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STUDIES ON THE DIFFERENTIATION OF CRANIO-VISCERAL CARTILAGE IN NORMAL AND PREMATURE DEATH MUTANT EMBRYOS OF *Ambystoma mexicanum*.

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Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the
Ph.D. degree in Biology.

Université d'Ottawa/University of Ottawa

ISBN 0-315-60035-7

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ACKNOWLEDGEMENTS

I wish to thank some of the many people who have provided invaluable assistance in the preparation of this thesis.

I am especially indebted to my supervisor, Dr. John B. Armstrong. His patience and guidance were indispensable during the course of this study, and prevented me from drowning in a sea of useless detail. More importantly, however, he has shown me an outlook and approach to experimental research that I will strive to emulate.

The members of my research committee, Drs. James Neelin, Pierre Moreau, and Roberto Narbaitz, also deserve thanks, as they provided me with excellent scientific advice, as well as solutions to the seemingly endless list of technical problems.

William S. Fletcher (D.O.L.) is certainly deserving of his title, as demonstrated by his ability to fulfill my many requests for numbers, types and times, despite uncooperative animals and changing parameters.

I wish to thank Jacques Hélie, whose technical assistance was appreciated during the production of the drawings. The photographs presented in this thesis are the products of the generosity and expertise of many: George Ben-

tchavtchavadze, Paul Brunon, Tom Devecseri, Mary Whiteley, Steven Smith, the University of Ottawa Media Production Services, and the Ottawa-Carleton Centre for Biological Microscopy.

Merci à mes parents. Quand on aime ça, ça en vaut la peine.

Finally, special thanks go to Steve, the best ground support anyone could ever hope to have.

ABSTRACT

A great amount of controversy exists in the literature concerning the determination and inductive processes which occur prior to the differentiation of cartilage from cranial neural crest cells. Therefore, an *in vitro* culture system was used to investigate various aspects of the induction and differentiation of cartilage from the cranial neural crest in the Mexican axolotl, *Ambystoma mexicanum*.

The self-differentiative ability of the cranial neural crest cells was tested at various developmental stages between early neurulation, and the stage at which these cells complete their migration between the ectoderm and mesoderm and begin their medial migration to surround the mesodermal cells and form the visceral arches. Even at the latest of these stages, the processes through which cartilage determination occurs are far from complete; only a very small number of these cultures contained cartilage, although all appeared healthy and contained melanocytes and abundant mesenchyme.

At all of these stages, pharyngeal endoderm is the only inductor required for cranial neural crest cells to differentiate into cartilage. Over 90% of cultures containing neural crest and pharyngeal endoderm produced

mature cartilage nodules. The time course for chondrogenesis *in vitro* is similar to that which occurs *in situ*, which suggests that the culture system provides an accurate assessment of what occurs *in vivo*.

Only those cells which normally contribute to skeletal elements have the capacity to do so. Neither trunk neural crest cells nor the cells of the transverse (anterior-most) neural fold can produce cartilage, even when placed in intimate contact with inductive tissues. Similarly, inductive endoderm is only found in the head, which is the origin of, and ultimate location of, all chondrogenic cells of neural crest origin. A sharp boundary, which occurs at the junction of the pharyngeal wall and the pharyngeal floor, divides the inductive from the non-inductive endoderm. All anterior endoderm is capable of inducing mature cartilage formation in virtually all cases, whereas cartilage formation occurs only rarely in the presence of posterior endoderm.

Axolotl embryos which are homozygous for the premature death (*p*) gene do not develop past stage 37, which is well before cartilage differentiation usually occurs. They also display a variety of abnormalities in the gills, heart, pharynx, and liver, among others. Based on these and other observations, it was suspected that the mutation affected the endoderm. When mutant tissues were tested for the ability to form cartilage, however, the neural crest component of the

culture, and not the pharyngeal endoderm, was found to be defective. The lack of cartilage is not caused by an abnormal localization of chondrogenic neural crest, nor does the migration of the cranial neural crest appear to be affected.

The normality of the chordamesoderm and the ectoderm of mutant gastrulae was tested through the production of secondary neural structures. These studies determined that the chordamesoderm, which is the initiator of the events leading to neural crest specification, is normal in the mutant; the defect is in the ectoderm of mutant embryos.

The hypothesis that a number of neural crest functions are defective in the mutant appeared to be supported by the results of rescue attempts involving the transplantation of wild-type neural folds, and by the appearance of wild-type embryos which developed without neural crest cells. Thus, it would appear that the premature death mutation affects a subset of neural crest derivatives, and could therefore prove useful in the study of both the specification and differentiation of neural crest cells.

RESUME

Il y a beaucoup de controverse en ce qui concerne la détermination des cellules issues de la crête neurale céphalique. Un système de culture *in vitro* a donc été utilisé pour étudier l'induction et la différenciation de cartilage à partir des cellules de la crête neurale céphalique chez l'Urodèle *Ambystoma mexicanum*.

Les cellules de la crête neurale céphalique n'ont pas de capacité d'auto-différenciation pour le cartilage, quoique les prélèvements ont été faits à plusieurs stades embryonnaires, du jeune neurula jusqu'au stade où les cellules commencent leur migration médiale de la formation des arches viscéraux. Même lors de ces derniers stades, il est évident que les processus donnant lieu à la détermination pour la chondrogénèse ne sont qu'a peine commencés; très peu des cultures de crêtes neurales isolées avaient du cartilage, quoiqu'elles avaient toutes des cellules pigmentaires et mésenchymateuses.

Il n'y a qu'un seul tissu inducteur dans ce système, l'endoderme pharyngien. La présence de ce tissu provoque la formation de cartilage dans une vaste majorité de cultures de crêtes céphaliques. La chronologie de la chondrogénèse *in vitro* resemble beaucoup à celle *in situ*, ce qui indiquerait

la normalicité du système *in vitro*.

Les cellules de la crête neurale qui, normalement, ne donnent pas de dérivés squelettiques n'en ont pas la capacité, même lorsqu'elles sont en présence d'un inducteur actif. Il y a aussi une frontière distincte entre l'endoderme inductif et non-inductif, qui se trouve à la division usuelle de la tête et du corps de l'embryon. L'endoderme de la tête (l'endoderme pharyngien) induit la formation du cartilage dans presque tous les cas, alors que l'endoderme provenant de niveaux plus postérieurs n'en induit que rarement.

