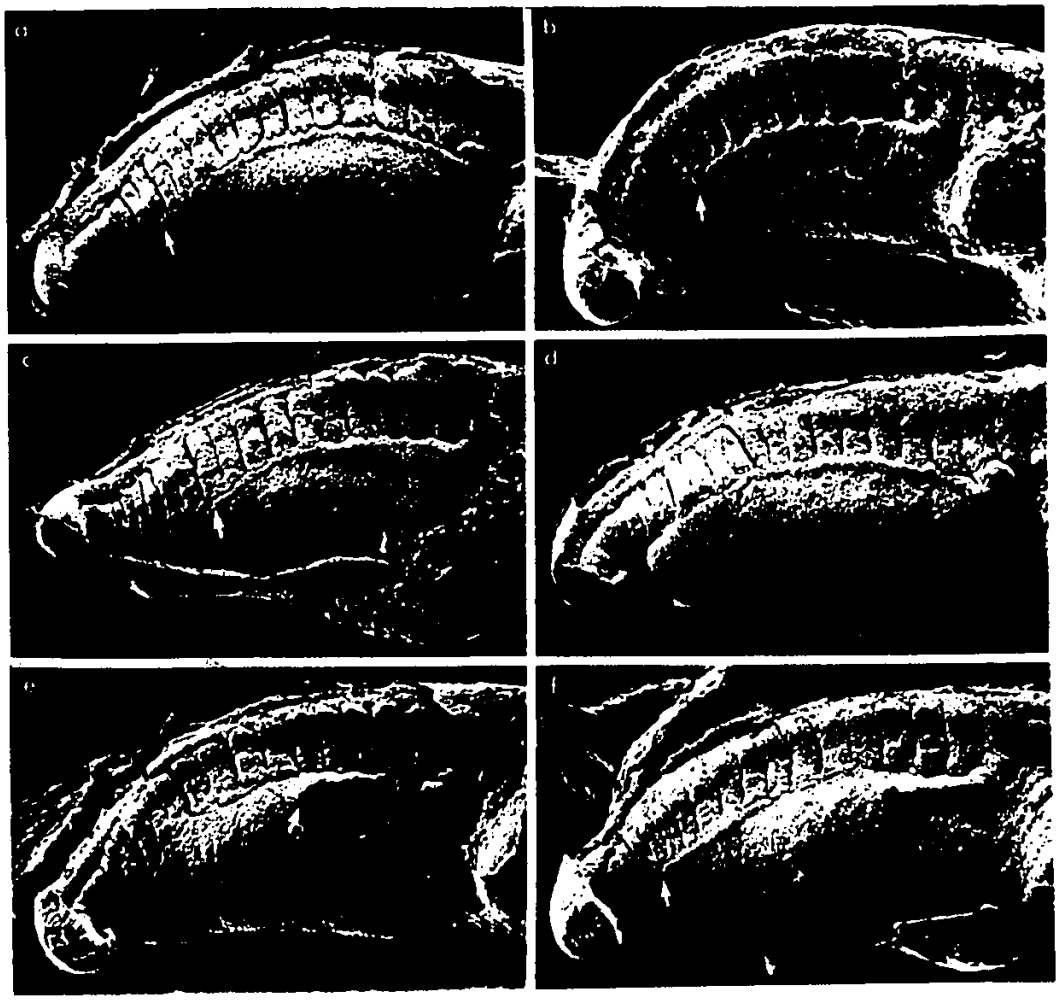
+ the position of the duct tip was measured in terms of somite number; B, M and E stand for beginning (anterior end), middle and end (posterior end) of the somite, respectively

++ number of somites posterior to the duct tip

* a somite that had not completely segmented at the time of fixation was counted as a half somite

Figure 11

Epidermis transplants. Embryos were fixed 24 hours after transplantation; the position of the duct tip is indicated by an arrow. (a) Control. (b) A-P reversal at the 7 somite stage. (c) A-P and D-V reversal at the 7 somite stage. (d) Old epidermis transplanted onto a younger embryo. (e-f) A-P and D-V reversal done at the 11 somite stage. In one case (e), the duct remained intact; in all other cases, the duct separated into 2 parts (f). (x28)



and D-V reversals, I decided to examine the effect at another stage. Transplants were performed at the 8 somite stage. In these embryos, the effect was similar to that seen at the 7 somite stage: the duct tip was located behind its normal position ($x = 12.6 \pm 3.2$; Table 4).

Reciprocal transplants were performed between young (7-8 somites) and old (11-12 somites) embryos. The epidermis was always transplanted in the normal orientation. In all 11-12 somite embryos that received a younger epidermis, the duct migrated normally (Table 2). In embryos of the reverse combination, however, the duct was always located behind its normal position (Table 5; Fig. 11d). The average duct position was below somite 12 ($x = 12.7 \pm 1.3$). Like the A-P and D-V reversals, one would require more cases in order to determine whether the duct had actually stopped migrating.

3.2.1.2.2 Discussion

The control transplants show that the observed effects were not due to the operation itself, but rather, to the abnormal orientation of the epidermis.

Pronephric duct migration was unaffected when the D-V axis was reversed, suggesting that the polarity of this axis is not important for duct migration. This suggests that the epidermis does not contain guidance information because one would expect a D-V reversal to lead to deviation of the duct ventrally over the lateral mesoderm towards the original dorsal side of the epidermis.

The effect on migration in the other experiments depended on the stage at which the transplant was done. Due to the complicated nature of these results, the following hypothesis is proposed in order to explain all the effects on duct migration. A wave of change is postulated to pass through the epidermis. The following properties of this wave were postulated in order to explain the results obtained in this section:

1) since the duct was never found to deviate from its path, this wave is not directly responsible for guiding migration.

2) in order to explain the duct's behaviour when both axes of the epidermis were reversed at the 11 somite stage, I am postulating that the duct can migrate on either side of the wavefront but cannot cross it in reverse A-P orientation.

3) in order to explain the results of the young to old transplants, I postulate that this wave represents the epidermal equivalent of the mesodermal **post-migration** wave and that it can be transfered in some way to the lateral mesoderm in regions where the mesodermal post-migration wave has not already passed. This will initiate a second post-migration wave which will proceed posteriorly. When the duct reaches the point of initiation of the second wave, it will be unable to continue migrating. These properties are consistent with all the results of the epidermis transplants.

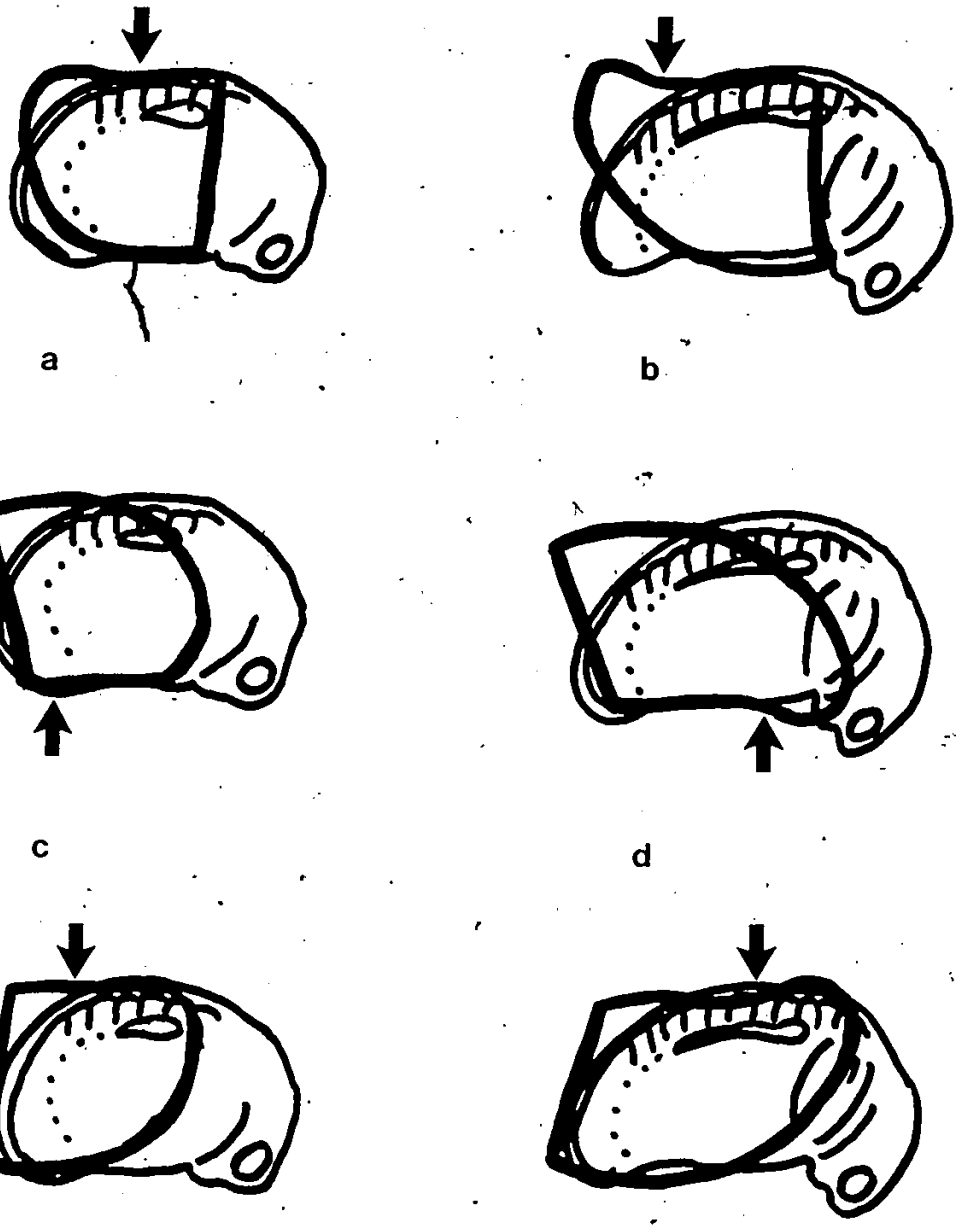
The A-P position of the wave would depend on the developmental age of the embryo, thus accounting for the

different effects observed at the 7 and 11 somite stages. In A-P reversals, no effect on migration was observed in 11 somite stage embryos because the transplanted epidermal wave would be located over the pronephros (Fig. 12). In 7 somite stage embryos, the transplanted epidermal wave would be located posterior to the host's post-migration wave (Fig. 12). The epidermal wave would be transfered to the lateral mesoderm, thus initiating a second post-migration wave. Once the duct reaches this point, it would be unable to migrate further.

When both axes are reversed, the transplanted epidermal waves would be in the same positions as in the A-P reversals. Therefore, one would expect to see the same effect in both types of transplants. This was the case when 7 somite embryos were used but not when 11 somite embryos were used. This difference can be explained by realizing that the irregular shape of the embryo as well as the shrinkage of the epidermis after dissection results in the placing of the epidermal wave in slightly different A-P locations, depending on whether the D-V axis is also reversed (Fig. 12). One can see that when both axes are reversed, the epidermal wave would be located over the anterior end of the duct rather than over the pronephros. By assuming that duct cells can migrate on either side of the wavefront but cannot cross it in reverse A-P orientation, one can explain the effect as follows: that part of the duct located anterior to the wave is stopped while those cells

Figure 12

Diagramatic illustration of proposed epidermal wave. Although the exact position of this wave could not be determined, the results of the epidermis transplants are consistent with a location in the vicinity of the duct tip. Thus, if one uses the position of the duct tip (arrow) as a reference point in the donor epidermis, one can see that in D-V reversals (a,b), the position of the wave in donor and host would coincide. In the A-P reversal at the 7 somite stage (c), the position of wave in the donor would be posterior to that of the host. The same is true for A-P and D-V reversals done at the same stage (e). In an 11 somite embryo, an A-P reversal would place the reference point over the pronephros of the host (d), while an A-P and D-V reversal places this same point over the anterior end of the duct (f).


a
b
c
d
e
f

located posterior to the wavefront continue to migrate normally. The continued migration of the posterior tip results in a tugging on the anterior portion, causing the latter to pile up at the epidermal wavefront. Eventually, the posterior portion must separate from the anterior part in order to continue migrating.

The results of the reciprocal epidermal transplants between young and old embryos can be explained by assuming that the epidermal wave can be transfered to the lateral mesoderm in regions where the post-migration wave has not already passed. If this is correct, then one should observe an effect only in young embryos with an old epidermis. In these embryos, the duct should stop when it reaches the point where the second post-migration wave has been initiated and therefore be located behind its normal position. One would not see an effect on old embryos with a young epidermis because the epidermal wave would be located anterior to the host's post-migration wave. This is exactly what was observed.

In embryos with an older epidermis, the duct was not always located in exactly the same spot. This could be due to inaccuracy in placing the epidermis in identical positions and/or to slight variations in the actual stage of the embryo. On the other hand, it could mean that the duct is still migrating. As a result of this variation, one would require more transplants of this kind and statistical analysis of the results.

The results presented in this section have led to the conclusion that the epidermis does not guide the duct but can inhibit duct migration. They have also suggested the existence of a wave that passes through the epidermis.

## 3.2.2 CHANGES IN THE MORPHOLOGY OF THE LATERAL MESODERMAL CELLS

The results from section 3.2.1.1 provide evidence that a specific region of the lateral mesoderm contains guidance information for duct migration. We also know that the lateral mesoderm is not a static substratum for migration. There are changes taking place, not only in the shape of the tissue as a whole, but also in the shape and position of the individual cells. The lateral mesoderm itself increases significantly in length along the A-P axis, while the individual cells change from being relatively round to being elongated along the D-V axis. Therefore, I decided to examine the lateral mesoderm for morphological changes that might be correlated with duct migration.

In this study, I will show that the cells of the lateral mesoderm undergo a series of shape changes that might be causally related to duct migration. Possible models are discussed.

### 3.2.2.1 Results

The cells of the lateral mesoderm were examined in light microscope sections as well as in the scanning electron microscope. Electron micrographs of the fractured edge of the lateral mesoderm below the duct tip revealed differences in both cell shape and orientation at different A-P levels in the embryo. These differences were not evident

at stages prior to duct migration. At these early stages, the lateral mesoderm consists of a 2 cell layer and all the cells appear columnar (Fig. 13a-b). During duct migration stages, cells in an anterior position with respect to the duct tip are relatively flat, with one cell sitting on top of another (Fig. 14a, 15b). Cells posterior to these appear to be triangular, with the top and bottom cells interdigitated (Fig. 13d, 14b, 15c). In the region of the pronephric duct tip, the lateral mesoderm appeared to be composed of columnar cells that form a single cell layer (Fig. 13c-d, 14c, 15a,d). Posterior to this, the cells are cuboidal and form a 2 cell layer (Fig. 13c, 14d). By far the most obvious difference among the cells of this region was their difference in height.

In order to determine whether these shape changes take place in the cells of the duct path and to obtain quantitative information about these shape changes, measurements of cell height were made from serial sections. In all four stages examined, the same profile was observed. The cells increased in height from 20 to 50 um as they went from a 2-cell to a single cell layer and then decreased in height (to 20 um) as they went back to a 2-cell layer (Fig. 16-19). At each stage, the height of the tallest cell was found to be significantly different ($P < 0.05$) from the height of either the most anterior or the most posterior cell measured. The heights of the most posterior and most anterior cell, however, were not significantly different ($P >$

a
b
c
d

Figure 13

Morphological changes in the cells of the lateral mesoderm. A total of 24 embryos were examined. The anterior end of the embryo is on the right hand side in each micrograph. The lateral mesoderm has been fractured along the A-P axis. (a) a 3 somite embryo; the lateral mesoderm is composed of a 2-cell layer of columnar cells. (b) a 5 somite embryo; the lateral mesoderm still consists of a 2-cell layer, although cell height has decreased slightly. (c) an 11 somite embryo; note the position of the duct tip (black arrow) with respect to the columnar cell (white arrow). Posterior to the columnar cell are cuboidal cells. (d) a 14 somite embryo; note the position of the duct tip (black arrow) with respect to the columnar cells (white arrow). Triangular shaped cells are found anterior to the the columnar cells. (a-b, x134; c, x625; d, x500)