Les embryons d'axolotes qui sont homozygotiques pour le gène *p* (*premature death*) arrêtent de se développer au stade 37, qui est bien avant la différenciation de cartilage. Ces embryons ont aussi des difformités aux niveaux des branchies, du coeur, du pharynx, et du foie, entre autres. Ces observations, et les résultats d'autres expériences, ont mené à la conclusion que la mutation affect l'endoderme. La formation de cartilage a donc été mis à l'épreuve, en utilisant le système *in vitro* avec les tissus de mutants. Les résultats de ces cultures ont démontrés que la crête neurale, et non l'endoderme pharyngien, est défectueuse. Le manque de cartilage n'est dû ni à une mauvaise localisation de la crête chondrogénique, ni à une migration anormale des cellules de la crête céphalique.

La formation de structures neurales secondaires a été effectuée avec des tissus de gastrulae mutants. Le chordamésoderme, qui est l'initiateur des événements qui spécifient la crête neurale, est normal chez le mutant; le défaut se trouve dans l'ectoderme des mutants.

La supposition que, à part la chondrogénèse, d'autres fonctions de la crête neurale serait affectées chez le mutant a été appuyée par les résultats des transplantations de bourrelets médullaires de type sauvage chez le mutant, et par l'apparence d'embryons de type sauvage dépourvus de bourrelets médullaires. Ainsi, la mutation *premature death* semble affecter plusieurs, mais pas toutes, des dérivées de la crête neurale, et donc, pourrait s'avérer très utile pour l'étude de la specification et la différenciation des cellules de la crête neurale.

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CHAPTER I

INTRODUCTION

I-1. The neural crest:

The amphibian nervous system develops from presumptive ectoderm, due to the inductive influence of the underlying tissues (see Spemann, 1962; Saxén and Toivonen, 1962; Nieuwkoop *et al.*, 1985, for reviews). The neural folds are raised structures found at the periphery of the neural plate, at the boundary between the neurectoderm and ectoderm. The neural crest is a population of cells first found within the neural folds, although the cells do not remain with the other neurectoderm cells, which are destined to form the central nervous system. The neural crest cells undergo extensive migration following specific pathways to their final destinations, where they differentiate into an amazingly diverse array of derivatives. In urodeles, migration begins after the folds have begun to fuse to form the neural tube. The cells begin migrating first from the mesencephalic regions, and then in more anterior and posterior regions.

Neural crest cells have been the subject of much study and controversy since their discovery in the chick by His in 1868 (reviewed by Hörstadius, 1950; Hall and Hörstadius, 1988). The neural crest cells disperse throughout the embryo and are

extremely invasive, which has complicated the study of their migration and differentiation. To overcome these difficulties, a variety of markers has been used to follow the cells and determine their derivatives (see Weston, 1967; 1970; Le Douarin, 1982).

The first such markers were based on the intrinsic differences in pigmentation and yolk platelets between the neural crest cells and mesoderm in amphibians, and on vital stains applied *in situ* (Detwiler, 1937; Detwiler and Kehoe, 1939; de Beer, 1947). These caused the least amount of disruption to the embryos, but did not always allow positive identification of the neural crest cells (particularly individual cells) or their derivatives, since these markers changed with time, faded, and sometimes labelled neighboring cells through diffusion of the dye.

Xenoplastic transplantations of neural folds between species with cells of different sizes and pigmentation (Raven, 1937; LeDouarin, 1982) and different nuclear staining patterns (Sadaghiani and Thiébaud, 1987; Krotoski *et al.*, 1988) have also been used. The origins of the cells were easily identified, although the results may have been affected by developmental differences between the species or even by actual interactions between the cells of the two species (Chibon, 1967; Krotoski *et al.*, 1988).

Extirpation of neural crest has also been used

(Hörstadius, 1950; Chibon, 1966). Although this method did not allow migration to be followed, it did allow for the identification of some cell functions. The deficiencies and abnormalities seen after neural crest removal were indicative of neural crest involvement, although it was impossible to distinquish between structures which were derived from neural crest and those which were merely dependent on the neural crest for their proper development through inductive interactions. The analysis of these experiments was further complicated by regulative events, which could partially or completely correct defects resulting from some of the less extreme operations (Hörstadius, 1950; Chibon, 1966).

Chibon (1966) performed an extensive study of the neural crest in *Pleurodeles waltl*, using a marking technique which yielded much more reliable results. Segments of tritiated thymidine-labelled neural folds were transplanted onto unlabelled host embryos of the same species. Not only were the normal neural crest derivatives of different axial levels established, by homotopic transplantations, but the developmental potential of the segments was tested by performing heterotopic transplantations. The use of this method resolved many of the controversies caused by the limitations of the other techniques.

According to Chibon (1966), the neural crest gives rise to most of the pigment cells, major portions of the

peripheral nervous system (both sensory and autonomic), including such supporting cells as Schwann sheath cells and parts of the meninges, and chromaffin (adrenal medullary) cells. In a role usually associated with mesoderm, neural crest cells also give rise to mesenchyme.

Quail-chick chimeras have been extensively used to expand the list of known neural crest derivatives, in birds, to include portions of the endocrine and paraendocrine systems (carotid body type I and II cells and calcitonin cells), and head connective tissues (muscles and the connective tissue components of the pituitary, lacrymal, salivary, thyroid, parathyroid and thymus glands; see Le Douarin, 1982). It is not yet known whether amphibian neural crest is capable of forming all of these derivatives.