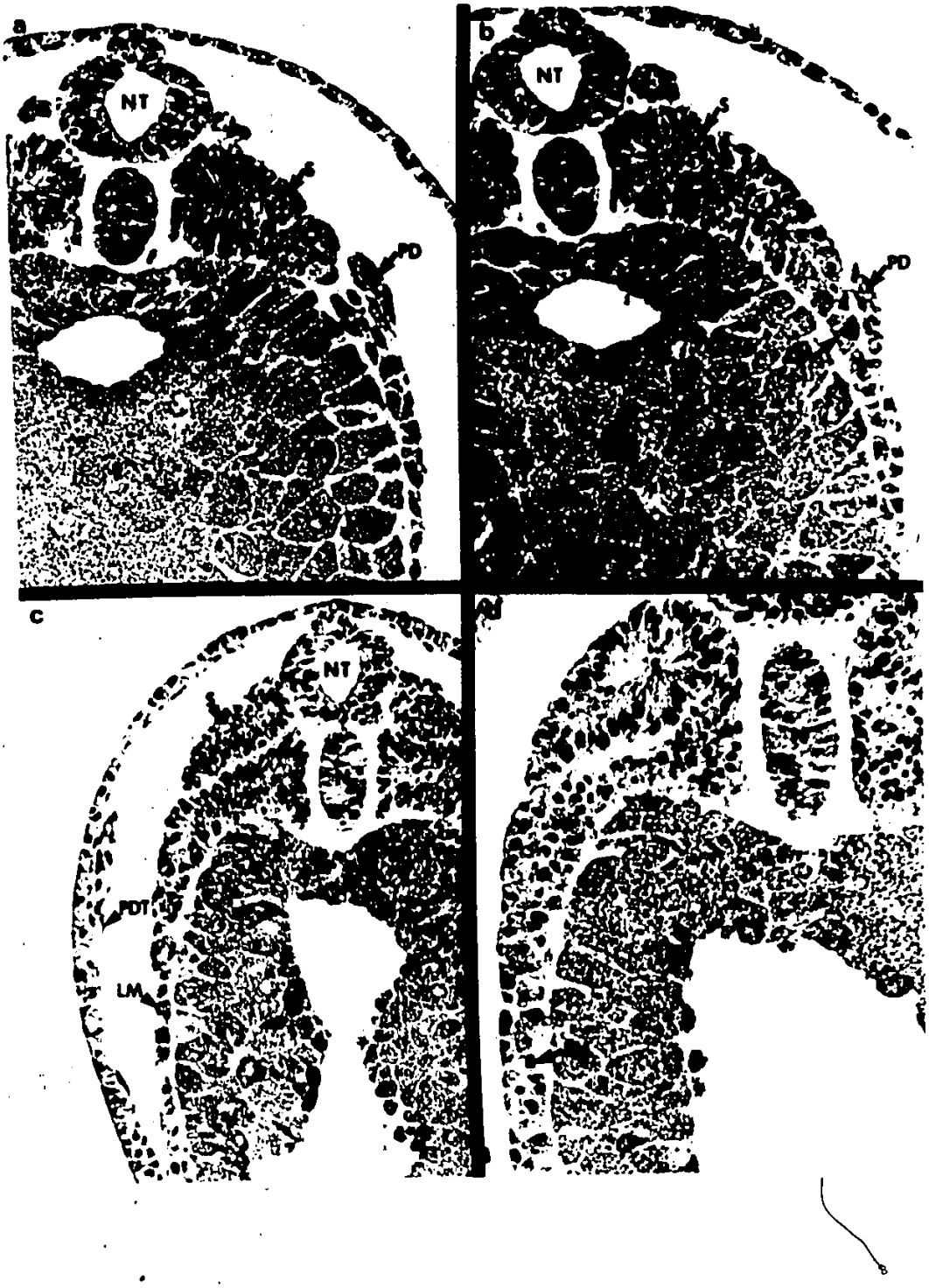

a
NT
PD
b
NT
PD
c
NT
PDT
LM
d

Figure 14

Morphological changes in the cells of the lateral mesoderm. A total of 18 embryos were examined. Cross-section through a 10 somite embryo showing (a) 2-cell layer of the lateral mesoderm (LM) anterior to the pronephric duct (PN) tip; (b) triangular cells of the lateral mesoderm in a section posterior to that in (a); (c) columnar cells in the lateral mesoderm under the pronephric duct tip (PDT); (d) 2-cell layer of the lateral mesoderm at a level posterior to the duct tip. E=endoderm, NT=neural tube, N=notochord, S=somite. (a, x108; b, x103; c, x80; d, x127)

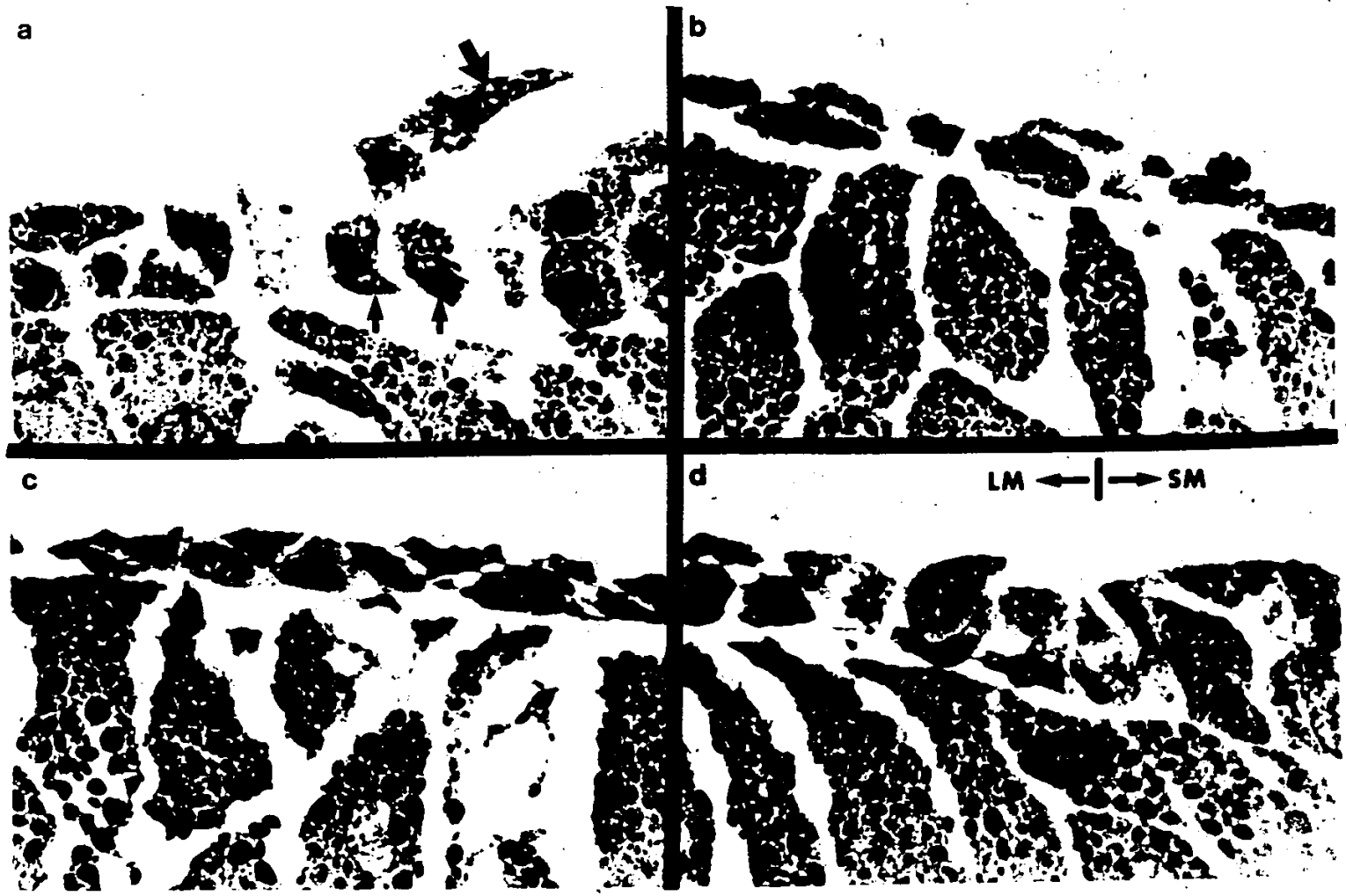

a
b
c
d
LM
SM

Figure 15

Morphological changes in the cells of the lateral mesoderm. The anterior end of the embryo is on the left hand side of each photograph. Frontal section through a 14 somite embryo. (a) Pronephric duct tip cells (large arrow) sitting on columnar cells (small arrows) of the lateral mesoderm. (b) Flat cells of the lateral mesoderm in the anterior region of the same section. (c) Triangular cells located immediately anterior to the duct tip cells in (a). (d) Columnar cells in the lateral mesoderm (LM) immediately posterior to the duct tip in (a). The juncture between lateral and somitic mesoderm (SM) is indicated. (x296)

Figure 16

Changes in lateral mesodermal cell height in 9 somite stage embryos. The height of eight contiguous duct path cells in the vicinity of the duct tip were measured. Each value is averaged from four cases; the standard deviations are indicated. In regions where the lateral mesoderm consists of a two cell layer (stippled bars), the average height was used. The horizontal line represents the duct tip cell in each case.

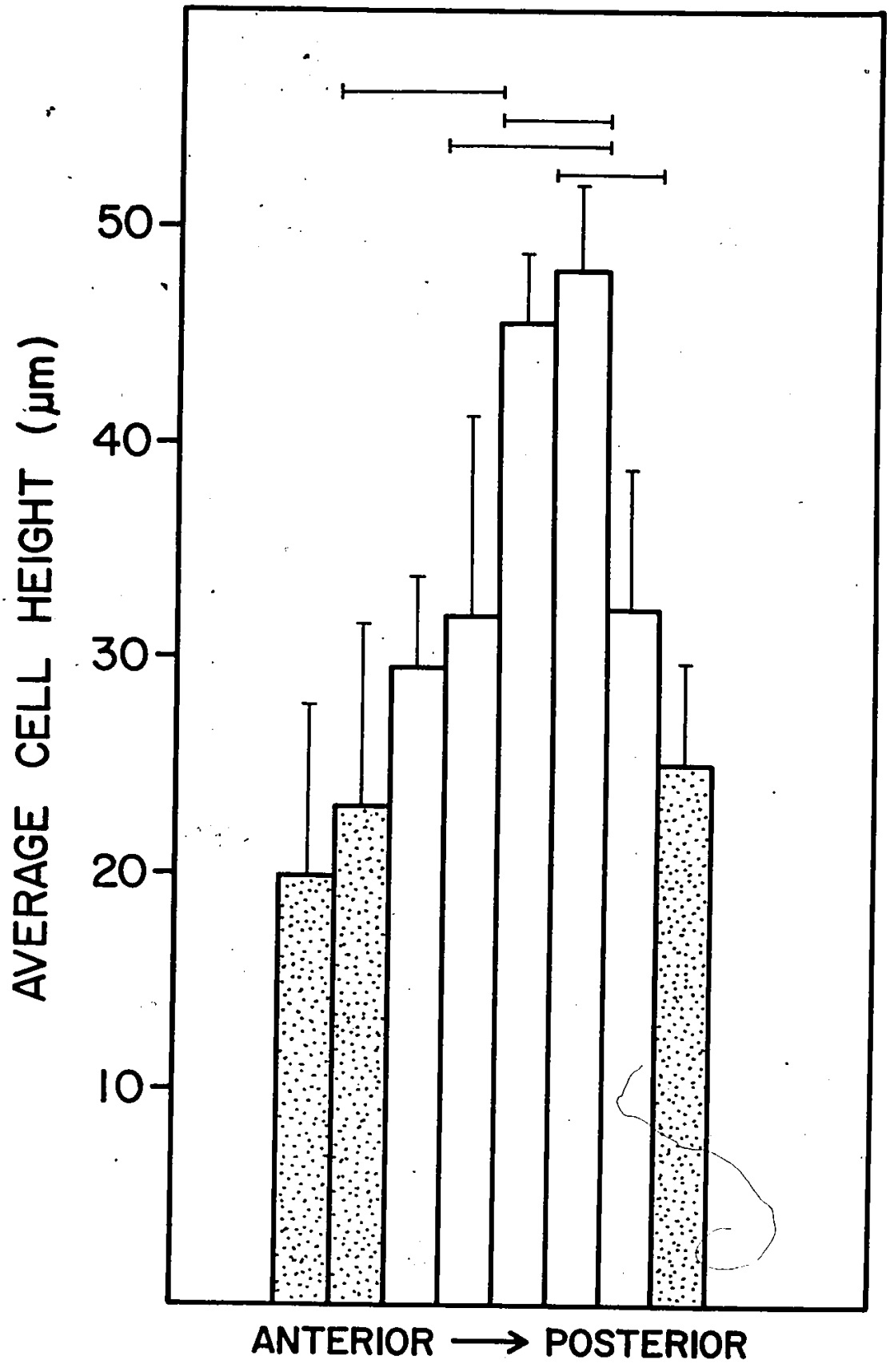

AVERAGE CELL HEIGHT (μm)
50
40
30
20
10
ANTERIOR → POSTERIOR

Figure 17

Changes in lateral mesodermal cell height in 10 somite stage embryos.

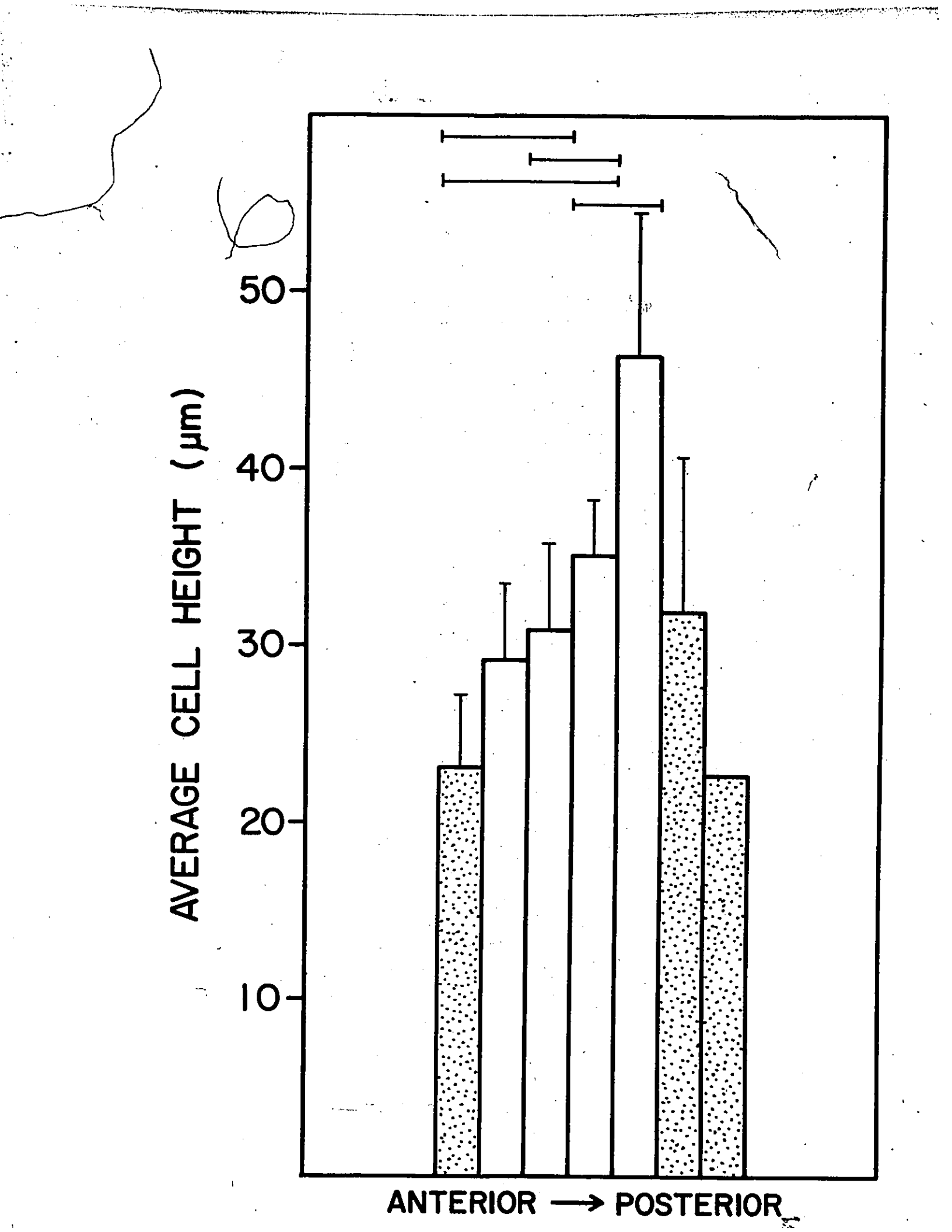

50
40
30
20
10
AVERAGE CELL HEIGHT (μm)
ANTERIOR → POSTERIOR

Figure 18

Changes in lateral mesodermal cell height in 11 somite stage embryos.