It has been proposed that the final fate of neural crest cells is determined through a series of stepwise restrictions of developmental potential (Bronner-Fraser and Cohen, 1980; Nieuwkoop *et al.*, 1985; Le Douarin, 1986). Various workers have characterized possible intermediates in birds, using a variety of techniques. For example, Ciment and Weston (1985) found that, while premigratory rhombencephalic neural crest could give rise to connective, neural, and glandular tissues, as well as melanocytes, by the time they had colonized the branchial arches, the cells no longer had melanogenic potential. By this time, anterior and posterior branchial

arch cells were different with respect to their immunoreactivity to the monoclonal antibody E/C8, and their differentiative potentials (connective tissues can form from both populations, though the neuronal and glandular derivatives are only formed by the posterior cells). Le Douarin (1986) has suggested a sequence of restriction steps for trunk neural crest cells, from the results of heterochronic transplantations of quail peripheral ganglia into chick hosts (Le Lièvre *et al.*, 1980; Le Douarin, 1986). Soon after their emigration from the neural tube, the sensory and autonomic precursors would be segregated from a common precursor. In the ganglia developing in close proximity to the central nervous system (CNS), the sensory precursors would become post-mitotic within seven days, while the autonomic precursors would persist without differentiating. In the autonomic ganglia, farther from the CNS, some autonomic precursors would become post-mitotic, although some would remain in the undifferentiated state, at least until hatching. The sensory precursors which were initially present would rapidly disappear from these ganglia, possibly due to the lack of a growth factor being emitted by the CNS.

The environment through which the neural crest cells migrate and localize appears to play an important role in their differentiation. Culture conditions have affected the cell types which differentiated from chick neural crest cells

in vitro (see Le Douarin, 1986; Bronner-Fraser and Cohen, 1980). Furthermore, when avian or amphibian neural crest cells are transplanted to other axial levels, the cells give rise to derivatives not normally formed by cells of the donor axial level, but which are normal for neural crest cells of the host site (Chibon, 1966; 1970; Le Douarin, 1982; Noden, 1978a, b).

While these models address the multipotentiality of the neural crest cell population, the status of the individual cells is unknown. Each cell may indeed be multipotent, and external factors would therefore be directly responsible for the developmental decisions made by the cell. Alternatively, each cell may already be determined prior to migration (although the population would be heterogeneous). The pathways and final locations could allow the expression of only one of the possible cell types, or they could be preferentially chosen by the appropriate precursors. There is evidence that at least some neural crest cells are multipotent. Some clones of individual neural crest cells have given rise to both melanocytes and neurons (Sieber-Blum and Cohen, 1979). Even more striking were the results of microinjecting single neural crest cells with a fluorescent lineage marker *in situ*; as many as 4 different cell types differentiated from a single precursor cell (Bronner-Fraser and Fraser, 1988).

While some neural crest cells are multipotent, not all neural crest cells are identical. In the heterotopic transplantations mentioned above, not all grafted cells developed according to the host site. In some cases, the localization and differentiation was normal for neural crest cells, although at a non-host axial level. In other cases, the development was abnormal for neural crest cells, such as the participation of neural crest cells in vertebral cartilage formation (Le Douarin, 1982). In amphibians, only trunk neural crest cells have the ability to form Rohon-Béard cells, whereas chondrocytes and odontoblasts are strictly cranial derivatives (Hörstadius, 1950; Chibon, 1966).

1-2. Contributions of cranial neural crest to the amphibian cranio-visceral skeleton:

The ability to form mesenchyme differs between head and trunk neural crest in amphibians (Hörstadius, 1950; Chibon, 1966). Trunk neural crest normally gives rise only to dorsal fin mesenchyme *in situ*, and cannot produce cartilage and odontoblasts (normal derivatives of the cranial neural crest) when transplanted to the head. The majority of cranial neural crest cells, however, normally differentiate into mesenchymal derivatives, although they also possess the ability to form both neural tissues and pigment cells.

The cranial neural crest is the source of the entire

visceral skeleton, except for the second basibranchial. In the cranial skeleton, the anterior portions of the trabeculae and the palatoquadrates are neural crest-derived. The neural crest also contributes to the formation of the posterior trabeculae, basal plate, and parachordal cartilages. Table 1.1 correlates these skeletal elements with the axial levels of cranial neural crest which form them, according to the notation devised by Chibon (1966; see Fig. 1.1).

During development, the proper differentiation of many tissues and organs is dependent on interactions with other tissues. At the end of the induction(s), as these interactions are called, the tissue is said to be determined, and is capable of final differentiation even if removed from its normal environment (see Hamburger, 1988).

While many neural crest cell derivatives have been identified, the timing of their determination to form these structures has been more difficult to ascertain. The stage at which cranial neural crest is determined for differentiation into cranio-visceral cartilage has been tested (in amphibians) by transplanting it to the flanks of host embryos (Raven, 1935; Hörstadius, 1950; Newth, 1954). The presence of ectopic cartilage in host larvae was taken as an indication that the neural crest cells had already received all the inductive signals required for differentiation. The critical assumption of this system was

TABLE 1.1

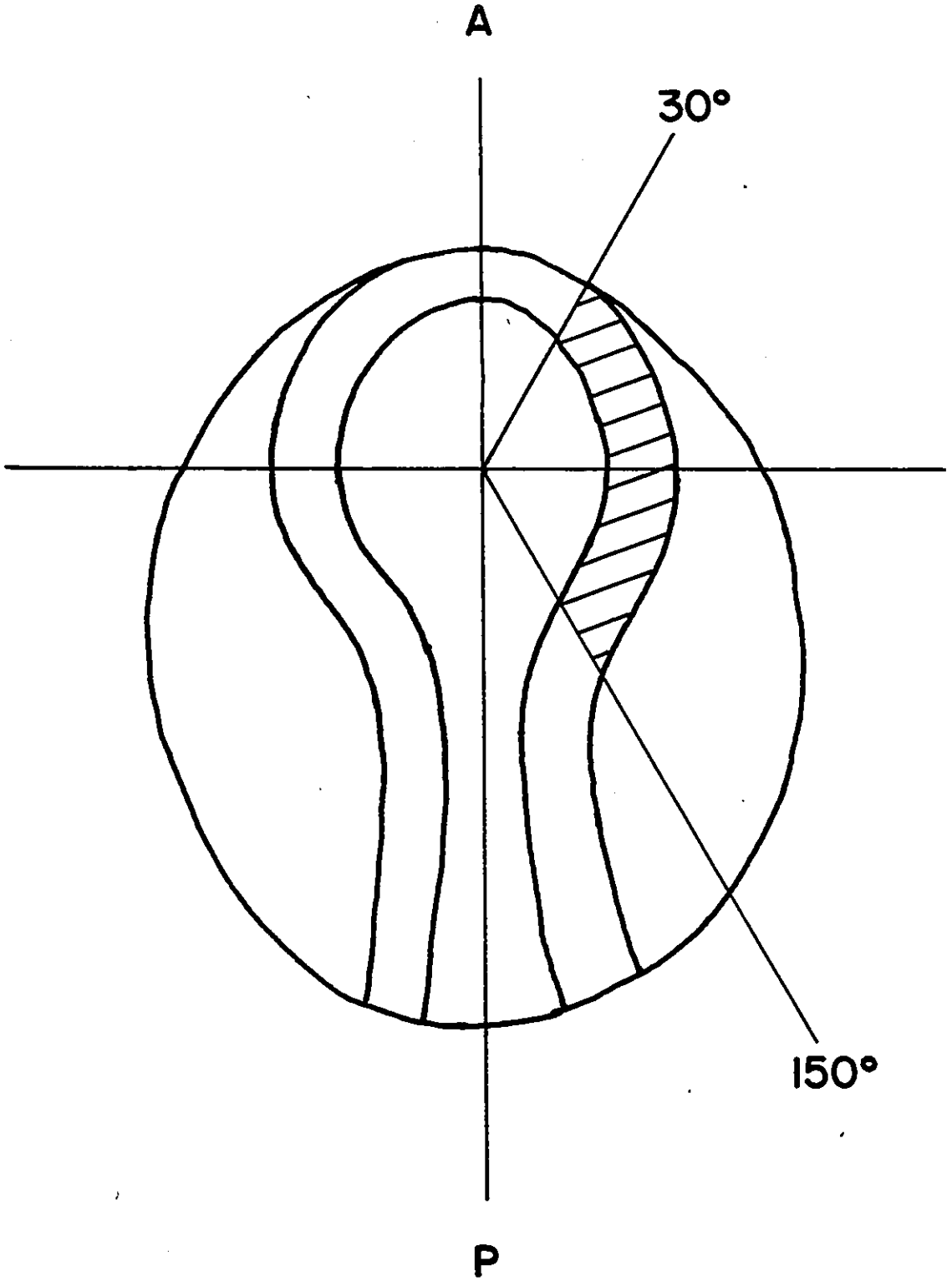
Skeletal Neural Crest Derivatives[1]

Neural Crest Region[2]	Skeletal Elements
0° - 30°	No skeletal elements
30° - 50°	Anterior portions of trabeculae
50° - 70°	Posterior portions of trabeculae Basal plate Palatoquadrates
70° - 100°	Meckel's cartilage Hyoid arches
100° - 120°	Basibranchial I Hypobranchials Anterior branchial arches
120° - 150°	Posterior branchial arches

[1] Translated from Chibon (1966).

[2] Axial levels as defined by the system depicted in Figure 1.1.

Figure 1.1: Dorsal view of a stage 17 embryo indicating the notation used to describe axial levels of cranial neural crest cells which have been found to contribute to the cranio-visceral skeleton of amphibians (adapted from Chibon, 1966).


A
30°
150°
P

that the graft site did not affect the differentiation of the transplanted cells.

Even when this technique was repeated within one species, however, different workers obtained very different results. Raven (1935) found ectopic cartilage in nearly all cases of transplanted neural folds from mid-neurulae of *Ambystoma mexicanum*. According to Hörstadius (1950), however, ectopic cartilage was only seen when specific tissues were co-transplanted with the cranial neural folds, or when certain host tissues (somites) were damaged in the vicinity of the transplant. Newth (1954) repeated these studies, again using axolotl neurulae, and was able to show that the longer culture periods used by Raven (1935) were not the cause of the discrepency. Newth's (1954) results, however, were at neither of the two extremes of the previous studies; approximately 19% of the host larvae contained ectopic cartilage.

Another source of concern is that the graft sites contained mesoderm, which is known to have chondrogenic potential (it forms the entire axial and appendicular skeleton). Therefore, the ectopic cartilage could have been derived from host mesoderm due to the unnatural influence of either the grafted tissues or the surgery itself (as suggested by Hörstadius, 1950). Thus, it would appear that the embryonic flank may not be the neutral environment which

is required for testing the self-differentiative capacities of the cranial neural crest.

As mentioned previously, Hörstadius (1950) obtained cartilage formation when the neural folds were co-transplanted with other tissues. When the other tissue was pharyngeal endoderm from the gill area, not only was cartilage formed in the large majority of cases (88%), but the morphology greatly resembled that of the branchial area, with rods of cartilage found between structures which resembled gill slits. The suspicion that this endoderm might be the normal inductor was strengthened by the finding that when the pharyngeal wall was unilaterally removed, there was a lack of cartilagenous arches on the operated side. (The term "inductor", in this thesis, will be used according to the definition of Holtfreter, 1968; the inductor is the tissue which produces the agent of induction, the "inducer".)

In addition to the transplantation experiments, *in vitro* (explant) cultures have been used to investigate the determination and induction of cartilage from cranial neural crest. Using this method, a variety of urodele species have been examined, although *Ambystoma mexicanum* was not among them. The results of these studies were, for the most part, in accordance with those of Hörstadius (1950). Cranial neural crest cells from neurulae were not determined for cartilage formation, as this tissue did not form when neural

folds were cultured in isolation. These cultures did give rise to pigment cells and abundant mesenchyme (Corsin, 1975), and, in addition, neurons and Schwann sheath cells have also been reported (Wilde, 1955; Epperlein, 1974; 1978).

Pharyngeal endoderm was present in all cases in which chondrocytes developed. The extent of its involvement, however, appeared to be variable. In *Triturus alpestris*, cartilage, including intermediate phenotypes as well as mature cartilage nodules, was found in cultures of cranial neural folds and pharyngeal endoderm (Seno and Nieuwkoop, 1958; Drews *et al.*, 1972; Epperlein and Lehmann, 1975). Although the reported numbers of positive cases was rather low (55% for Seno and Nieuwkoop, 1958, and 60% for Drews *et al.*, 1972), pharyngeal endoderm appeared to be the only inductor required. Similar results have been reported for *Ambystoma tigrinum*, although only 5 of 26 cultures (19%) contained cartilage (Holtfreter, 1968).