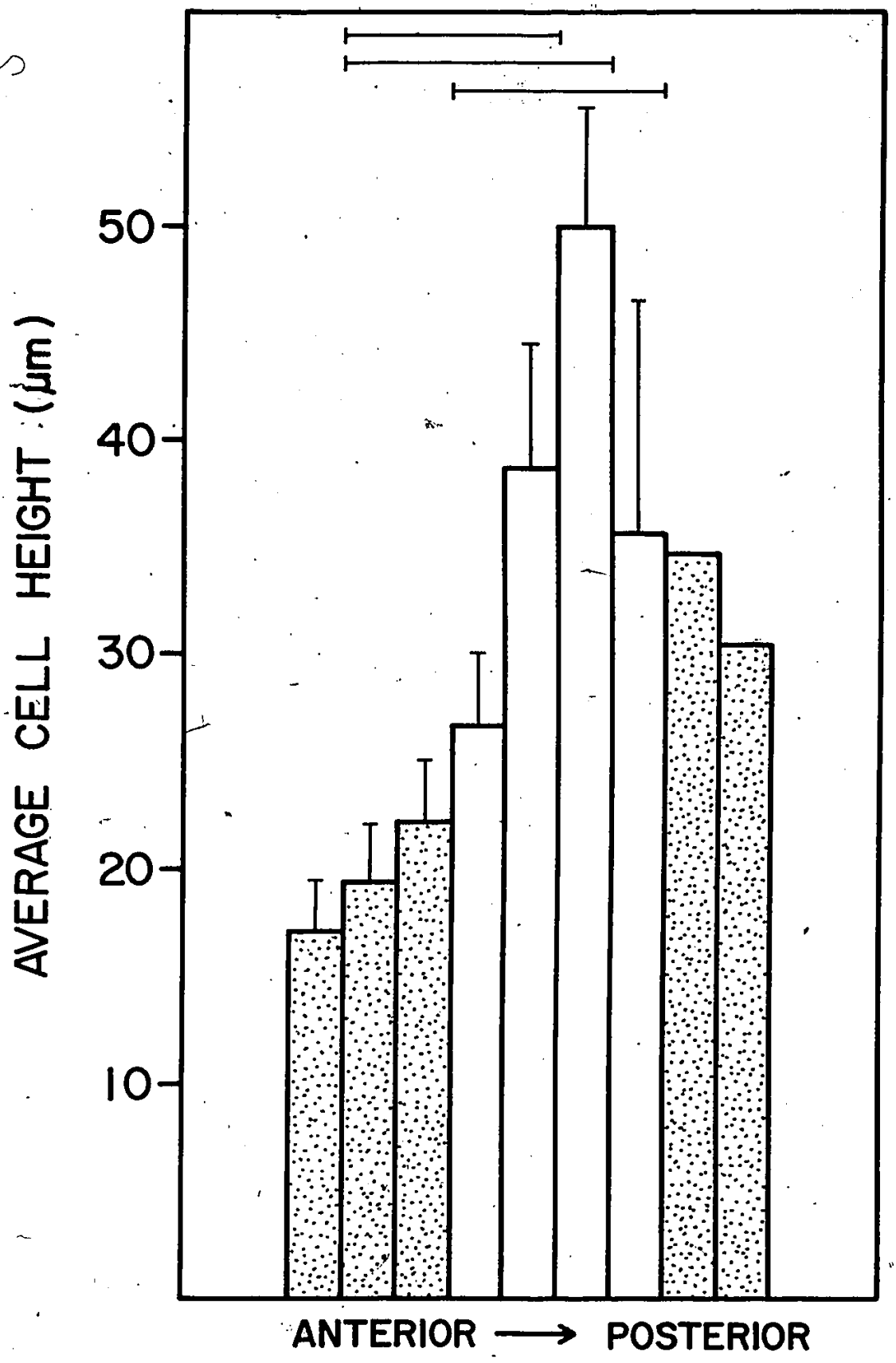
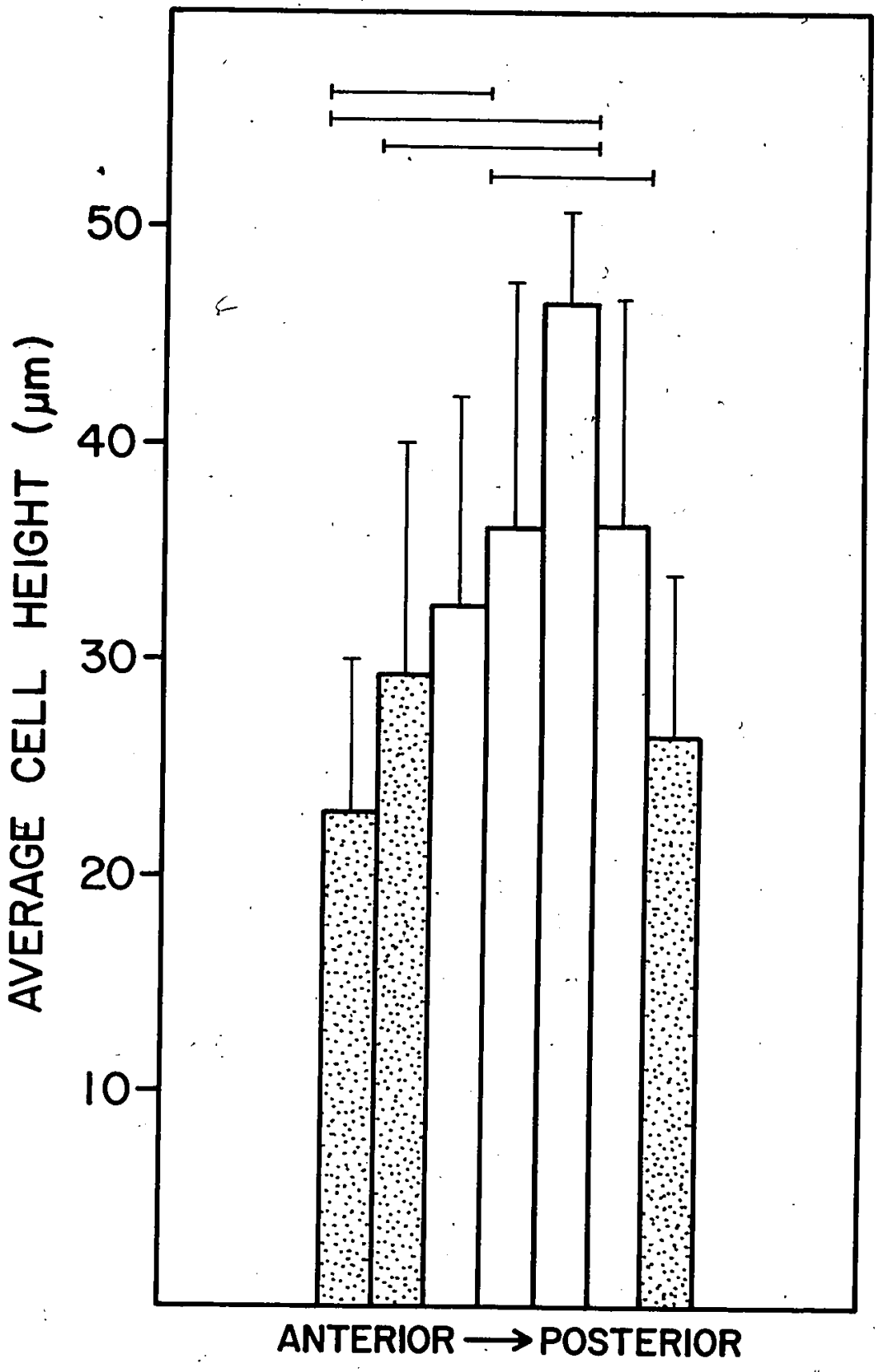

AVERAGE CELL HEIGHT (μm)
50
40
30
20
10
ANTERIOR → POSTERIOR

Figure 19

Changes in lateral mesodermal cell height in 13 somite stage embryos.


AVERAGE CELL HEIGHT (μm)
50
40
30
20
10
ANTERIOR → POSTERIOR

0.05) from each other.

The tip cell was always located in the region of this increase in cell height. The posterior end of the tip cell was always located over a single cell whereas the anterior end was sitting on either a 2-cell or a single cell layer.

These shape changes occur not only in the duct path but along the entire dorso-lateral axis (Fig. 14c), thus forming a wavefront. The position of this wavefront of shape change corresponds to that of the **post-migration** wave. Though it is not possible at this time to prove that the two represent the same morphogenetic process, one can obtain indirect evidence that supports this idea. For example, one can calculate the angle that the wave of shape change makes with the D-V axis and compare it to the angle calculated for the **post-migration** wave from the secondary duct transplants. The former was calculated from cross-sections rather than from frontal sections because the latter would require three dimensional re-construction. Angles were measured using the shape of the notochord to determine the angle at which the sections were cut. The average angle $40^{o}$ (4 cases) corresponds very closely to the $41^{o}$ angle calculated from the secondary duct transplants.

### 3.2.2.2 Discussion

Due to the spatio-temporal correlation between these shape changes and duct migration, one can argue that either the two are causally related or they are both the direct

result of a third causal agent or process. If the latter is the case, then one should be able to uncouple them. At the moment, however, I am unable to suggest a method of inhibiting one without directly affecting the other.

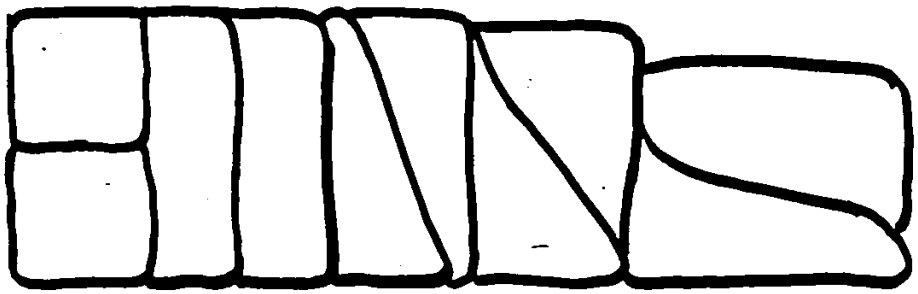
If these changes in morphology are causally related to duct migration, then one must provide an explanation of how such changes could cause the duct to migrate. There are at least two possible models. In the first, illustrated in Fig. 20, one could imagine that the change from a two cell to a one cell layer in the region of the duct tip would result in temporary exposure of the inner (medial) cells to the surface of the lateral mesoderm, and consequently, expose cell surface molecules that are either quantitatively or qualitatively (or both) different from those on the outer (lateral) cells. It would not be unreasonable to expect such differences to exist since the inner and outer cells have different microenvironments; the inner cells are in contact with the endodermal cells while the outer cells are sandwiched between the inner cells and the epidermis.

In this model, there are two possible types of interaction between these exposed inner cells and the pronephric duct tip. The exposed inner cells provide either a more suitable or a less suitable substratum for migration than the outer cells. In the first case, the duct tip would adhere preferentially to the inner cells. Return of these cells to the inner surface with concommittant exposure of inner cells in an adjacent region would cause the duct to

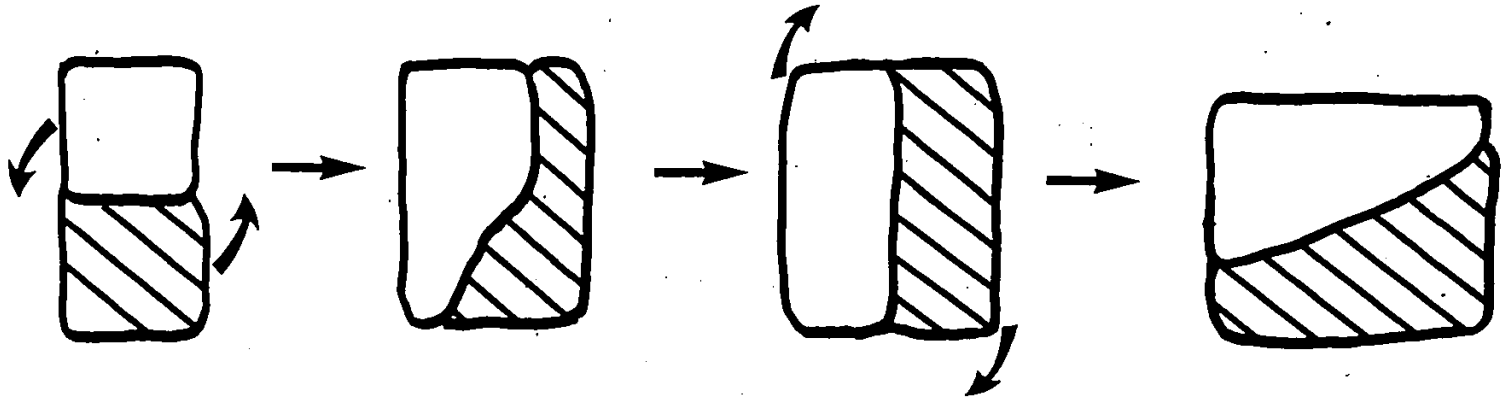
Figure 20

Model illustrating relationship of lateral mesodermal cell shape change to duct migration. A general scheme of the morphological changes is illustrated in (a). Cuboidal cells posterior to the duct tip (b) interdigitate (c) to form two columnar cells (d). This exposes the medial cell (hatched) to which the duct tip would adhere preferentially. Gradually this cell returns to the inner layer (e), leaving the duct tip to form contacts with another cell.

POSTERIOR ANTERIOR



a



b c d e

migrate posteriorly to that spot. This model implies that the duct cells are being **pulled** along the duct path. If, on the other hand, the duct tip cannot adhere as well in this region, then the duct is being **pushed** along the duct path in order to remain ahead of this region. At the moment, it is not possible to distinguish between the two.

A second possibility is that these cells are preparing to undergo mitosis. This model does not require a difference between inner and outer cells. One must next explain how mitosis could cause the duct to migrate. It is well known that culture cells undergoing mitosis detach from their substratum and become rounded. One can see the same process in sections of axolotl embryos. Cells undergoing mitosis are rounded and are quite separate from surrounding cells. If the lateral mesodermal cell beneath the duct tip becomes dettached, then the duct tip would have no choice but to adhere to some other cell. Since the duct tip cannot backtrack, it must either go posteriorly or ventrally. It cannot migrate ventrally because the cell below it would also be undergoing mitosis (due to the angle of the wavefront calculated in section 3.2.1.1). Therefore, the duct tip would have no choice but to move posteriorly.

These observations have illustrated morphological changes in the cells of the lateral mesoderm that are spatio-temporally linked to duct migration. Although a causal relationship has not been established, these changes may represent a morphogenetic process that could be

important for directing duct migration.

## 3.2.3 DISTRIBUTION OF EXTRACELLULAR MATRIX DURING PRONEPHRIC DUCT MIGRATION

Extracellular matrix has been implicated in growth regulation, cell adhesion, alteration of biosynthethic patterns and cell motility (for review, see Hay, 1981; Yamada, 1983). For example, components of the ECM have been shown to play a role in gastrulation in chick embryos (Wakely and England, 1979). In addition, the localization of ECM fibrils on the blastocoelic wall in several amphibian species (Nakatsuji et al., 1982; Komazaki and Hirakow, 1982; Komazaki, 1983) has led authors to suggest that the fibrils act as a contact guidance mechanism for cell migration during gastrulation.

As a preliminary to the study of the importance of this matrix in duct migration, the spatio-temporal distribution of ECM was examined in light microscope sections and the scanning electron microscope.

In this section, I will demonstrate that ECM fills the space between the mesoderm and epidermis and that the duct migrates through this material. In addition, I will show that the posterior limit of visible ECM is spatio-temporally correlated with duct migration.

### 3.2.3.1 Results and Discussion

During the course of examining light microscope sections and embryos in the scanning electron microscope, I

observed large quantities fibrous material which, based on its structure and location, I suspected was extracellular matrix. This material was located between the mesoderm and the epidermis.The first step in this study was to determine whether this material was in fact ECM and not a fixation artifact.

Sectioned material was specifically stained for polysaccharides using the PAS method. This staining procedure has been used routinely to stain ECM due to large quantities of glycosylated compounds present (e.g. glycoproteins, glycosaminoglycans). Sections prepared in this fashion displayed staining throughout most tissues (Fig. 21a). This was to be expected since the yolk platelets contains substantial amounts of glycolipids. In addition to this, the material between the epidermis and mesoderm was found to stain quite intensely. Control slides, on the other hand, showed virtually no staining (Fig 21b). One can conclude from this that this fibrous network is indeed ECM.

The distribution of ECM can be observed most clearly in the SEM as a thick web-like network covering the mesoderm (Fig. 22). Frequently the pronephric duct tip cell could be seen embedded in the matrix (Fig. 22d).

Embryos were examined at several different stages and the posterior limits determined. It was found that the posterior limit was always located posterior to the duct tip and, in most cases, the ECM ended anterior to the last formed somite (Table 6; Fig. 22a-c). The average position

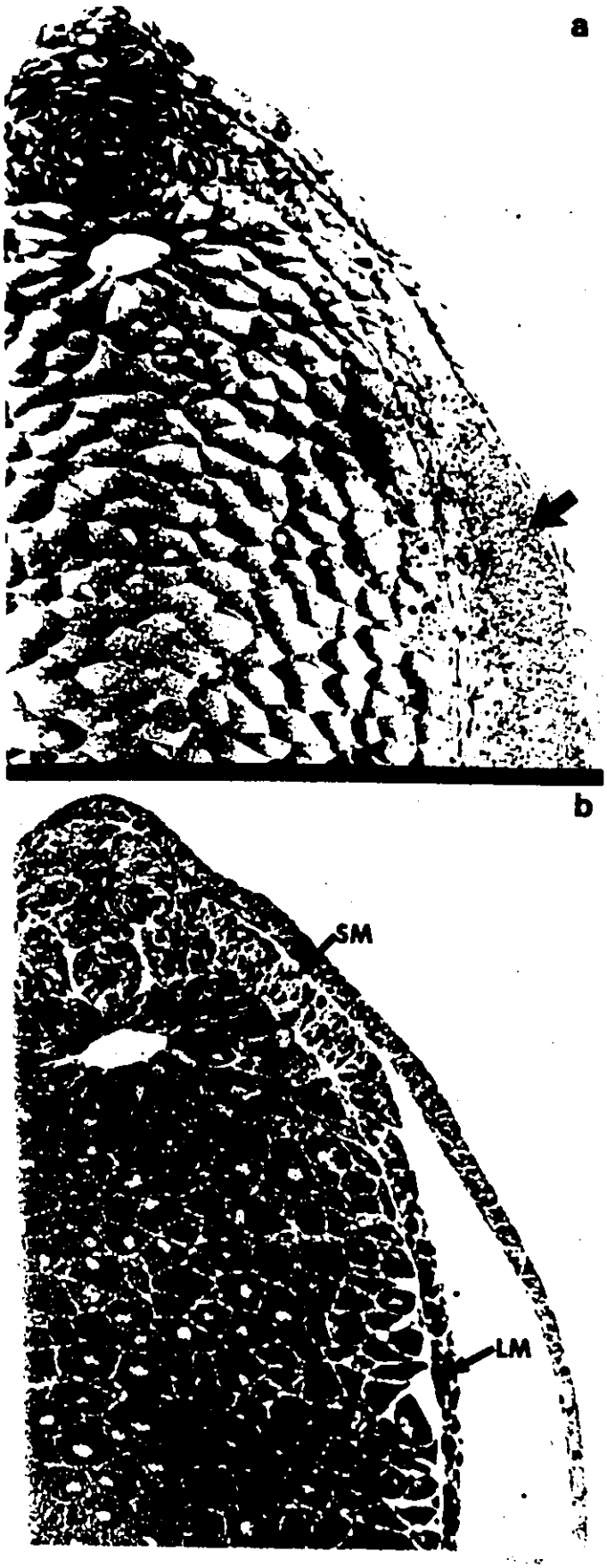

a
b
SM
LM

Figure 21

Periodic-acid Schiff staining of sectioned embryos. (a) PAS staining showing the presence of ECM (arrow) in the subepidermal space. (b) Control (no periodic acid treatment); no staining was observed. LM=lateral mesoderm, NT=neural tube, N=notochord, SM=somitic mesoderm. (x100)