Results from similar studies performed in other urodeles have indicated that, although the pharyngeal endoderm is a necessary inductor, other tissues are also required for complete differentiation of cartilage from the cranial neural crest of neurulae. The additional inductor was found to be stomodeal ectoderm in *Ambystoma maculatum* (Wilde, 1955), and dorsal mesoderm in *Pleurodeles waltl* (Corsin, 1975). In both of these studies, exposure to pharyngeal endoderm alone only

induced procartilage formation from the cranial neural crest of neurulae. (Procartilage was defined as being composed of condensations of cells with the morphology of chondrocytes, but with little or no extracellular matrix.) When the neural crest cells were only exposed to the second inductor, no evidence of chondrogenesis was seen, suggesting a sequential set of inductions.

In *Pleurodeles waltl*, the induction by pharyngeal endoderm is completed by mid-neurulation; neural folds from late neurulae yielded procartilage in isolation, and such cultures containing dorsal mesoderm produced mature cartilage nodules (Corsin, 1975). The inductions in *Ambystoma maculatum* appear to occur much later, however. Neural crest cells from embryos as old as stage 23 (initiation of migration) still required the presence of pharyngeal endoderm to produce procartilage (Wilde, 1955).

It is obvious from the preceding discussion that many questions concerning the timing of determination and the inductive processes required for the differentiation of cartilage from cranial neural crest cells remain unanswered. Furthermore, species differences are quite apparent, even among urodeles. Therefore, the original purpose of this study was to investigate various aspects of the induction process(es) believed to occur in this system, using the axolotl (*Ambystoma mexicanum*). The results of this portion

of the study have been partially reported by Graveson and Armstrong (1987).

1-3. The axolotl:

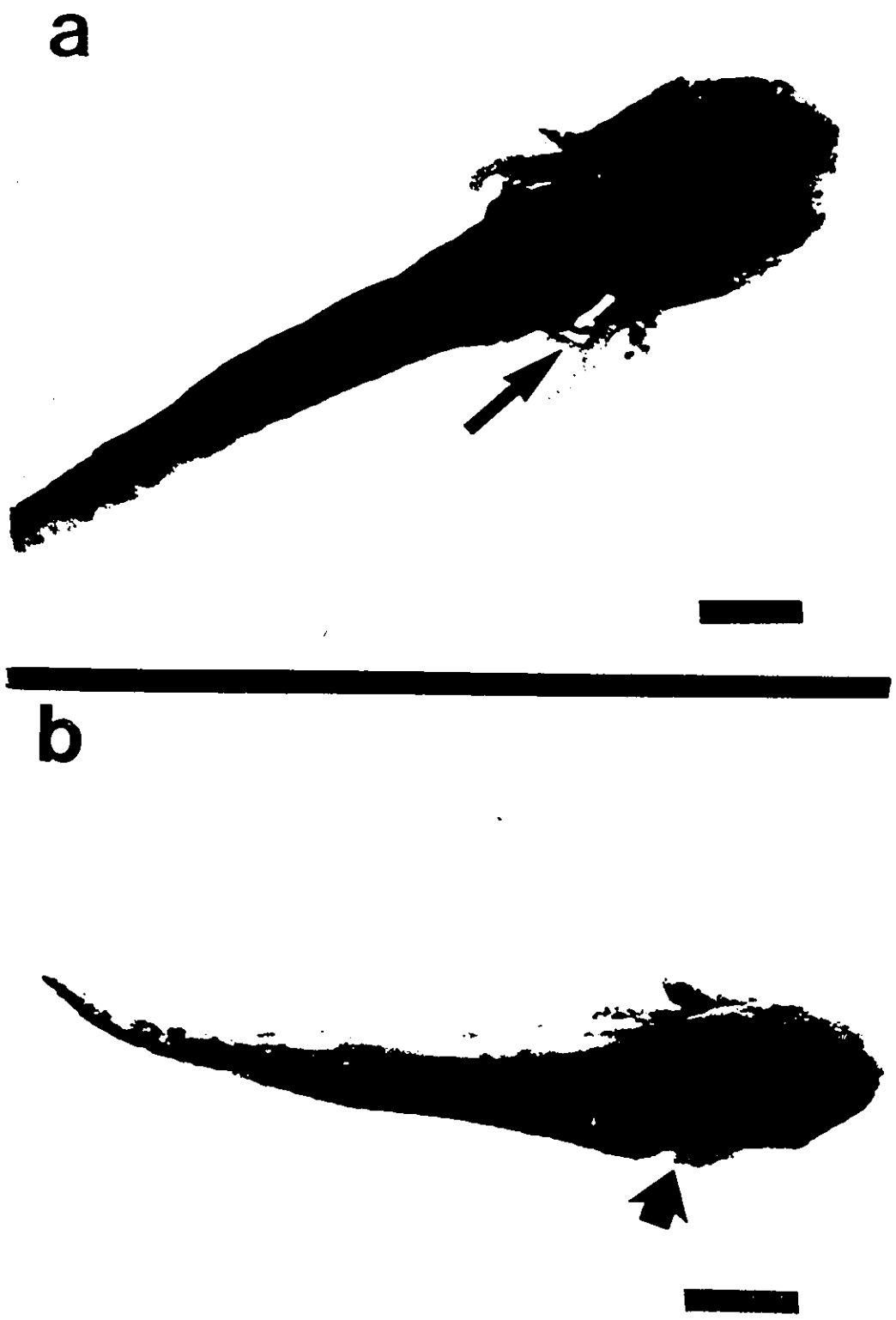
The Mexican axolotl, a neotenous urodele, is particularly well suited to developmental studies. Spawnings can be obtained throughout the year, and each can produce several hundred fertile eggs of known genealogy (Armstrong and Fletcher, 1984). The eggs are large (approximately 2 mm in diameter), allowing surgical manipulations to be performed with relative ease. The development is sufficiently slow (11 days to hatching at 18°C; Bordzilovskaya and Dettlaff, 1979; Bordzilovskaya *et al.*, 1989) that specific developmental stages can be obtained with certainty. Furthermore, the rate of development can be controlled by varying the temperature, allowing the simultaneous use of different stages from a single spawning, or allowing different spawnings to be synchronized. Axolotls have been used as research subjects for over one hundred years, and many aspects of their anatomy, physiology, and development are therefore well known (see Armstrong and Malacinski, 1989). Finally, several dozen developmental mutations (which can be used to study the development of specific organ systems and tissues) and colour variants (which can be used either as markers or to examine pigment cell migration and differentiation) have been

described in the axolotl (Armstrong, 1985; Malacinski, 1989).