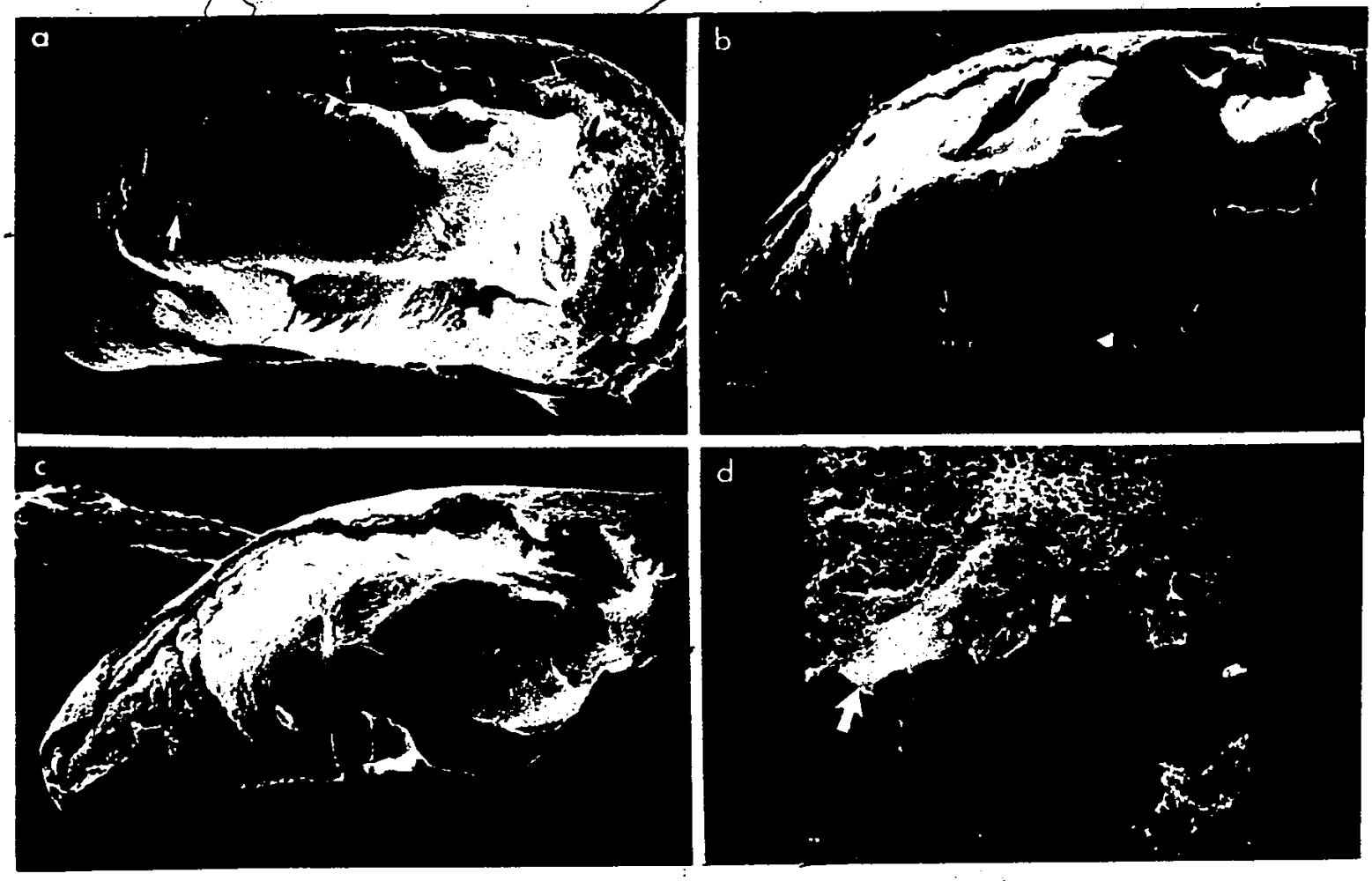

a
b
c
d

Figure 22

ECM distribution. A thick layer of ECM covers the mesoderm in stage 28 (a) and stage 32 (b-c) embryos. (d) A higher magnification of the region indicated by the arrow in (a) reveals the pronephric duct tip cell (arrow) embedded in the ECM. (a-c, x41; d, x1,100)

* the position of the posterior limit was measured in terms of somite number; B, M and E stand for beginning (anterior end), middle and end (posterior end) of the somite, respectively

** a negative value indicates that this limit is posterior to the last formed somite

+ a somite that had not completely segmented at the time of fixation was counted as a half somite

The average number of somites posterior to the limit of visible ECM was 1.2 $\pm$ 0.7

Table 6. Distribution of extracellular matrix over mesoderm

| No. of Somites | Posterior limit of visible ECM* | No. of somites posterior to the limit |
|---|---|---|
| 8.0 | 8B | +1.0 |
| 10.0 | 9M | +1.5 |
| 10.0 | 9M | +1.5 |
| 14.0 | 14E | -0.5** |
| 14.0 | 13M | +1.5 |
| 15.0 | 14M | +1.5 |
| 16.0 | 15E | +1.0 |
| 16.5+ | 16B | +1.5 |
| 17.0 | 17M | +0.5 |
| 18.0 | 17B | +2.0 |
| 18.5 | 17M | +2.0 |

was approximately one somite width anterior to the last formed somite.

The fact that no ECM was visible in extreme posterior regions of the embryo does not necessarily mean that no ECM is present in live embryos but that if ECM is present, it was not preserved by the fixation procedure used. Thus, one may being observing either a quantitative or a qualitative difference in ECM.

The results of this study demonstrate that at all stages examined, a layer of ECM covers all of the lateral and somitic mesoderm up to the last somite where it ends abruptly. Thus, the spatio-temporal distribution of ECM parallels that of somite segmentation and pronephric duct migration.

## 3.3 ROLE OF CELL SURFACE PROTEINS IN DUCT MIGRATION

One of my objectives has been to obtain information about the biochemical nature of the guidance mechanism. Since many developmental processes are thought to be mediated by cell surface molecules, one biochemical approach would be to perturb the system either by inhibition of synthesis or by enzymatic digestion of these cell surface molecules.

In this section, I will describe the effects of tunicamycin and trypsin on pronephric duct migration which will serve to demonstrate the importance of cell surface proteins in duct migration.

### 3.3.1 Effect of tunicamycin on duct migration

In order to examine the involvement of cell surface proteins, embryos were treated with tunicamycin, a drug that specifically inhibits glycosylation of N-linked glycoproteins (Takatsuki et al., 1975), thus interfering with the production of cell membrane and extracellular glycoproteins.

The results of these experiments demonstrate the importance of cell surface glycoproteins for migration along the normal pathway. The observed effects are consistent with the proposed existence of a segmental wave. In addition, they provide information about the angle of this wave with

respect to the D-V axis as well as the distance between the post-migration and segmental waves.

3.3.1.1 Results

These preliminary experiments were performed by microinjecting embryos with 10-25 ng of tunicamycin. The embryos were injected during gastrulation since one would expect the synthesis of surface proteins required for duct migration to take place prior to duct migration. Gastrula were carefully staged according to the shape and size of the blastopore, in order to pinpoint the sensitive stages. The results of five experiments were combined and are reported below.

None of the controls or tunicamycin treated embryos that developed normally showed an effect on duct migration (Fig. 23). Embryos injected at the beginning-middle of gastrulation (stage 9-10 3/4), continue to gastrulate but fail to complete neurulation. Therefore, the possible effect on duct migration could not be studied. In embryos injected at later stages of gastrulation (stage 11-11 1/2), development continued beyond neurulation, however, pronephric duct migration was normal in all cases.

When injected with tunicamycin at the end of gastrulation (stage 12), a small proportion of the injected embryos displayed abnormal duct migration (8/82). In these embryos, the pronephric duct was found to deviate from its normal path and migrate ventrocaudally over the lateral

Figure 23

Effect of tunicamycin on embryonic development. The percentage of abnormal embryos was plotted for each group. The hatched region represents the percentage of embryos that survived past neurulation; the stippled region represents the percentage of embryos that displayed abnormal duct migration. The number of embryos injected in each group is indicated above the bar.

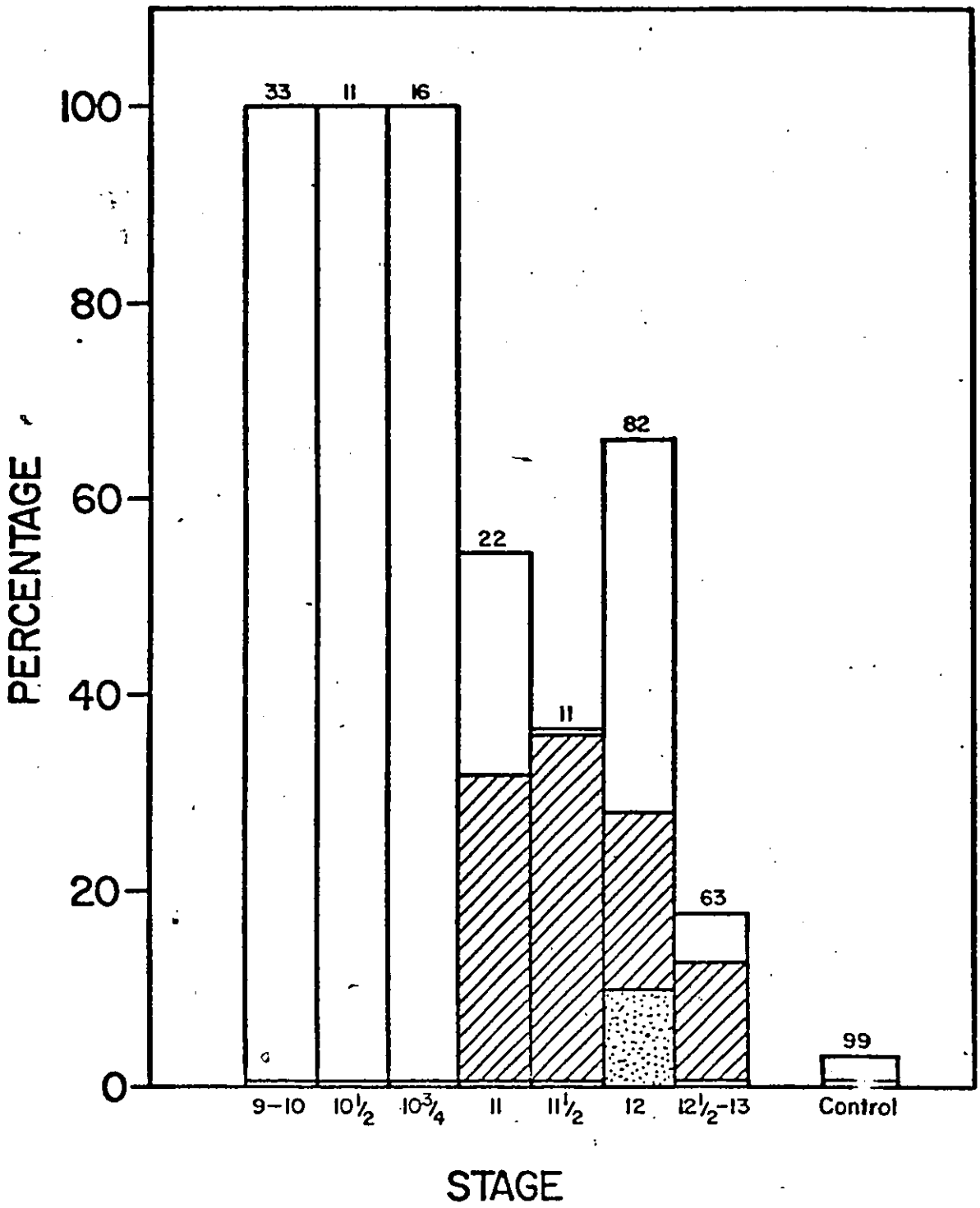
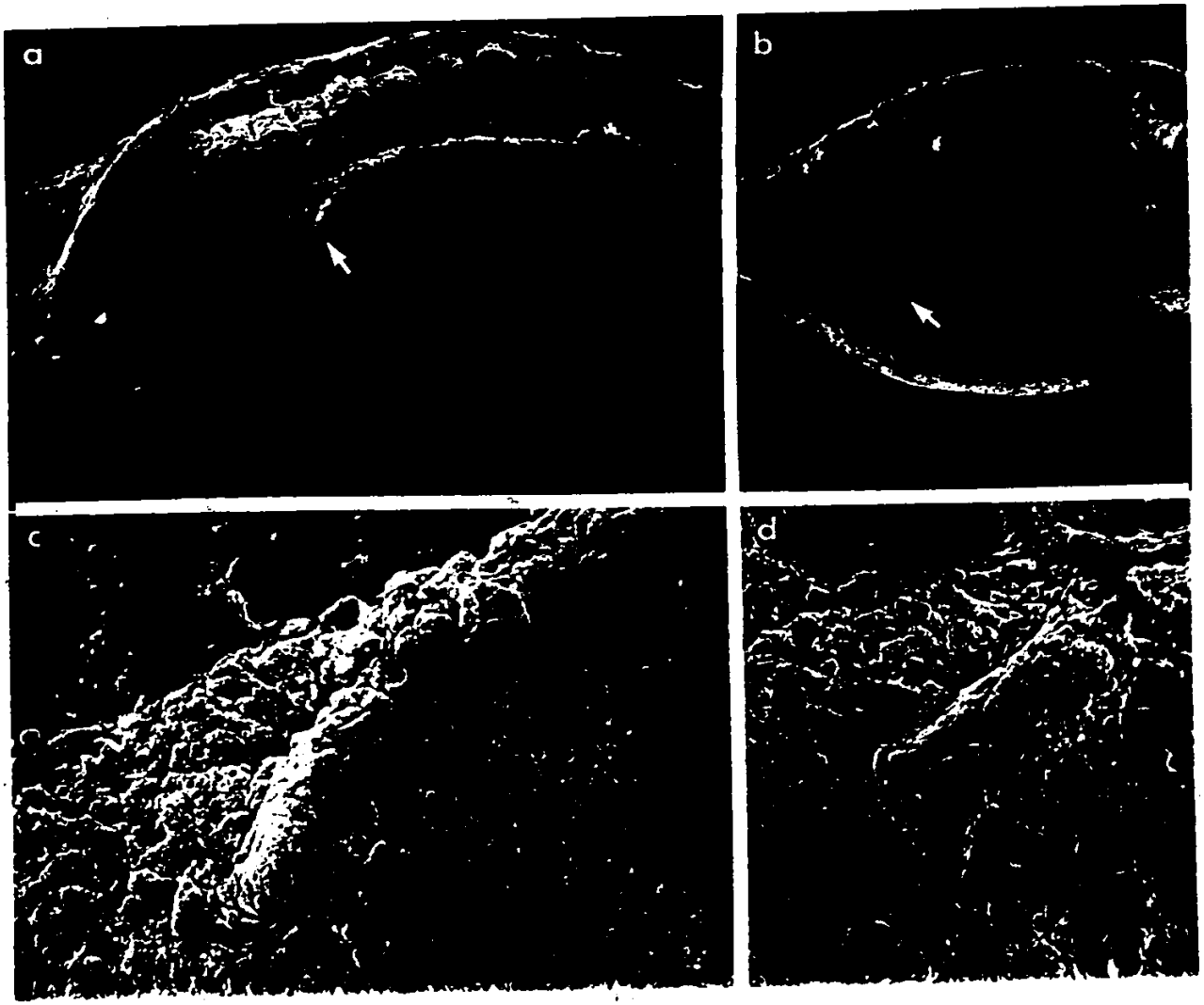

100
80
60
40
20
0
PERCENTAGE
33
11
16
22
11
82
63
99
9–10
10½
10¾
11
11½
12
12½–13
Control
STAGE

Figure 24

Effect of tunicamycin on duct migration. The anterior end of the embryo is on the right hand side of each micrograph. Embryos were injected with 25 ng of tunicamycin at stage 12. The duct (arrow) deviates laterally (a-b). Higher magnification of the duct tip (c-d) reveals its blunt appearance. (a, x66; b, x41; c, x241; d, x156)


a
b
c
d

mesoderm (Fig. 24).

Very few of the embryos injected post-gastrulation (stage 12 1/2-13) displayed abnormalities. Of the few that did develop abnormally, none showed abnormal duct migration.

3.3.1.2 **Discussion**

Only one of the injection stages showed subsequent abnormal duct migration. Perhaps too few stage 11 1/2 embryos were injected to observe an effect. A large enough sample was examined at later stages (12 1/2-13), but it is likely that the mesoderm was no longer accessible to drugs injected into the archenteron since the endoderm almost completely encloses the gut by the end of gastrulation.

In addition to the effect being localized to one stage, only a small percentage of embryos displayed abnormal migration. This could be due to the use of a sub-optimal dose of tunicamycin. However, the highest dose used, 25 ng/200 nl, is the maximum amount soluble in a sub-teratogenic concentration of DMSO (Woo Youn, personal comunication). Regardless of whether these explanations are correct, additional studies of the tunicamycin effects on duct migration are not practical because of the small number of affected embryos. In order to continue this line of study, one would have to find an alternate method of treating the embryos, perhaps by direct exposure of the mesoderm to the drug.

In spite of the small number of affected embryos, one

can obtain some important information about duct migration. The fact that the duct continues to migrate, albeit in an abnormal direction, suggests that the substratum rather than the duct is affected. In addition, if tunicamycin was interfering with the duct cells such that they could no longer recognize their path, one would expect the duct to migrate in a random fashion. This is not what was observed. In all cases, the duct migrated along the duct path to somite 8-9 and then headed ventrocaudally at a $52^{o} \pm 6^{o}$ angle to the D-V axis.

One explanation for this ventral deviation is that the tunicamycin-affected proteins are required for the segmental wave and thus required for duct migration. Since this wave travels in a craniocaudal direction, one might expect the synthesis of proteins involved to take place in the same sequence. Injection of tunicamycin at a specific time would affect only those cells that have not synthesized the important proteins, leading to a sharp boundary between the posterior affected and anterior unaffected cells of the lateral mesoderm. When confronted with this boundary, one might expect the duct to migrate ventrally along this boundary since it cannot migrate posteriorly into the affected area. This explanation is consistent with what is observed when the duct path of normal, untreated embryos is blocked by a dorsal incision (Holtfreter, 1944). The duct migrates ventrally around the obstruction. Thus, the migration path of the duct would represent the wavefront of

changes occuring in the lateral mesoderm.

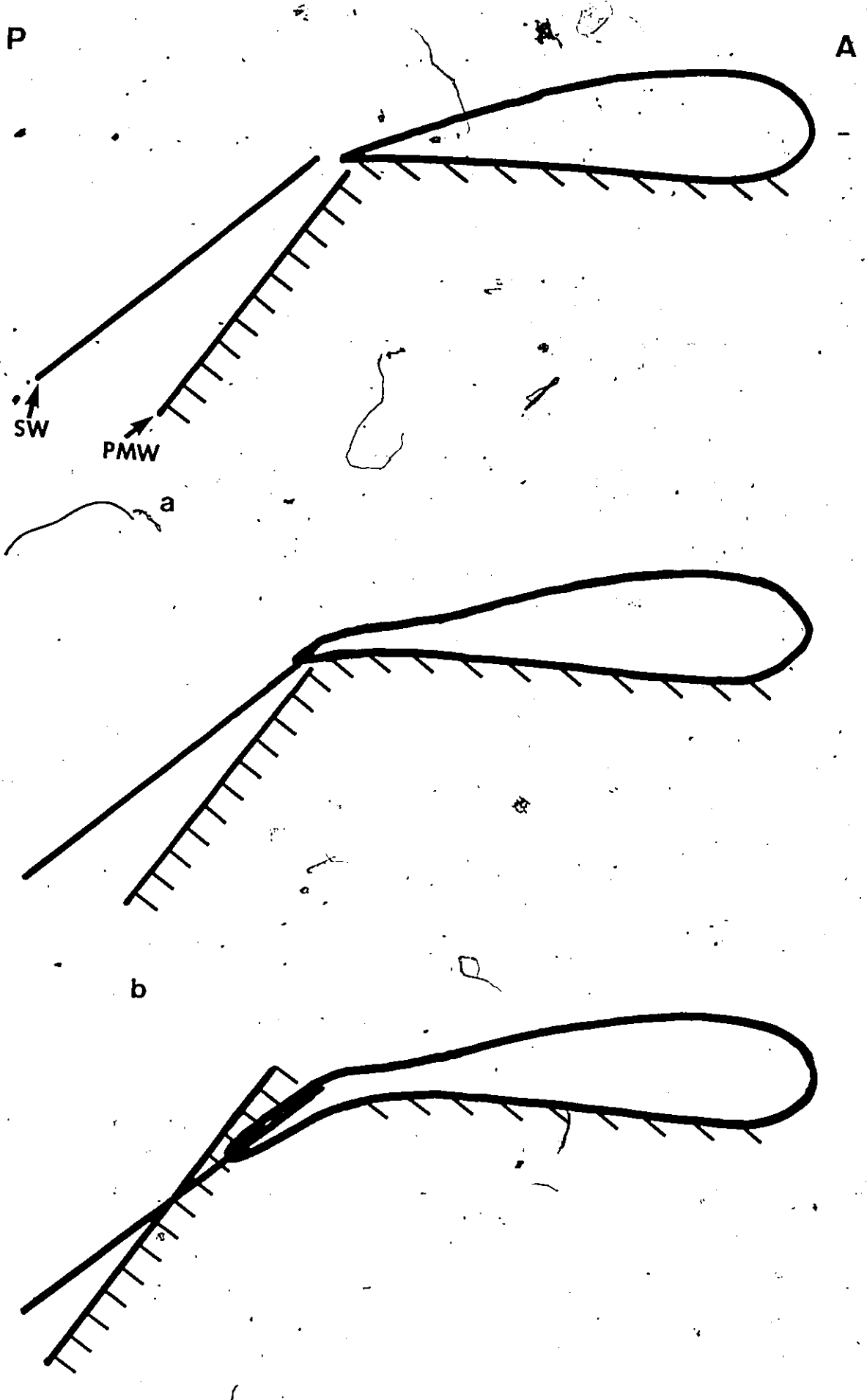
As mentioned above, the duct deviates ventrally at an angle of $52^{o}$. If the duct is migrating ventrally along the posterior wavefront, then the angle at which it migrates represents the angle that the wavefront makes with the D-V axis. This angle ($52^{o}$) is significantly different ($0.02 < P < 0.05$) from the angle that the anterior boundary makes with the D-V axis ($41^{o}$).

In all cases, the duct stopped after migrating a short distance ventrocaudally. This was evidenced by the rounded, blunt appearance of the duct tip. The average distance migrated was $0.26 \pm 0.04$ mm. There are two possible explanations for the duct's limited migration over the lateral mesoderm. The first is that only the dorsal portion of the lateral mesoderm is a suitable substratum for migration. This, however, is inconsistent with observations made from secondary duct transplants by both Poole and myself. Our combined data indicate that only the extreme ventral portions of the lateral mesoderm are not able to support migration.

An alternate explanation is that the anterior boundary of the active region continues to move normally through the lateral mesoderm after the duct begins to deviate ventrally. As mentioned previously, this anterior boundary is equivalent to the wave described by Poole and Steinberg (1982) and results in a change such that the lateral mesoderm could no longer support migration. Thus, the duct

Figure 25

Diagram illustrating the movement of the post-migration and segmental waves. The anterior (A) and posterior (P) axes of the embryo are indicated. In untreated embryos, the duct tip would migrate ahead of the post-migration wave (PMW) but behind the segmental wave (SW) (a). In tunicamycin treated embryos, the segmental wave is arrested; upon encountering the arrested wave, the duct tip begins to migrate ventrally along the wavefront (b). The post-migration wave eventually overtakes the duct, causing the latter to stop migrating (c). The hatched area represents the region through which the post-migration wave has passed.


P
A
SW
PMW
a
b
c

in tunicamycin treated embryos would migrate ventrally until this wave passed through the region where the tip is located; in other words, until it collapses onto the posterior boundary of the active region (Fig. 25). Assuming that the anterior wave proceeds at the same rate as duct migration, the distance migrated ventrally by the duct would represent the distance between the two waves.

The observed effect of tunicamycin has demonstrated the importance of cell surface glycoproteins for duct migration.

### 3.3.2 Trypsin-sensitive cell-surface proteins required for duct migration and somite segmentation

A second approach to the study of cell surface molecules in duct migration is to examine the effects of enzymatic digestion of such molecules. Trypsin, a proteolytic enzyme commonly used to analyse cell surface molecules (Takeichi, 1977; Urushihara et al., 1977; Atsumi et al., 1979; Magnani et al., 1981), was used in this study.

One problem encountered when utilizing enzymatic treatment as a means of obtaining information about morphogenesis of the embryonic mesoderm is that enzymes are unlikely to pass through the epidermis to the underlying mesodermal target, and complete removal of the epidermis results in arrested development of the mesodermal tissue (unpublished observations). This problem can be circumvented by temporary removal of the epidermis during a brief

enzymatic treatment. The epidermis is peeled back on one side of the embryo, but left attached ventrally. Thus, the mesoderm is exposed for treatment but the epidermis is easily replaced afterwards.

In this section, I will report the effects of trypsin on somite segmentation and pronephric duct migration. I have found that both the somitic mesoderm and the lateral mesoderm along the duct path contain trypsin sensitive regions that travel synchronously through the embryo in advance of somite segmentation in a manner similar to the heat-shock wave discovered by Elsdale et al. (1976). However, calcium protects the pronephric duct guidance information from the effects of trypsin, whereas somite segmentation is still affected.

### 3.3.2.1 Results

Studies on other systems have shown that the trypsin sensitivity of some cell surface proteins is calcium dependent (Takeichi, 1977; Urushihara et al., 1977; Atsumi and Uno, 1979; Takeichi et al., 1979; Magnani et al., 1981; Hyafil et al.,1981). Therefore, the first set of experiments was carried out in the absence of calcium while the second set was carried out in the presence of 340 uM calcium. In the first series, there was a striking effect on somite segmentation and pronephric duct migration. Several somites were found to segment either incompletely or in a non-linear

fashion (Fig. 26b, 27c-d; Table 7). Abnormalities were restricted to a specific region spanning several somite widths. This region was located 3.55 $\pm$ 0.69 somites posterior to the last formed somite at the time of treatment. Therefore, the affected somites were not those already segmented or those that formed immediately after the treatment, but those that formed several hours later. Only 3-5 ($x=3.91 \pm 1.04$) somites segmented abnormally, after which normal segmentation resumed. As shown in Table 7, this region of trypsin sensitivity was located progressively more posterior with increasing developmental age. This wave of trypsin sensitivity is highly reminiscent of the wave of heat shock sensitivity discovered by Elsdale et al. (1976).

After the treatment, the pronephric duct migrated a short and relatively constant distance of 5-7 somites before stopping (Table 7; Fig. 26b, 27c). The final position of the duct tip was independent of the time that the embryo was left to develop, as shown by a regression analysis of the distance migrated by the duct versus the total number of somites formed at the time of fixation (Fig. 28b). A t-test showed that there is a significant difference between the mean duct position of treated and normal embryos ($P << 0.001$).The region where the duct had stopped migrating corresponded to the beginning of the region of somite abnormalities (Table 7; Fig. 26b, 27c-d). This was true no matter which embryonic stage was used. These results show that the duct path contains a trypsin sensitive region

Experimental embryos were treated with 0.01% trypsin in calcium-free Steinberg's solution for 3 min. Control embryos were treated with calcium-free Steinberg's solution. The first three are representative control embryos; a total of 12 control embryos were included in this experiment. No abnormalities were found in any of the controls. In the diagram on the right, the boxes represent individual somites present at the time of fixation, beginning with somite 1 on the left. The hatched boxes represent those somites already formed at the time of treatment; the black boxes indicate the abnormal somites. The line represents the pronephric duct; the vertical bar indicates the position of the duct tip. A total of 15 embryos were treated. Pronephric duct migration proceeded normally in two of these (not shown) and somite segmentation was normal in one of the two.

* The position of the duct was measured in terms of somite number; B,M and E stand for beginning (anterior end), middle and end (posterior end) of somite, respectively.

+ The pronephric duct stops migrating once it reaches the cloaca which is normally located between somites 19 and 20.

+ A somite that had not completely segmented at the time of fixation was counted as a half somite.

# TABLE 7

| No. of somites at time of treatment | No. of somites at time of fixation | Abnormal somites | Position of duct |
|---|---|---|---|
| 7 | 20 | none | cloaca* |
| 8 | 21 | none | cloaca |
| 11 | 23 | none | cloaca |
| 6 | 15 | 11-13 | 11B |
| 7 | 16 | 11-15 | 10E |
| 7 | 16.5 | 11-14 | 11B |
| 7 | 20.5 | 12-15 | 12E |
| 8 | 17 | 13-16 | 13B |
| 8 | 17 | 13-17 | 13M |
| 8 | 20 | 13-16 | 13M |
| 8 | 20 | 14-16 | 13E |
| 10 | 19 | 14-17 | 14E |
| 10 | 19.5 | 14-17 | 14B |
| 10 | 22 | 15-17 | 15E |
| 11 | 22 | 15-18 | 15B |
| 12 | 22 | 16-18 | 16B |

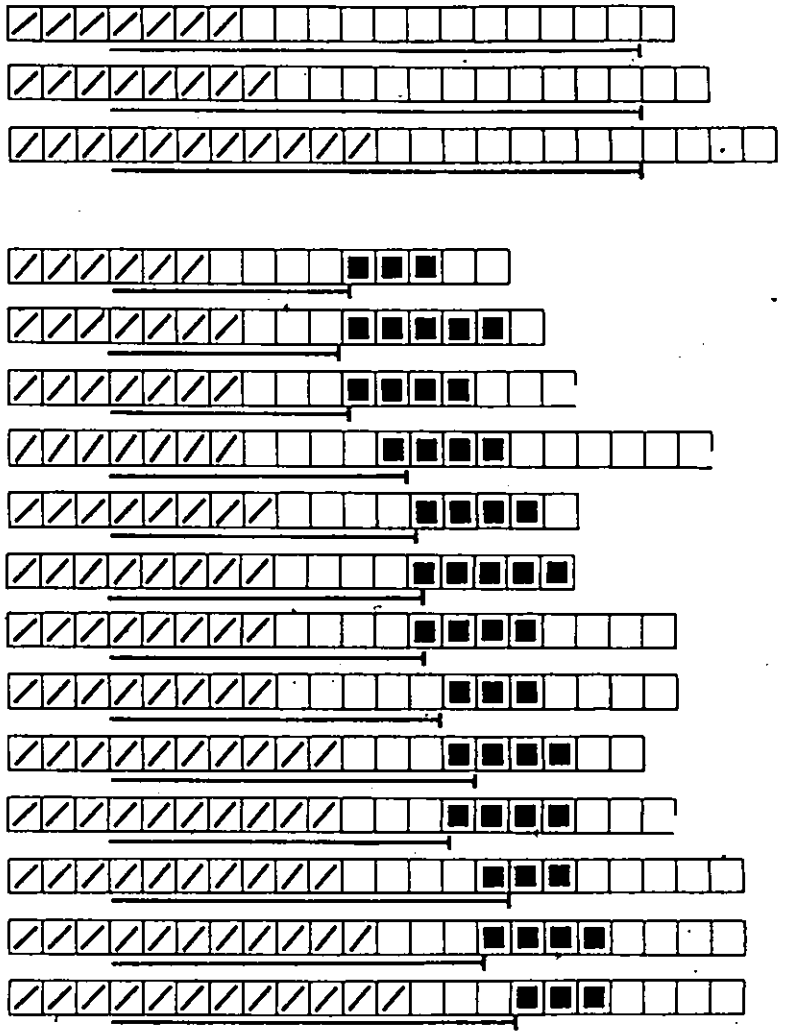


Figure 26

Effect of trypsin treatment on somite segmentation and pronephric duct migration. (a) Control embryo; the duct tip (arrow) was located in a normal position, approximately 2.5 somites behind the last formed somite. No obvious abnormalities in somite segmentation were observed. (b) Embryo trypsin treated at the 7 somite stage in the absence of calcium; the duct tip (arrow) was located at the anterior end of the region of abnormal somites (11-14). (c) Embryo trypsin treated at the 6 somite stage in the presence of 340 uM calcium; the duct tip (arrow) in this case had migrated through the region of abnormal somites (10-15). (36X).

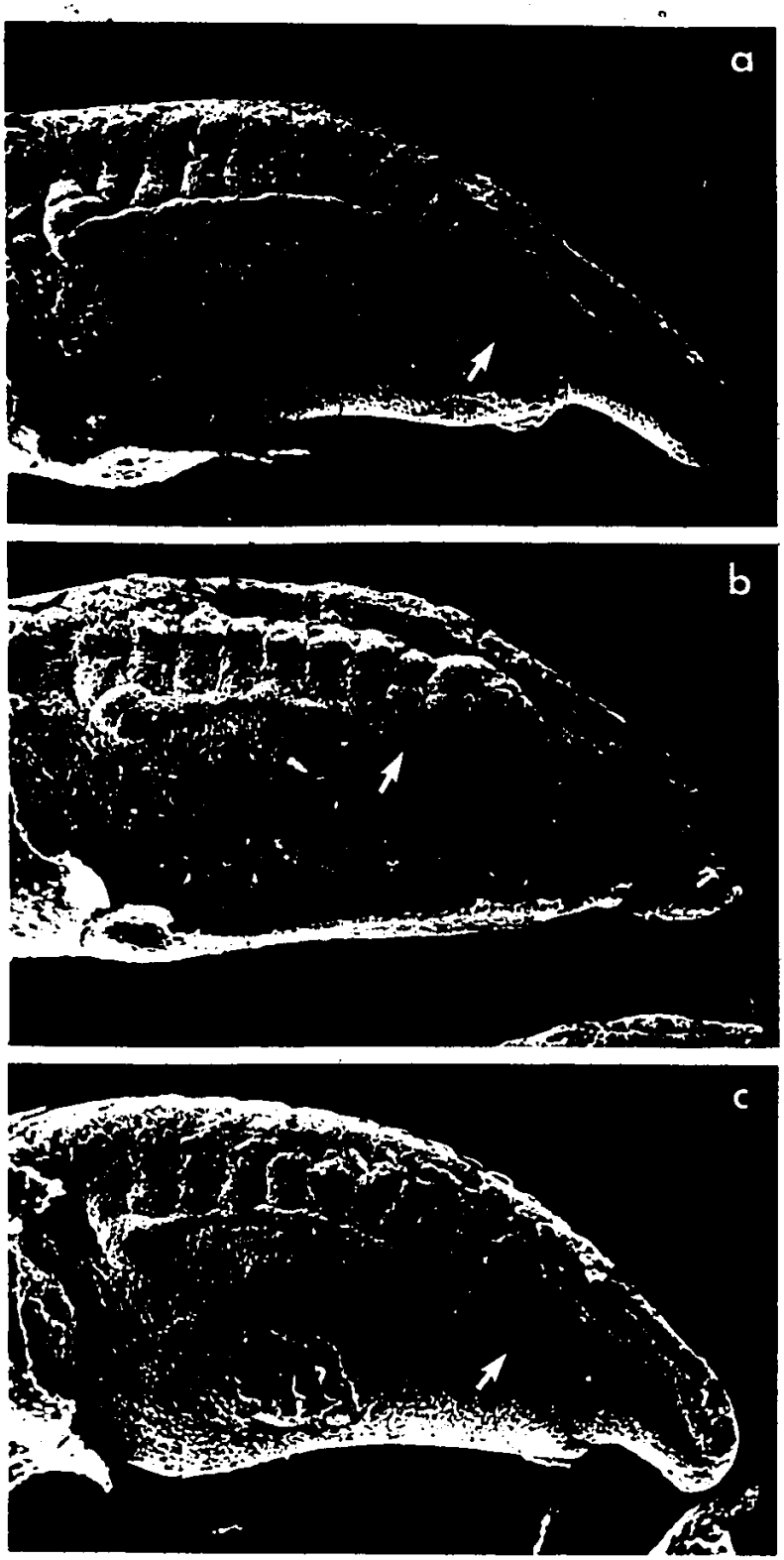
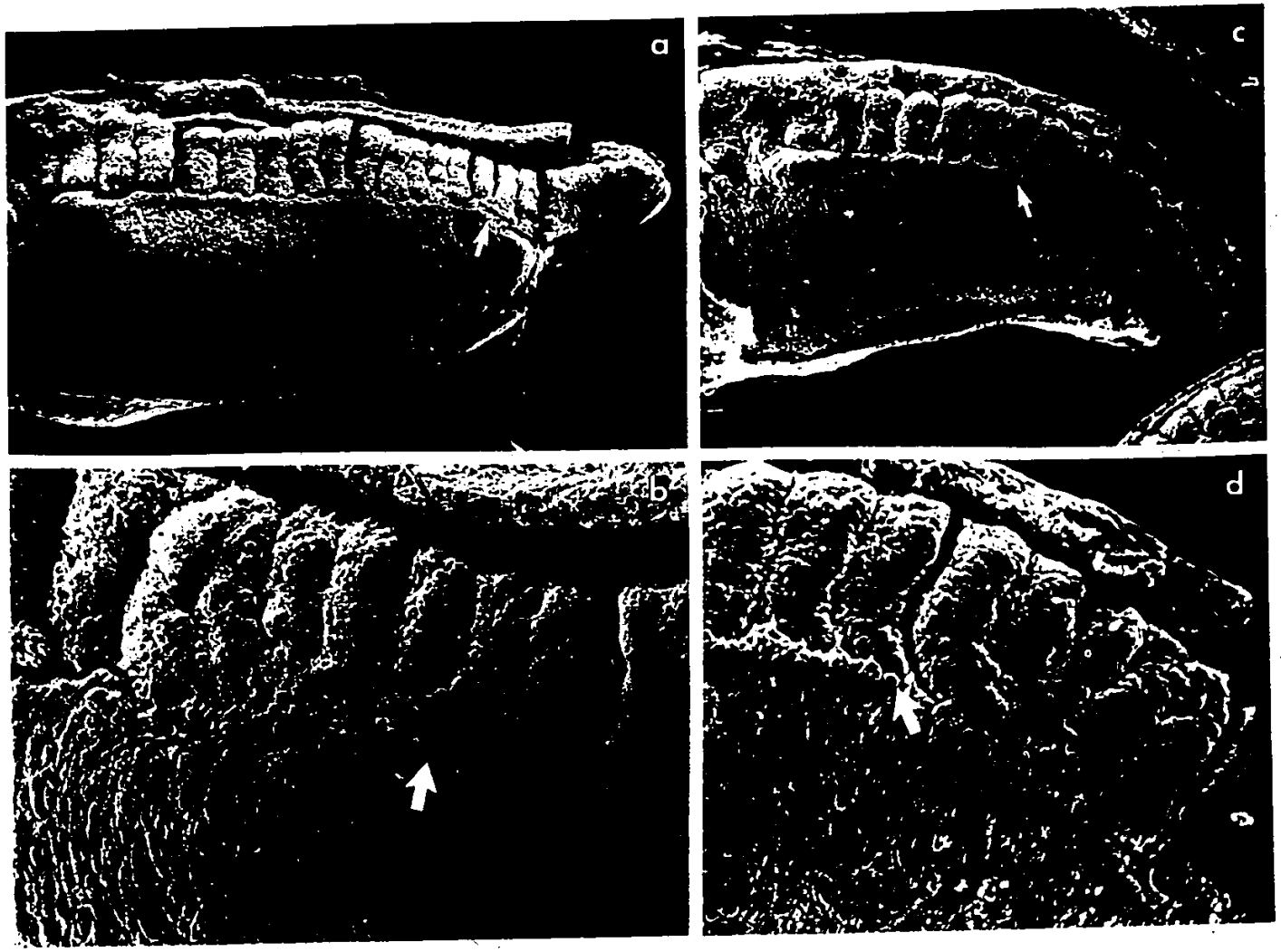

a
b
c

Figure 27

Effect of trypsin treatment on somite segmentation and pronephric duct migration. (a) Embryo treated at the 7 somite stage in the presence of calcium; the duct tip (arrow) had migrated through the region of somite abnormalities (somites 11-15). (b) Higher magnification of the duct tip in (a). (c) Embryo treated at the 7 somite stage in the absence of calcium; the duct tip (arrow) did not migrate into the region of abnormal somites (11-15). (d) Higher magnification of the duct tip (arrow) in (c). (a,c 36X; b,d 94X).


a
c
b
d

adjacent to the trypsin sensitive somitic region. Both trypsin sensitive regions travel synchronously in a caudal direction through the embryonic mesoderm.