1-4. The premature death mutant:

One mutation in the axolotl, the premature death (*p*) mutation, was first described by Trottier and Armstrong (1977). Embryos homozygous for this gene develop apparently normally until stage 37, when development arrests. The gills on these mutants never develop secondary filaments, and bulb-like structures are often seen at the distal ends of the primary filaments (Fig. 1.2). The eyes tend to be underdeveloped, although this trait is variable. The hearts do beat in mutant embryos, but only weakly, and circulation is not established. The blood cells remain in the blood islands, where they originate. Weak tactile reflexes can be observed, although righting and swimming reflexes are absent in these embryos. This suggests the presence of functional Rohon-Béard cells, which are the initial sensory neurons of larval amphibians. Epidermal blistering and disintegration of superficial tissues begins within days. The developmental arrest apparently does not affect the pigment cells of the mutant. The timing and pattern of their appearance is the same as that seen in wild-type embryos.

Histological examination of the internal structures revealed a wide variety of abnormalities in mutant embryos (Trottier and Armstrong, 1977). Particularly striking were:


a
b

a plug of undifferentiated cells, instead of endocardium, in the anterior regions of the heart (ventricle and conus arteriosus), the underdeveloped state of the liver, and the abnormal morphology of the pharynx and associated structures (gill pouches). The myotomes of mutant embryos appeared normal, though underdeveloped, but degenerated rapidly after stage 37.

The most obvious defective organs in these embryos depend on the endoderm for their development. The liver and pharynx are endodermally derived, whereas the gills and heart require the endoderm as an inductor. This suggested that endoderm is the target tissue for the *p* mutation (Trottier and Armstrong, 1977). This hypothesis was apparently supported by the results of transplantations of mutant tissues onto wild-type hosts. The survival and development of these grafts appeared to be correlated with their requirement for endoderm. Transplants of eyes, limbs, and epidermis were successful, whereas gill transplants were resorbed by the host (Mes-Hartree and Armstrong, 1980).

If defective endoderm were indeed the cause of the abnormalities seen in the *p* mutant, then other functions of the endoderm might also be affected. Since pharyngeal endoderm is probably involved in the induction of cartilage formation by cranial neural crest cells, it seemed probable that the mutant could provide an ideal model system for

studying the role of the inductor in more detail than could be done using wild-type embryos alone. Therefore, the effects of the *p* mutation on the differentiation of cranial neural crest into cartilage were also examined. Some preliminary findings of this study have been reported in abstract form (Graveson and Armstrong, 1988).

CHAPTER II

MATERIALS AND METHODS

2-1. Animals:

The Mexican axolotl (*Ambystoma mexicanum*) was used for all experiments. The embryos used in this study were obtained from spawnings of animals raised and bred at the University of Ottawa Axolotl Colony. The adults were kept in individual plastic mouse cages containing 50% modified Holtfreter's solution (Table 2.1). This solution was changed three times a week, after which the animals were fed strips of beef heart.

Females were injected with 250 I.U. of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, Mo.) approximately 16 hours prior to spawning. Occasionally, the males were also injected with the same dosage of the hormone.

2-2. Embryos:

Fertilized eggs were deposited within 24 hours of the spawning. They were removed from the mating pan and placed in small bowls containing 25% modified Holtfreter's solution (Table 2.1).

The rate of development of the embryos can be slowed by lowering the temperature. Post-gastrula embryos were kept at

TABLE 2.1

Culture Media

Holtfreter's medium:

NaCl	3.46 g
KCl	0.05 g
$CaCl_2$	0.10 g
$MgSO_4 \cdot 7H_2O$	0.20 g
$NaHCO_3$	0.20 g

per litre dechlorinated tap water.
pH 7.4.

Steinberg's medium:[1]

NaCl	3.40 g
KCl	0.05 g
$CaCl_2$	0.05 g
$MgSO_4 \cdot 7H_2O$	0.21 g
Tris	0.56 g

per litre distilled water.
pH 7.7

[1] Calcium-free Steinberg's medium was made by omitting the $CaCl_2$.

20°, 10°, or 4°C, as required. Embryos which had not completed gastrulation were not placed at temperatures lower than 10°C, as these low temperatures appeared to cause abnormal gastrulation in a large proportion of embryos.

Embryos were staged according to the morphological characteristics diagrammed and described in the staging table of Bordzilovskaya and Dettlaff (1979; see also Bordzilovskaya *et al.*, 1989; Appendix I).

2-3. *Surgical equipment:*

Most of the instruments used for operating on the embryos could not be purchased, and were therefore made as required. The following is a brief list of the instruments used in this study, and their uses.

Forceps: Watchmaker's forceps (Dumoxel No. 5) were used for dejelling embryos, removing vitelline membranes, and manipulating glass bridges. The tips of the forceps were sharpened on an oilstone to fine, matching, points.

Needle: The main cutting implement was a 5 mil thick tungsten wire with an electrolytically sharpened point bent at a 45° angle.

Hair Loops: Small (1-3 mm) loops of fine blond hair,

attached to a handle, were used to move embryos and tissues, and to cut tissues.

Glass bridges: Rectangles of #1 coverslip glass (approximately 1 x 2 mm) were used to hold grafted tissues in place on the host during the healing process.

Operating Dishes: Pyrex 3- and 9-well spot plates were used for all surgical procedures with the exception of the dorsal lip transplantations (see below). The wells of the plates were lined with a 4:1 (w:w) mixture of Permoplast (American Art Clay Co., Inc., Indianapolis, Ind.) and Paraplast (Lancer, St. Louis, Mo.). At the end of each operating session, the used wells were rinsed successively with tap water and 95% ethanol. Prior to use, the air-dried dish surfaces were smoothed, filling in all previous depressions, and sterilized by heating over the flame of an alcohol lamp until the surfaces were shiny.

Dorsal lip transplantations were performed in disposable plastic Petri dishes 1/3 filled with sterile 1% Noble agar (containing 0.05 mg/ml gentamicin sulphate). These plates were made several hours before use.

Micropipettes: Spemann micropipettes (Hamburger, 1960) were modified and used to transfer explants between operating

dishes and culturing vessels. To make each pipette, the narrow portion of a Pasteur pipette was cut approximately 2 cm from the taper and smoothed in a flame so as to leave an opening about 1 mm in diameter. The wide portion of the pipette was drawn, cut, and flamed smooth approximately 5 cm from the taper. This end was then inserted into a length of rubber tubing (3 mm bore) attached to a mouthpiece. Solution was drawn into the micropipette by capillary action, and the fine control required for manipulating the tissues was attained by applying and releasing pressure on the rubber tubing.

Media: The various media used during the operations and the embryos and explants are described in Table 2.1. All media were filter-sterilized through filters with 0.22 µm pores (Nalgene or Millipore). As well, 0.05 mg/ml gentamicin sulphate (Sigma, or Schering Co., Kenilworth, N.J.) was added to all media.

2-4. Procedures for explantations:

Jelly coats and vitelline membranes were manually removed using sharpened watchmaker's forceps. Each embryo was then passed through three rinses of sterile 100% Steinberg's solution before being placed in the Permoplast/Paraplast operating dish which also contained 100% Steinberg's

solution. The embryo was placed in a depression designed to hold it snugly. The desired tissues were removed using a tungsten needle and hair hoop, and brought to another, shallower depression. The tissues were placed in this depression with the ectoderm-side down. The explant was left undisturbed for approximately 30 minutes, during which time it balled up, and dead cells fell away. The explant was then transferred to the appropriate culturing container using a modified Spemann pipette.

For most experiments, the explants were to be processed for light microscopy. Such explants were cultured in approximately 150 µl of sterile 100% Steinberg's solution in BEEM capsules (J.B. EM Services, Inc., Montréal, Qué.) lined with 1% Noble agar. In this way, the explant could be processed directly in the culturing container, eliminating the need for transferring it at the end of the culture period.

Explants of presumptive heart tissue, which were examined daily for beating tissue, were cultured in hanging drops. Drops (10-20 µl) of sterile 100% Steinberg's solution were placed in the lids of plastic Petri dishes. One explant was placed in each drop. The bottom of the Petri dish contained a few millilitres of sterile distilled water, in order to provide enough humidity to prevent evaporation of the drops. Petroleum jelly (Vaseline) was used to seal the

lid to the bottom of the dish.

For some experiments, the embryo from which the tissues were removed was kept for phenotype identification. If it was not sufficiently healed after the transfer of the explant from the operating dish, most of the solution in the operating dish was replaced with calcium-free Steinberg's solution. After about 30 minutes, this solution was replaced with 100% (calcium-containing) Steinberg's solution. After a few minutes, the embryo was transferred to its culturing container.

2-5. Procedures for transplantations:

The initial preparation of the embryos for transplantations was identical to that for explants. The desired tissues were removed from the donor, and placed next to the host, carefully maintaining the orientation of the graft. The host site was then prepared by removing tissue. For heterotopic transplants, usually done to test the development of organ primordia in abnormal environments, the area of tissue removed was slightly smaller than the graft. This ensured faster and better healing of the graft. For homotopic transplants, usually performed as reciprocal operations between mutant and wild-type embryos, the tissue removed from the host corresponded exactly to that taken from the donor.

The graft was placed in the host site, and the edges of the graft were tucked under the edges of the ectoderm of the host. A glass bridge was placed over the graft, completely covering it, with some of the weight of the bridge being supported by the Permoplast. Most of the operating solution was replaced with calcium-free Steinberg's solution. After 30 minutes, this solution was replaced with 100% Steinberg's solution. Several minutes later, the glass bridge was carefully removed, and the embryo gently transferred from the operating dish. The usual culturing containers for the experimental embryos were 24-well dishes (Corning Glassworks, Corning, N.Y. or Falcon, Becton Dickinson and Co., Lincoln Park, N.J.). Each well was lined with 1% Noble agar to prevent the embryo from adhering to the dish. Following surgery, the embryos were maintained in 100% Steinberg's solution for about 2 days, after which 25% Holtfreter's solution was used, and changed every 2 days.

2-6. Procedures for dorsal lip transplantations:

These operations were not performed in the same manner as the other transplantations, and will therefore be described separately. After removal of their jelly coats, the embryos were passed through the series of rinses of sterile media with the vitelline membrane intact. The operating dish was a plastic Petri dish, as described above. The operating

solution was 100% Steinberg's medium. The embryo was placed on the agar and left undisturbed for a few minutes, while it rotated within the vitelline membrane so that the animal pole was uppermost. The tip of the tungsten needle was poked through both the vitelline membrane and the cells forming the roof of the blastocoele, usually causing the animal cap to collapse. The vitelline membrane was removed by placing one tine of each of two pairs of forceps into the perivitelline space, through the hole made by the needle, and then pulling the forceps apart. The host was placed such that the animal pole was uppermost, while the donor was placed such that the dorsal lip of the blastopore was uppermost. The piece of dorsal lip was removed, using a tungsten needle and hair loop, and placed on top of the flattened host. The needle was inserted into the hole made for removing the vitelline membrane, and one side of the blastocoele roof was gently lifted. A hair loop was used to slide the tissue into the blastocoele, towards the opposite side from the host's dorsal lip. The size of the hole was minimized by pushing the tissues together with hair loops. The embryos, both donor and host, were left undisturbed for at least 24 hours, when the survivors were handled as previously described for explant removal or phenotype identification.

2-7. Processing for light microscopy:

Explants were processed for light microscopy in the culturing BEEM capsules, thereby eliminating the possibility of loss and damage during transfer.

Explants and embryos were fixed overnight in 2% glutaraldehyde, 0.5% cetylpyridinium chloride, 0.5% polyvinylpyrrolidone in 0.1 M sodium cacodylate buffer, pH 7.7. Following several cacodylate buffer rinses, the specimens were dehydrated in a graded ethanol series. The embedding medium was glycol methacrylate (Sorvall/Dupont Instruments or JB-4 from J.B. EM Services Inc.). Infiltration with the monomer was done at room temperature, with at least one change of fresh solution per day over the course of several days.