When the trypsin treatment was repeated in the presence of calcium, the effect on somite segmentation was the same (Table 8; Fig. 26c, 27a-b). The width and position of the sensitive region were not statistically different in the two treatments. In this case, however, pronephric duct migration proceeded normally, as in control embryos (Table 8; Fig. 26c, 27a-b). Regression analysis clearly demonstrates that, after trypsin treatment in the presence of calcium, the distance migrated by the pronephric duct is correlated with the number of somites that have formed at the time of fixation (Fig. 28a). Also, there was no significant difference between the mean duct position of treated and normal embryos. However, the duct tip was actually located ahead of the last formed somite in one case. This was never observed in control or normal embryos.

The duration of the trypsin treatment was varied in order to determine the effect of duration on the severity of somite abnormalities. When the treatment was increased to 4 min (either with or without calcium), there was extensive damage to the epidermis and subsequent healing was not complete. In contrast, a decrease in duration to 2 min resulted in normal segmentation and duct migration (results not shown)

In order to determine whether the observed effects were

Experimental embryos were treated with 0.01% trypsin in 100% Steinberg's solution for 3 min. Control embryos were treated with 100% Steinberg's solution. The first three are representative control embryos; a total of 11 control embryos were included in this experiment. No abnormalities were found in any of the controls. In the diagram on the right, the boxes represent individual somites present at the time of fixation, beginning with somite 1 on the left. The hatched boxes represent those somites already formed at the time of treatment; the black boxes indicate the abnormal somites. The line represents the pronephric duct; the vertical bar indicates the position of the duct tip. All treated embryos displayed somite abnormalities.

* The position of the duct was measured in terms of somite number; B,M and E stand for beginning (anterior end), middle and end (posterior end) of somite, respectively.

+ The pronephric duct stops migrating once it reaches the cloaca which is normally located between somites 19 and 20.

++ A somite that had not completely segmented at the time of fixation was counted as a half somite.

# TABLE 8

| No. of somites at time of treatment | No. of somites at time of fixation | Abnormal somites | Position of duct |
|---|---|---|---|
| 7 | 21 | none | cloaca* |
| 9 | 22 | none | cloaca |
| 11 | 23 | none | cloaca |
| 6 | 17 | 10-15 | 15E |
| 7 | 15.5 | 11-14 | 14B |
| 7 | 17 | 11-15 | 15B |
| 7 | 18 | 11-15 | 17B |
| 7 | 18 | 12-15 | 19M |
| 8 | 18.5 | 13-15 | 18M |
| 8 | 20 | 13-16 | cloaca |
| 8 | 20 | 13-16 | cloaca |
| 9 | 20 | 13-16 | cloaca |
| 9 | 21 | 14-17 | cloaca |
| 10 | 21 | 16-17 | cloaca |
| 11 | 20.5 | 15-17 | cloaca |
| 11 | 20.5 | 15-18 | 18M |

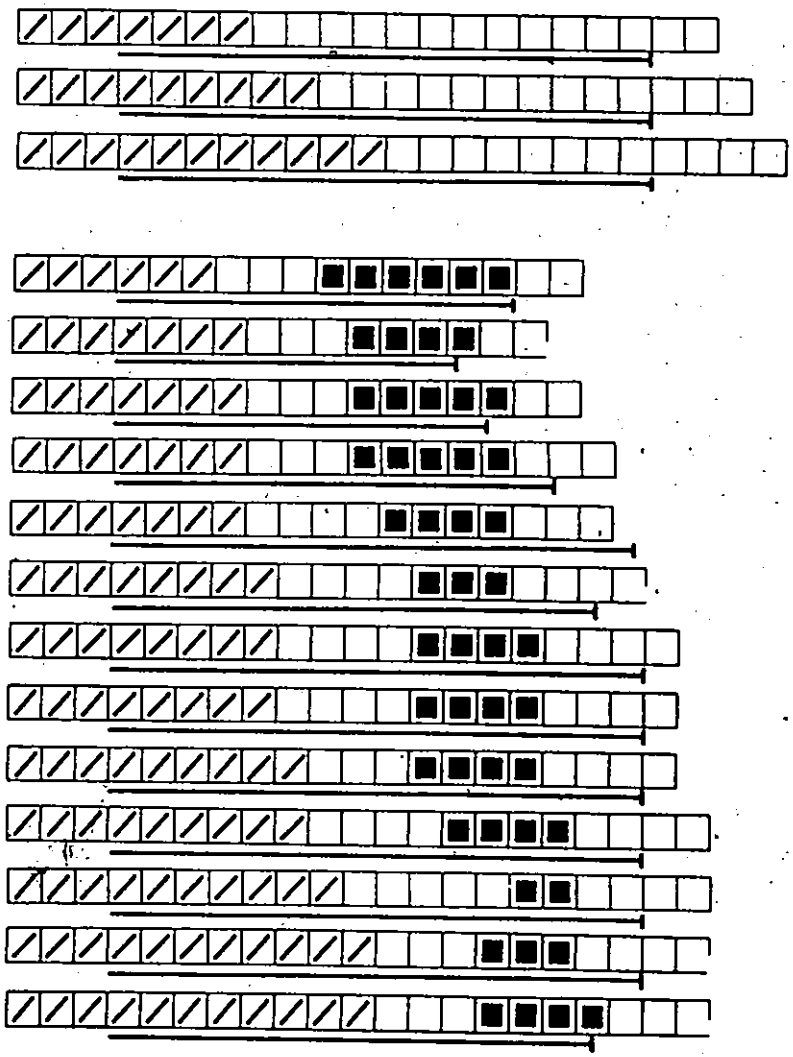
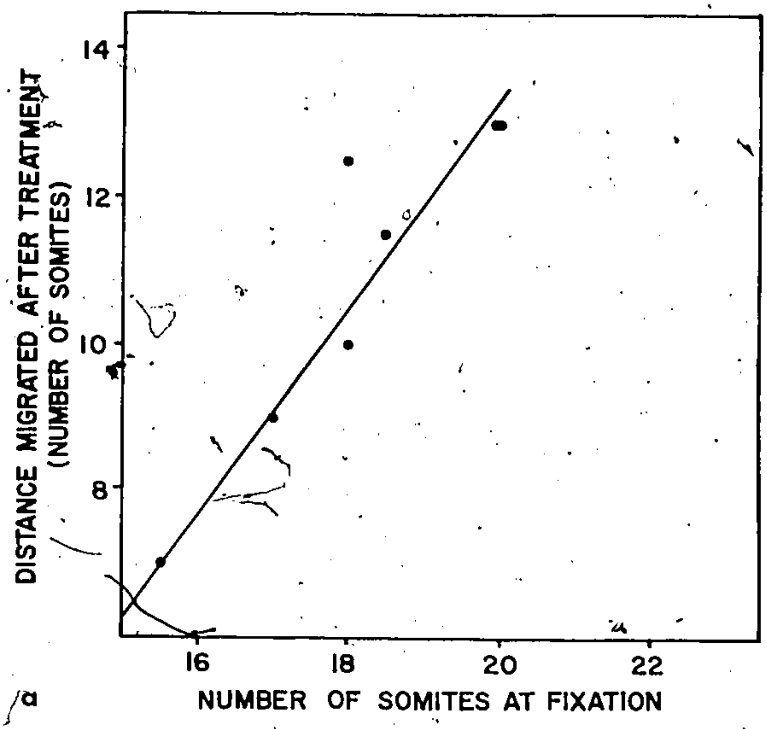
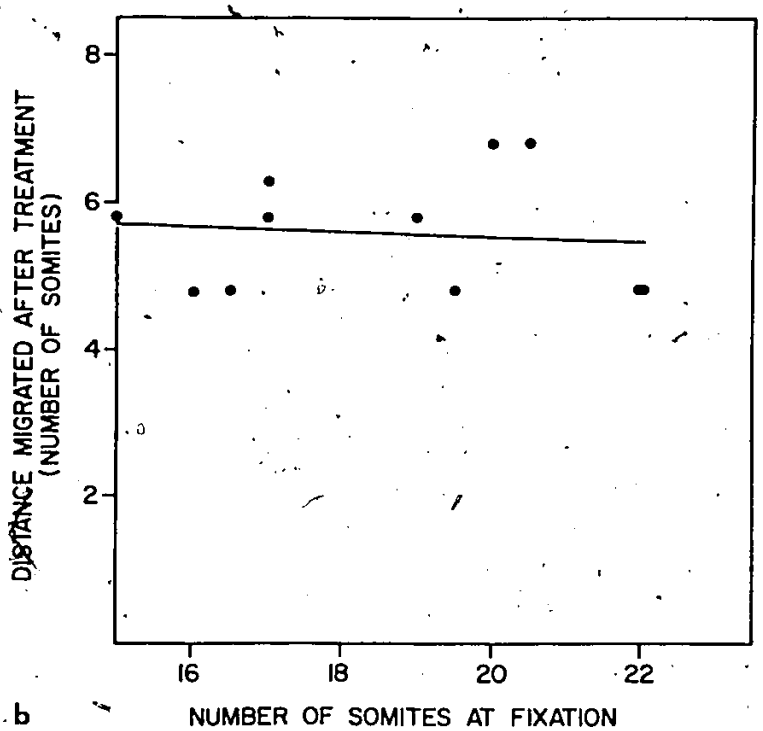


Figure 28

Correlation between the distance migrated by the pronephric duct and the number of somites at fixation in trypsin treated embryos; (a) in the presence of 340 uM calcium and (b) in the absence of added calcium. In the latter case, there was no correlation between the two as the slope of the regression line was not significantly different from zero.


DISTANCE MIGRATED AFTER TREATMENT
(NUMBER OF SOMITES)
14
12
10
8
16
18
20
22
NUMBER OF SOMITES AT FIXATION

a


DISTANCE MIGRATED AFTER TREATMENT
(NUMBER OF SOMITES)
8
6
4
2
16
18
20
22
NUMBER OF SOMITES AT FIXATION

b

in fact due to proteolysis, embryos were treated with trypsin (in calcium-free Steinberg's) in the presence of aprotinin (a serine protease inhibitor). In all 5 cases, no effect on either somite segmentation or pronephric duct migration was seen. This confirms that the effects are due to proteolysis. The possibility remains that this effect could be due to proteolysis by chymotrypsin rather than by trypsin, since the trypsin preparation used may contain chymotrypsin as a contaminant.

In view of the results of the epidermis transplants described previously, one must consider the possibility that the trypsin was not directly affecting the mesoderm but rather, the effects were mediated through trypsinization of the epidermis. This possibility was examined by performing reciprocal epidermis transplants between untreated and trypsin treated embryos. These experiments were performed in the absence of calcium and the results of 4 sets of reciprocal transplants were obtained.

All 4 embryos that received a trypsin treated epidermis developed normally. Two of the trypsinized embryos that received an untreated epidermis displayed the typical trypsin effect. The other two embryos were not sufficiently advanced in development to see an effect. Although more cases are necessary in order to draw firm conclusions, these experiments support the idea that trypsin acts on somite segmentation and duct migration by a direct effect on the mesoderm.

### 3.3.2.2 Discussion

In the present study, I have found that a brief trypsin treatment of the mesoderm, during the period when somite segmentation is taking place, disrupts the normal segmentation over a band 3-5 somites wide. In all embryonic stages studied, the first abnormal somite is always the 4th-6th somite formed after trypsinization. Thus, the pre-segmental wave of trypsin sensitivity moves caudally along the embryonic axis at the same rate as and in advance of segmentation.

The lag between the time of trypsinization and the appearance of the first abnormal somite indicates that cell surface protein(s) required for normal segmentation are present many hours prior to the actual participation in somite formation. Since the first few somites formed after treatment segment normally, these cell surface proteins are either no longer required or no longer susceptible to cleavage by trypsin. The region posterior to the last abnormal somite segments normally, suggesting that the required proteins are either resistant to trypsin cleavage or not yet present. Alternatively, the cells in this region may be affected but have sufficient time to recover before participation in somite segmentation.

Unlike somite segmentation, duct migration was affected only when the trypsin treatment was done in the absence of calcium. In this case, the pronephric duct migrated a short

distance to the region below the first abnormal somite. The fact that the duct initially continued to migrate indicates that trypsin affects the cells of the duct path rather than the duct itself. This also indicates that the wave of trypsin sensitivity travels through the duct path in advance of the wave of guidance information previously described by Poole and Steinberg (1982).

These results demonstrate the existence of a trypsin sensitive region in the somitic mesoderm as well as the lateral mesoderm of the duct path, that travel synchronously in advance of somite segmentation. In addition, I have shown that the trypsin sensitivity of the duct path is calcium dependent whereas that of the somitic mesoderm is not.

# 4. GENERAL DISCUSSION

## 4.1 Origin of the pronephros and pronephric duct

The studies on the formation of the pronephros and pronephric duct led to the conclusion that intermediate mesoderm, as defined by Balfour (1876), does not exist as a distinct tissue in the axolotl embryo. In 1950, this tissue classification was applied to all vertebrates by Fraser (1950), even though all of the amphibian literature cited in her review clearly stated that the pronephros and duct are derived from the somatic layer of the lateral mesoderm (Mollier, 1890; Field, 1891; O'Connor, 1938). In fact, none of these papers even mentioned the presence of intermediate mesoderm. Clearly, an incorrect generalization was made by Fraser (1950) in claiming that intermediate mesoderm is the origin of the nephric system in all vertebrates.

My observations have led to the suggestion that the pronephros is derived from somitic mesoderm. Although I am proposing a different origin for the pronephros than earlier workers, my interpretation is consistent with their experimental results. The difference in suggested origin most likely comes from the difference in technology. All of the earlier studies on the origin of the urodelan pronephros and duct rely on vital staining and examination of cross-sections in order to determine the origin of these structures. While vital staining has been very useful in

studying embryonic development, one must realize the limitations of this methodology. Loss of precision arises from inaccuracy in placing the stain, as well as diffusion of the dye after the tissue has been marked. Thus, it would be impossible to distinguish between a somitic and a somatic origin for the pronephros.

After examination of cross-sections through axolotl embryos, it was clear that one cannot distinguish between a somitic and a somatic origin for the pronephros in this way. However, one can easily understand why, in the absence of evidence to the contrary, earlier workers chose the latter. In other vertebrates, such as the chicken, the nephric system was known to arise from the tissue immediately adjacent to the somites, namely the intermediate mesoderm (Balfour, 1876). Thus, there was no precedent for a somitic origin for the pronephros. It was only after examination of the developing pronephric rudiment using the high resolving power of the scanning electron microscope, that I became aware of the possibilty of a different origin. Of course, one cannot completely rule out a genuine difference in origin since my observations were made on different Ambystoma species than that examined by Field (1891) or O'Connor (1938).

In spite of the morphological evidence presented here in favour of a somitic origin for the pronephros, it would be premature to draw firm conclusions in the absence of experimental evidence. Classically, scientists have excised

the suspected source tissue in order to determine its contribution to a particular structure. There are two problems, however, with this type of experiment. The first results from regulation that one frequently observes when a piece of tissue is excised. The surrounding cells will "fill in" and form the missing structure even though they would normally form a different cell type. A typical example of this is lens regeneration in urodeles (for review, see Yamada, 1967). After excision, a new lens can be regenerated from cells of the dorsal iris epithelium. The second problem arises from the difficulty in excising only somitic mesoderm and none of the adjacent lateral mesoderm since there are no clear boundaries between the two.

The best method of conclusively determining the origin of the pronephros would be to look for the presence of somite specific markers in the presumptive pronephros. Classical markers include tissue specific enzymes, cytoskeletal components and cell surface antigens. Such studies will have to wait until tissue specific markers have been identified in the axolotl somitic mesoderm.

Although my studies agree with earlier studies as to the origin of the pronephric duct, the observed position of the lateral mesodermal cells that give rise to the duct is different. O'Connor (1938) concluded from his vital staining studies that in A. punctatum the duct arises from cells beneath somites 5 to 7. My observations indicate that the duct cells originate below the pronephros in segments 4-5

and migrate dorsally to take up a position below somites 5 to 7. This was only observable in the scanning electron microscope and, therefore, could be the reason why this was not seen by earlier workers. Another reason could be the short period of time necessary for this dorsal migration to take place. The duct cells first appear at the 7 somite stage and by the 8 somite stage, 1-2 hours later, are located on the duct path below somites 5 to 7.

## 4.2 Relationship between somitogenesis and pronephric duct migration

The evidence presented in this thesis clearly demonstrates that the process of forming a segmentation furrow is not causally linked to duct migration, since the latter can proceed in advance of segmentation. Instead, some other change that occurs in the somitic cells just prior to somite formation appears to be necessary in order to provide a suitable substratum for migration.

Possible candidates for the changes that correspond to the **segmental wave** in the axolotl mesoderm are increased cellular adhesiveness or ECM production. I would like to point out that these two cellular events are not necessarily separate; for example, a change in ECM components could lead to increased adhesiveness.

The first possibility is that these changes could

represent an increase in adhesiveness between the duct cells and the duct path as proposed by Poole and Steinberg (1982). Their hypothesis is based on a study by Bellairs et al. (1978) showing that the cells of the somitic mesoderm undergo an increase in adhesiveness prior to somite formation. Poole and Steinberg propose that the lateral mesoderm would contain a gradient of increasing adhesiveness along the anterio-posterior and latero-dorsal axes. That cells will migrate up a gradient of adhesiveness has been shown by Harris (1973) in culture. Harris used palladium coated plates to produce an artificial gradient and found that cells from a variety of different lines migrated up this gradient.

Although this adhesion gradient model is attractive, one must realize that this hypothesis contains several assumptions. The first is that an increase in adhesiveness between somitic cells will also mean an increase in adhesiveness between somitic and duct cells. Since Bellairs only measured intersomitic cell adhesion, there is no reason to expect increased adhesiveness between somitic cells and other cell types. The second assumption is that the change takes place in the form of a gradient. Although this is, in my view, the most likely possibility, the change could also be a step-function. Since Bellairs did not determine the spatial distribution of this increase in adhesiveness, it is not possible to determine how valid this assumption is. Nor is it possible to determine whether the increase in

adhesiveness corresponds to the active region.

A second possibility is that the segmental wave represents a change, either qualitative or quantitative, in the ECM. There are many systems in which the ECM has been implicated in cell migration. Two examples that are particularly relevant to my work are neural crest cell migration (Lofberg and Ahlfors, 1978; Lofberg et al., 1980; Spieth and Keller, 1984) and cell migration during gastrulation (Nakatsuji et al., 1982; Nakatsuji and Johnson, 1984)

Lofberg et al. (1980) reported a correlation among an increase in the number of ECM fibrils, expansion of the subepidermal space and emigration of crest cells from the neural tube. In addition, the ECM fibrils were aligned parallel to the direction of migration, thus leading to the suggestion that these fibrils serve to orient the cells by contact guidance (Lofberg and Ahlfors, 1978). In white axolotls where the trunk neural crest cells fail to migrate along the dorsal pathway, Spieth and Keller (1984) found structural differences in the subepidermal ECM.

Nakatsuji et al. (1982) reported the presence of blastopore-animal pole aligned ECM fibrils along the blastocoelic wall in A. maculatum embryos. These authors proposed that the involuting presumptive mesodermal cells migrate along these fibrils by contact guidance. More recently, Nakatsuji and Johnson (1984) have shown that the ECM fibrils can be aligned in vitro in any orientation by

mechanical tension. Furthermore, dissociated mesodermal cells were found to migrate along the tension axis, regardless of its orientation with respect to the blastopore-animal pole axis.

Taken together, these studies show that the ECM plays an active role in influencing cell migration. It is therefore possible that the ECM could either provide the proper receptors for attachment of the duct or it could provide aligned collagen fibers to guide duct migration. This possibility will be examined further in section 4.5

## 4.3 Relationship of spatio-temporal changes in the lateral mesodermal cells to duct migration

The results of my heterotopic duct transplants along with those of Poole and Steinberg (1982) show that at any one stage, there is a narrow window, the active region, in which the duct can migrate. This window consists of a strip of lateral mesoderm about two somites wide that moves caudally with increasing age. The posterior boundary of this active region represents cells that are changing in order to provide a suitable substratum for migration, while, at the anterior boundary, the lateral mesodermal cells are losing their ability to support migration.

In addition to the spatio-temporal dependent ability of the lateral mesodermal cells to provide a suitable substratum for migration, these cells also undergo a series

of shape changes. These changes in morphology occur over a very narrow region which corresponds in space and time to the post-migration wave. Although it is tempting to suggest that these shape changes occur concomittant with or directly result in the cells' inability to support migration, it is not possible at this time to determine whether there is a causal relationship between the two events. It is entirely possible that they are two totally independent developmental processes temporally coordinated by another process. Even if a causal relationship does indeed exist, one would have to distinguish between cause and effect.

In order to establish whether there is a causal relationship, one needs to answer both of the following questions: a) do the lateral mesodermal cells require the duct in order to undergo the shape changes? b) does the duct require the shape changes in order to migrate? One way of answering these questions would be to inhibit one process and see if the other still occured. One could examine the role of the duct on the shape changes by removing the entire duct and seeing if the shape changes still take place. Unfortunately, one cannot answer the second question directly since any drug used to inhibit the shape changes would also directly affect migration. However, one could obtain information on this question indirectly by a) examining embryos which have received a secondary duct transplanted at an A-P level posterior to the primary duct, and b) examining trypsin treated embryos for shape changes

in the lateral mesoderm.

In the first case, we know that these ducts will migrate ahead of the primary duct. We also know that in embryos without secondary ducts, there are no shape changes occuring at the site of transplantation. From this experiment, one could determine whether shape changes occur in the vicinity of the migrating secondary duct.

By examining trypsin treated embryos in the presence and absence of calcium, one could determine whether the duct's ability to migrate is correlated with the occurence of the shape changes.

If these shape changes are involved in guiding the migrating duct, this would not be the first case where shape changes were involved in a morphogenetic process. The classical example is that of neurulation (Baker and Schroeder, 1967; Burnside, 1971, 1973; Karfunkel, 1971; Brun and Garson, 1983). The cells of the neural plate elongate along the D-V axis into columnar cells. Sequential apical constriction of these cells beginning at the lateral edge of the plate, accompanied by flattening of the adjacent epidermal cells causes the plate to "roll up" into a tube.

A second example is the shape changes reported to take place in the process of epiboly during gastrulation in Xenopus (Keller, 1980). The animal region of the Xenopus blastula consists of three single cell layers: 1) the superficial layer 2) the inter deep layer and 3) the inner deep layer. The cells of all layers are originally cuboidal.

Keller (1980) has shown that the cells of the two deep layers interdigitate along the radial axis to form a single cell layer of columnar cells. These cells, along with the cells of the superficial layer, subsequently spread and flatten thus increasing the area occupied by these cells. The striking similarity between the shape changes observed by Keller and the shape changes documented in this thesis leads to the possibility that the lateral mesoderm is undergoing a similar process as the cells in the animal region of Xenopus blastula, and results in the increased length of this tissue as a whole.

Alternatively, these shape changes could be the result of the cells preparing to undergo mitosis. The spatio-temporal sequence of the shape changes could be the result of a wave of mitotic activity. There is certainly precedent for waves of mitotic activity. During blastula stages (5th to 13th cleavages) in several amphibian species (A. mexicanum; Hara, 1977; Bufo vulgaris; Sirakami, 1958; Xenopus laevis; Satoh, 1977), cleavage waves pass through the embryo from animal to vegetal pole. In all studies, this wave pattern gave way to asynchronous cell division just prior to gastrulation. Unfortunately, no one has examined later stage embryos to see if the mitotic waves resumed.

In order to test this model, one would first have to examine the distribution of mitotic cells in sectioned material. The problem with this approach is that the cell cycle is extremely long (52 h; Hronowski et al., 1979) at

this stage. One would have to examine a large number of embryos in order to observe cells in mitosis. One can arrest cells in mitosis, using colchicine; this would increase the proportion of cells at metaphase, thereby making an examination of cell cycle coordination more feasible.

The second step in determining the role of mitosis would be to inhibit mitosis and observe the effect on migration. Of course, one could not use drugs such as colchicine or cytochalasin that inhibit mitosis by interfering with microtubules or microfilaments since these drugs would also inhibit duct migration directly. Another method of arresting mitosis involves inhibition of DNA synthesis. Drugs commonly used for this purpose include hydroxyurea, an inhibitor of the enzyme ribonucleotide reductase, and cytosine arabinoside (Ara C), a cytosine analog.

## 4.4 Role of the epidermis in duct migration

The results presented here show that the epidermis influences the duct's ability to migrate. It does not, however, appear to provide the guidance mechanism for the duct. This conclusion is based on the fact that changing the polarity of the epidermis does not alter the migration path of the duct. However, when the polarity of both the epidermis and the mesoderm is reversed, the duct deviates to follow a very circuitous path to the cloaca (Holtfreter,

1944). In these experiments, Holfreter cut axolotl embryos transversely into an anterior part, containing the pronephric rudiment, and a posterior part, containing the cloaca. The latter portion was allowed to heal to the anterior part in reversed D-V orientation. In several of these embryos the duct continued to migrate beyond the level of the cut. In such cases, the duct changed its direction of migration upon entering the posterior portion and headed dorsally towards the duct path. Once the duct reached the duct path, it migrated caudally to the cloaca. These results, taken together with mine, lead to the conclusion that although the epidermis can affect the duct's ability to migrate, it is the mesoderm that serves to direct duct migration.

The region of the epidermis which does not support duct migration is different from that of the lateral mesoderm. According to Poole and Steinberg (1982), all of the lateral mesoderm anterior to the primary duct inhibits secondary duct migration. Yet this same region of the epidermis allows duct migration, as evidenced by the results of the A-P and the A-P, D-V reversals done at the 11 somite stage. In fact, my results indicate that only a very localized region of the epidermis can inhibit duct migration. Since the location of this region is age-dependent, I have postulated that this region represents an epidermal wave.

If my interpretation of the results of these epidermal transplant experiments is correct, then one should be able

to test predictions of the proposed model. For example, from the results collected so far, one could estimate the A-P position of the epidermal wave in the donor. One should then be able to predict where in the host this wave will contact the mesoderm and thus, where the duct should stop migrating. However, there is a problem with testing such predictions because the epidermis shrinks after dissection to about two thirds of its original size. Therefore, its A-P axis cannot be matched very accurately with the host's A-P axis. However, there are several experiments that one can perform in order to test my hypothesis. For example, if this proposed wave does exist and moves caudally through the epidermis, one should be able to show this by perfoming "old" to "young" transplants, using different stage embryos as the epidermis donor. With increasing age of the epidermis, the duct of the "young" host should migrate further.

Alternatively, one could perform additional A-P and D-V reversals at the 8 somite stage as well as at other stages. One would predict that the extent of duct migration would be inversely proportional to the age of the embryo at the time of transplantation.

One could conceivably get around the problem of shrinkage by transplanting small pieces of the epidermis instead. This way, one could accurately determine the A-P level from which the epidermis was taken as well as the level at which the graft is placed. This would lead to a

complete map of the areas of the epidermis that contain the proposed wave and allow one to determine the spatio-temporal relationship between mesodermal and epidermal waves.

Spieth and Keller (1984) reported that the structure of the ECM in white axolotls is abnormal. They proposed that this abnormality is responsible for the inability of the epidermis to support neural crest migration. This leads to the possibility that the effect of the epidermis on duct migration is in fact due to the ECM either produced by the epidermis or transplanted along with it. In order to explore this possibility, one would have to determine which tissue(s) produce(s) the ECM and whether extracellular material is included in epidermis transplants. This can be accomplished by performing epidermis transplants between embryos radioactively labelled at an early stage with proline, a major component of collagen, and unlabelled embryos. The distribution of the label would be examined later by autoradiography.

## 4.5 The role of the ECM in duct migration

The spatio-temporal distribution of ECM showed a strong correlation with duct migration. The average position of the posterior limit of visible ECM was 1.0 somite widths anterior to the last formed somite. This position does not correspond to either the post-migration or the segmental wave but is intermediate between the two. This means that

production of ECM is probably not related to the post-migration wave. A relationship to the segmental wave cannot be ruled out, however, since it is possible that the ECM was not completely preserved. One could explore this possibility by examining the distribution more precisely with antibodies against collagen, a major component of the ECM.

The next step in this study would be to establish whether the ECM is important for duct migration. The ECM could function as a contact guidance mechanism, as shown for gastrulating cells of A. maculatum (Nakatsuji and Johnson, 1984). However, no alignment of ECM fibrils was observed in my study.

A second possibility is that specific components of the ECM, such as fibronectin or hyaluronic acid, could be involved in guiding the duct. The role of hyaluronic acid could be examined by treatment with hyaluronidase. Other components, such as fibronectin, could be examined individually with antibodies. Labelled antibodies could be used to determine the distribution of the component while monovalent antibodies could be used to study the role of the component.

## 4.6 Importance of cell surface proteins in duct migration

The importance of cell surface proteins in duct migration was clearly demonstrated by the results of the

tunicamycin and trypsin experiments.

If my interpretation of the tunicamycin effect is correct, then tunicamycin affects glycoproteins that are necessary for the change in the lateral mesodermal cells that makes them a suitable substratum for duct migration. This could be examined further by transplanting secondary ducts onto the flanks of tunicamycin treated embryos. Due to the small proportion of injected embryos that display abnormal duct migration, additional experiments are not feasible unless an alternate method of treating the embryos is found. One possibilty is to expose the mesoderm by peeling back the epidermis. However, this kind of exposure would be too short for the drug to have an effect. One could increase the length of exposure by treating with tunicamycin-impregnated albumin beads. During healing of the epidermis some of these beads would be trapped between the mesoderm and the epidermis; the drug would gradually leach out, thus resulting in longer exposure. This method has previously been used to study the effect of long-term exposure to injected hormones (Lee et al., 1981).

If an appropriate treatment could be found, it would be interesting to examine the effects of tunicamycin on the other processes shown in this thesis to be spatio-temporally related to duct migration. For example, do the shape changes in the lateral mesoderm still take place after tunicamycin treatment? Is ECM production and distribution altered? In addition, one could examine the effect of tunicamycin in

combination with epidermis transplants.

Since tunicamycin inhibits glycosylation, one might be tempted to conclude that it is the carbohydrate moiety that is important. However, absence of the carbohydrate moiety has been shown to lead to increased proteolysis of the naked protein core (Overton, 1982). Overton found that desmosome formation was inhibited in the presence of tunicamycin. However, no inhibition was observed when the protease inhibitor leupeptin was present during treatment. Therefore, it is pre-mature at this time to speculate about the relative importance of the carbohydrate and protein components in duct migration.

This question could be examined by injecting leupeptin along with tunicamycin. If such experiments were to show that the carbohydrate moiety is of prime importance, then one could examine this further through the use of lectins.

If my interpretation of the tunicamycin effect is correct, then the glycoprotein profile of the lateral mesodermal cells posterior to the duct should differ from those anterior to the duct. These differences could be examined by gel electrophoresis.

The results of the trypsin experiments demonstrated the presence of a pre-segmental wave of trypsin sensitivity that travels caudally through the embryonic mesoderm in advance of segmentation. This is similar to the wave of heat shock sensitivity described by Elsdale et al (1976) in Xenopus. They found that heat shocks delivered just prior to or

during somite segmentation led to abnormalities in somites formed several hours after the treatment. Pearson and Elsdale (1979) suggested that heat shock acts by disrupting intercellular coordination. If one assumes that the trypsin sensitive target is a cell surface protein, then this protein may be required for such coordination. These proteins could either be glycoproteins or proteoglycans that form an integral part of the cell membrane or ECM.

Unlike somite segmentation, duct migration appeared normal when calcium was present during the treatment. This suggests that the protein(s) required for duct migration are different from those required for normal segmentation, though their synthesis is temporally coordinated. However, one cannot exclude the possibility that both processes require different portions of the same protein(s). In this case, the portion required for segmentation would be cleaved independently of calcium whereas that required for pronephric duct migration would be susceptible to cleavage only in the absence of calcium. There is good precedent for this model in the behaviour of uvomorulin, a cell surface glycoprotein involved in cell-cell adhesion between mouse blastomeres (Hyafil et al., 1981). In the presence of calcium, there is only a single tryptic cleavage of the protein, while in its absence more extensive proteolysis occurs. Hyafil et al. (1981) presented evidence that this differential sensitivity is due to a calcium induced conformational change.

From these experiments, I was unable to determine whether the width of the trypsin sensitive region of the duct path is the same as that of the somitic mesoderm. Grafts of normal ducts onto trypsin treated flanks will enable one to map out the trypsin sensitive region of the duct path as well as the rest of the lateral mesoderm.

The finding that duct migration is affected only in the absence of calcium could prove useful in attempts to isolate proteins required for duct migration. Pre-treatment of cells with trypsin in the presence of calcium would remove many background proteins. Isolation of the remaining cell surface proteins and subsequent identification of proteins that are degraded by trypsin in the absence of calcium could provide the first step towards purification of the protein(s) of interest. This is similar to the approach used by Yoshida and Takeichi (1982) to identify a cell surface protein involved in calcium dependent aggregation of teratocarcinoma cells.

The effects of trypsin differ from those of tunicamycin; in the latter, the duct deviates laterally before migration is arrested. One could imagine that tunicamycin inhibits synthesis of cell surface proteins that are necessary for the lateral mesodermal cells to respond to a morphogenetic cue, whereas trypsin is destroying cell surface proteins produced in response to this cue. Thus, the target proteins would be different in each case. This could either be due to the difference in the stages at which the

embryos were treated, or to the difference in the mode of action. Both glycoproteins and proteoglycans will be digested by trypsin, where as the synthesis of most proteoglycans will be unaffected by tunicamycin since the majority of the carbohydrates in these macromolecules are O-linked (Roden, 1980).

## 4.7 SUMMARY: MORPHOGENETIC WAVES

The work presented in this thesis provides evidence for two waves of change that pass through the mesoderm in a craniocaudal sequence. These are different from that described previously by Poole and Steinberg (1982) who presented evidence for a wave that passes through the embryo just behind the migrating duct. Their wave results in inability of the lateral mesoderm to support secondary duct migration. This **post-migration** wave defines the anterior boundary of **active** mesoderm.

The results of the secondary duct transplants presented in section 3.2.1.1 provide evidence for a posterior boundary to the **active** mesoderm that moves posteriorly approximately two somites ahead of the **post-migration** wave, positioning this boundary immediately posterior to the last formed somite. This boundary, or wavefront, represents a change that takes place in the lateral mesoderm that is a pre-requisite for duct migration. This wave must pass through both the lateral and somitic mesoderm since

secondary ducts would not migrate over any part of the posterior mesoderm. Due to its position in the mesoderm, I have named this wave the **segmentation** wave.

The presence of another wave that passes through the embryo in a craniocaudal sequence was brought into evidence by the results of the trypsin experiments. This wave passes through the mesoderm 3.5 somite widths ahead of the segmentation wave described above. This **pre-segmental** wave is not only required for duct migration, but also for a normal segmentation pattern.

Thus, we have three waves, a **post-migration**, a **segmentation** and a **pre-segmental** wave, separated in space and time. These could represent three totally independent events. Alternatively, all of these waves could be the result of a multi-step response triggered by some other process. The first step would involve the production of trypsin-sensitive cell surface proteins that are necessary for the setting up of a normal segmentation pattern and duct migration. This would be followed by cellular changes that result directly in segmentation in the somitic mesoderm and ability to support migration in the lateral mesoderm. Finally, the cells of the lateral mesoderm undergo a change such that they lose their ability to support duct migration. Thus, each mesodermal cell would undergo a smooth succession of changes as a result of a single trigger.

The shape of the wavefronts is not known; however, indirect evidence from the secondary duct transplants

suggests that the post-migration wavefront is not parallel to the D-V axis, but at a $41^{o}$ angle to it. This is the same angle as that between the wavefront of lateral mesodermal shape changes and the D-V axis. In addition, the spatio-temporal pattern of shape changes coincides with that of the post-migration wave, thus suggesting that the two result from the same morphogenetic process.

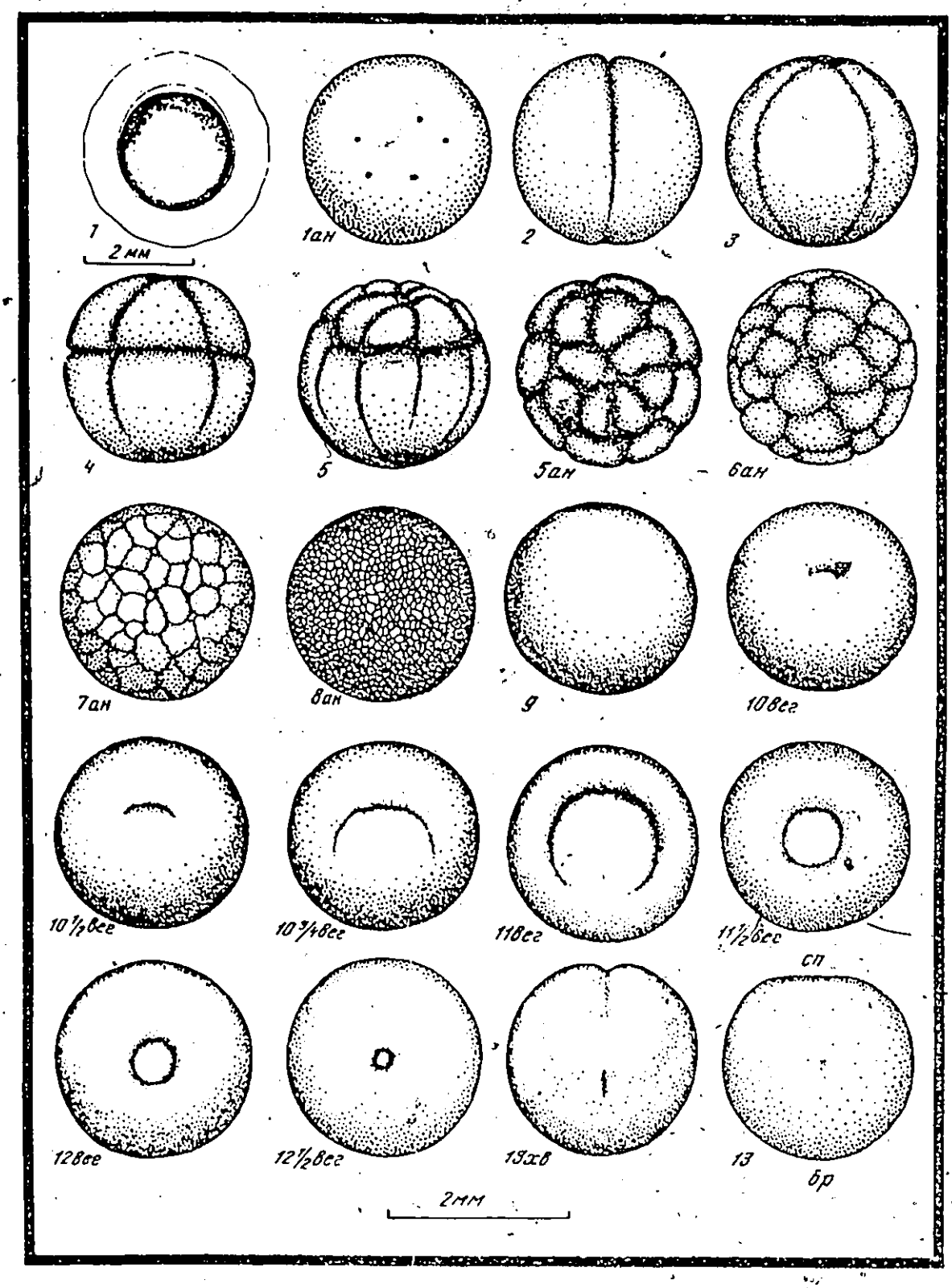
The evidence presented in this thesis suggests that a wave also passes through the epidermis in a craniocaudal direction. This epidermal wave does not appear to be directly involved in guiding duct migration, but it can inhibit migration. More information is required before the relationship between epidermal and mesodermal waves can be determined. It is possible that the two are causally related; for example, the epidermal wave could represent the trigger mentioned above.

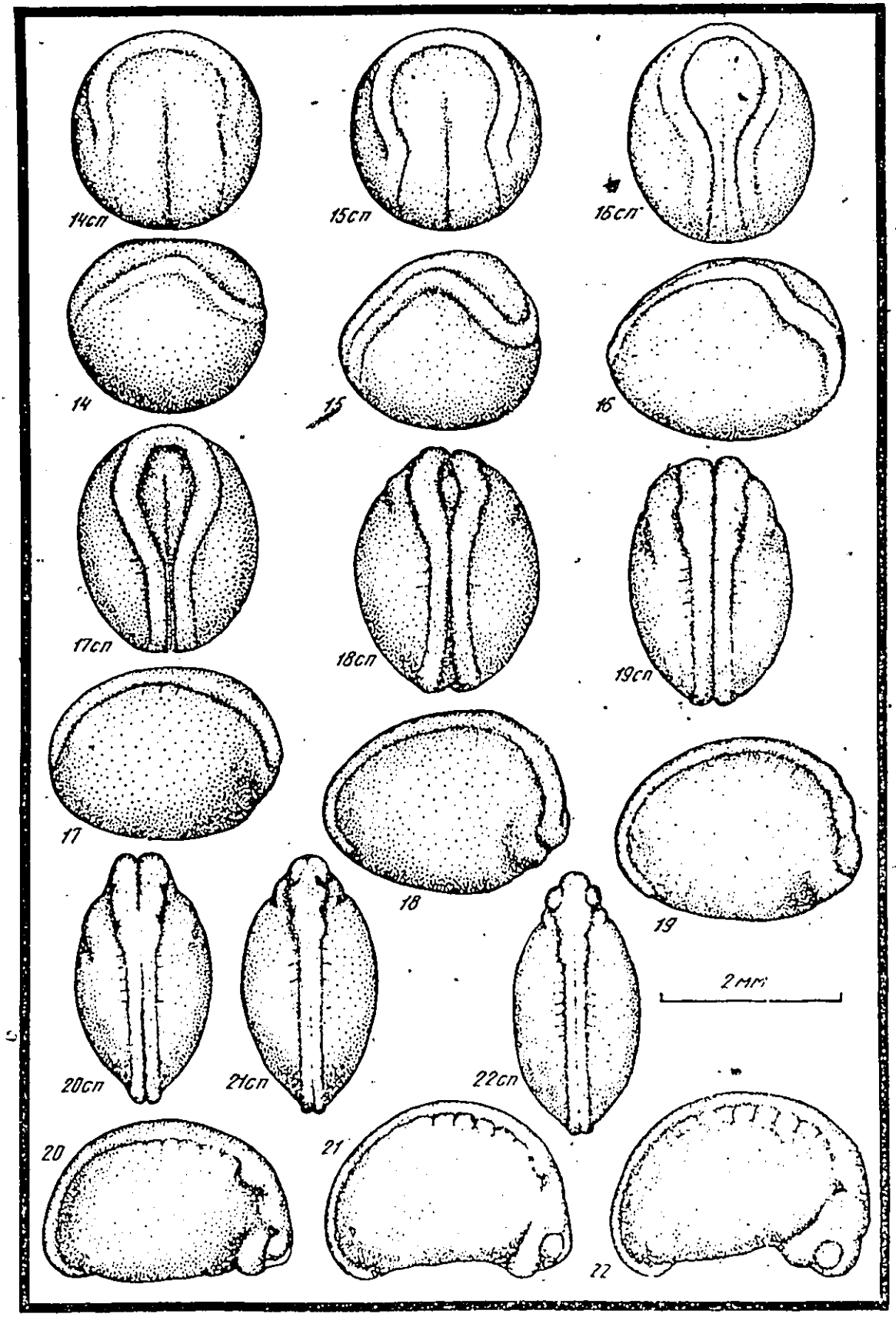
ECM production proceeded craniocaudally in advance of duct migration. Although the position of the posterior limit of visible ECM suggested a possible role in duct migration, its spatio-temporal pattern did not correspond to any of the mesodermal waves and its relationship to the epidermal wave remains to be determined.

Although the biochemical nature of the duct guidance mechanism is unknown, the results presented here have demonstrated the importance of a group of cell surface proteins, located in the ECM or cell membrane, for duct migration and have opened the door to biochemical analysis of these proteins.

# APPENDIX I

## STAGES OF NORMAL DEVELOPMENT OF AXOLOTL EMBRYOS

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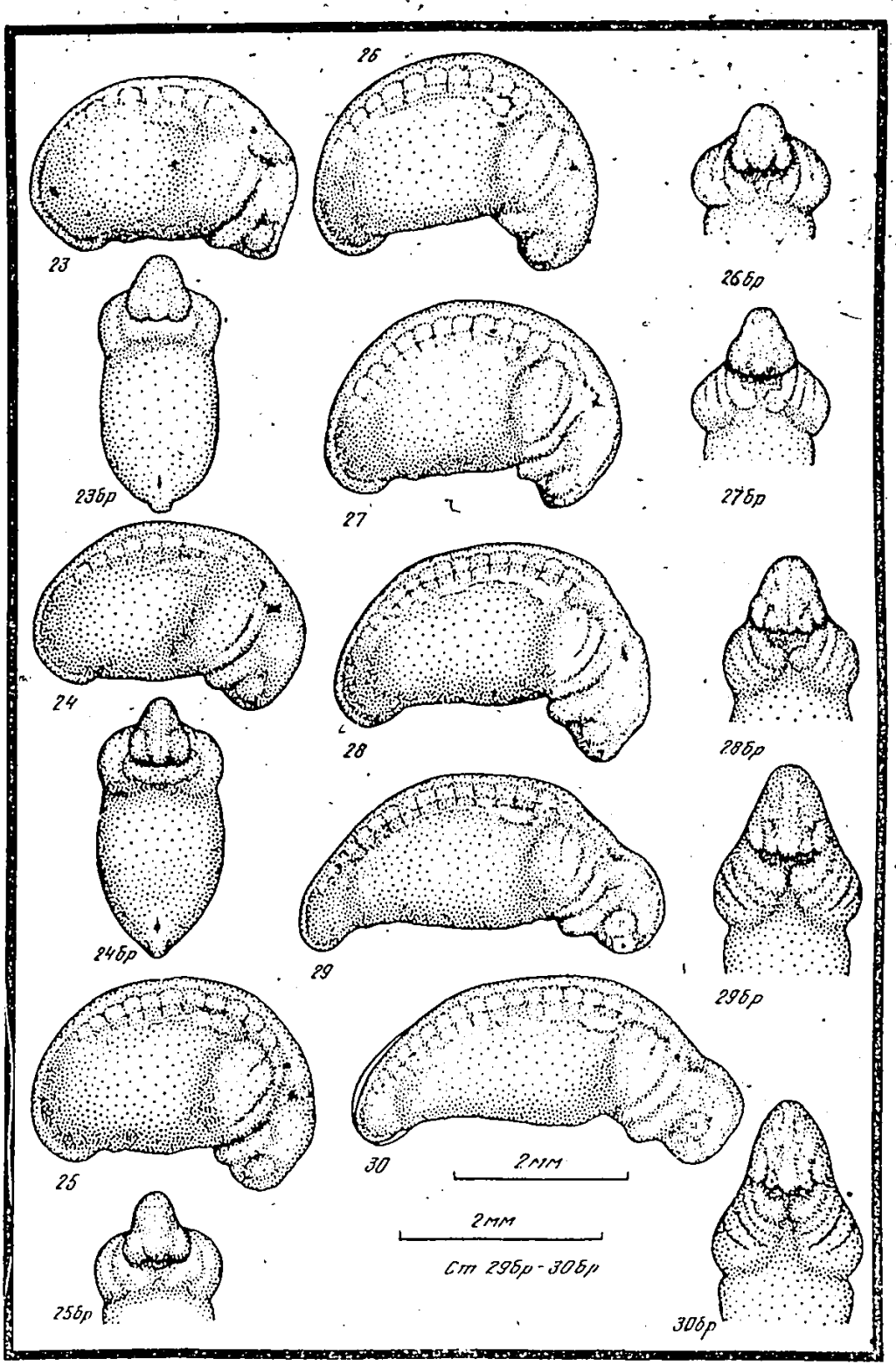
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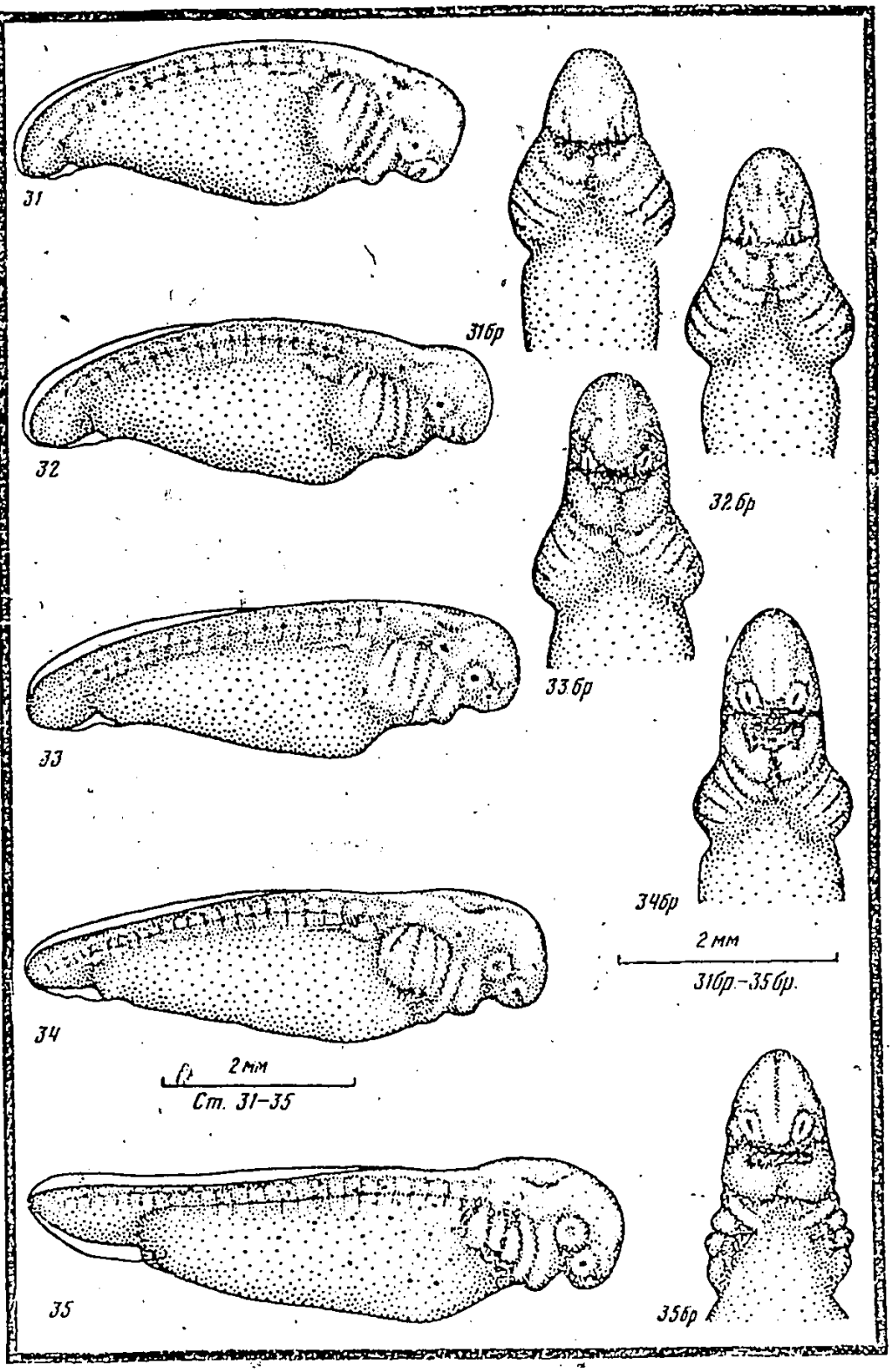
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