Serial 4 µm sections were made with a Sorval JB-4 microtome, using freshly made glass knives. The sections were placed in drops of water on ethanol-cleaned slides, which were then allowed to air dry.

The slides were stained for 15 minutes in 0.5% toluidine blue in 0.1 M sodium benzoate buffer, pH 4.4, rinsed in several changes of the benzoate buffer, dipped in acetone, and air-dried. Sections were examined with either a Wild M-11 or a Zeiss Standard RA microscope.

2-8. Statistical analysis:

All statistical analyses were carried out with Statistical Analysis System (SAS) programs, using Proc. Frequency. Likelihood ratio chi-square tests were performed in all cases. Identical superscripted letters indicate the classes of values which were compared for each test.

CHAPTER III

DIFFERENTIATION OF CARTILAGE FROM CRANIAL NEURAL CREST

As described in Chapter I, there are conflicting reports in the literature dealing with several aspects of the differentiation of cartilage from cranial neural crest in amphibians. This study focused on the inductive processes involved in cranio-visceral cartilage formation in *Ambystoma mexicanum*.

3-1. Self-differentiation of cranial neural crest:

An *in vitro* culture system was used to determine the stage by which cranial neural crest cells were determined for cartilage formation. According to Chibon (1966), the neural folds between 30° and 150° of Figure 1.1 contain all the skeletogenic neural crest cells of the amphibian embryo; these axial levels were therefore used for all the cultures.

For neurulae (stages 14 to 19), a cranial neural fold from one side of the embryo was explanted. Following neural tube closure, but prior to neural crest cell migration (stages 20-23), the dorsal half of the neural tube was explanted with the overlying ectoderm. The cranial neural crest cells migrate away from the neural tube, between the ectoderm and mesoderm, during stages 24-28. For these

stages, the tongues of migrating cells were removed from one side of the embryo, and enveloped with ectoderm for culturing. Usually, belly ectoderm from the same embryo was used, although ectoderm overlying the heart, gills, or brain, was also used in some cases.

The cultures were examined for the presence of cartilage after a 14-day culture period (Table 3.1). Cranial neural crest cells from neurulae are not determined for cartilage differentiation; only one culture contained cartilage, although all 94 appeared healthy, and contained melanocytes. After the completion of neurulation cartilage was seen in a small percentage of cases. The ability to self-differentiate was extremely weak, occuring in only 7 of the 52 post-neurulation cases. Furthermore, cultures from older embryos were not more apt to form cartilage. Embryos of stages 21-25 contributed 5 of the 7 positive cases, while the remaining 2 cases were from stage 27 embryos.

3-2. *Pharyngeal endoderm as inductor:*

In *Triturus alpestris*, pharyngeal endoderm is the only inductor required for mature cartilage formation from neurulae neural crest (Epperlein and Lehmann, 1973). Its inductive ability for chondrogenesis in *Ambystoma mexicanum* was tested, using an area of pharyngeal endoderm equivalent to that used by Epperlein and Lehmann (1975), but containing

TABLE 3.1

Self-Differentiation of Cartilage from Cranial Neural Crest.[1]

Stage of Development	Total no. of cultures	No. of cultures containing cartilage	Percent positive
14-16	53	0	0.0[a]
17-19	41	1	2.4[a]
20-24	24	4	16.7[b]
25-28	28	3	10.7[b]

[1] All explants were cultured for 14 days before processing.

[a] Not significantly different ($P_{.05}$).

[b] Not significantly different ($P_{.05}$).

more posterior material. This area corresponds to regions 1 and 2 of Figure 3.1 (pp. 40-41).

Cranial neural crest was removed from the embryos as described in the previous section, and placed ectoderm-side down. The piece of pharyngeal endoderm, taken from the same embryo, was centered on the other tissue. The ectoderm usually wrapped around the endoderm within 10-15 minutes, such that the different tissues did not readily separate during the transfer to the BEEM capsule.

The results are shown in Table 3.2. It is obvious that pharyngeal endoderm is indeed an inductor for the differentiation of cartilage from cranial neural crest; almost all the cultures (92.3%) contained cartilage after the two week culture period. Statistical analysis revealed a significant difference ($P < .001$) between the cartilage-forming abilities of cultures containing stage 20-27 neural folds with endoderm (Table 3.2) and those containing stage 20-28 neural folds alone (Table 3.1). Furthermore, it appears that it is the only inductor required after the onset of neurulation. Mature cartilage nodules, surrounded by perichondrial cells, were seen in all the positive cases, even when the tissues were removed from stage 14 neurulae (see Fig. 3.2g, pp. 45- 46).

TABLE 3.2

Inductive Ability of Pharyngeal Endoderm.[1]

Stage of Development	Total no. of cultures	No. of cultures containing cartilage	Percent positive
14-16	71	64	90.1*
17-19	51	50	98.0*
20-24	9	8	88.9*
25-27	13	11	84.6*

[1] The endoderm was removed from regions 1 and 2 of Figure 3.1. Both tissues of each explant were taken from the same embryo.

* Not significantly different ($P_{.05}$).

3-3. Inducing ability at different stages:

While pharyngeal endoderm was clearly the only inductive tissue required by cranial neural crest, it remained unknown whether it was necessary for the tissues to be from embryos of the same developmental stage. Therefore, cranial neural crest was cultured with pharyngeal endoderm of different developmental stages. From the results presented in Table 3.3, it is apparent that the tissues need not be of the same stage. For example, neural crest from neurulae (stage 18) gave rise to cartilage in the presence of endoderm from embryos whose own cranial neural crest cells have completed their lateral migration, and have begun the medial migration for visceral arch formation (stage 28). These two stages are normally over a day apart (see Appendix I). Pharyngeal endoderm which was developmentally younger than the neural crest was also effective as an inductor, as exemplified by the ability of late gastrula (stage 13) endoderm to induce cartilage formation from neurula (stage 16) neural crest. These results suggest that the duration of the inductive ability of the endoderm, the responding ability of the neural crest, or both, is extensive.

TABLE 3.3

Timing of the Induction.

Stage of neural crest	Stage of endoderm	Total no. of cultures	No. of cultures containing cartilage
15-16	12-13	3	3
15-16	17-18	5	5
17-18	12-14	2	2
17-18	20-28	7	7
23	18	1	1

3-4. Size of inductive tissue:

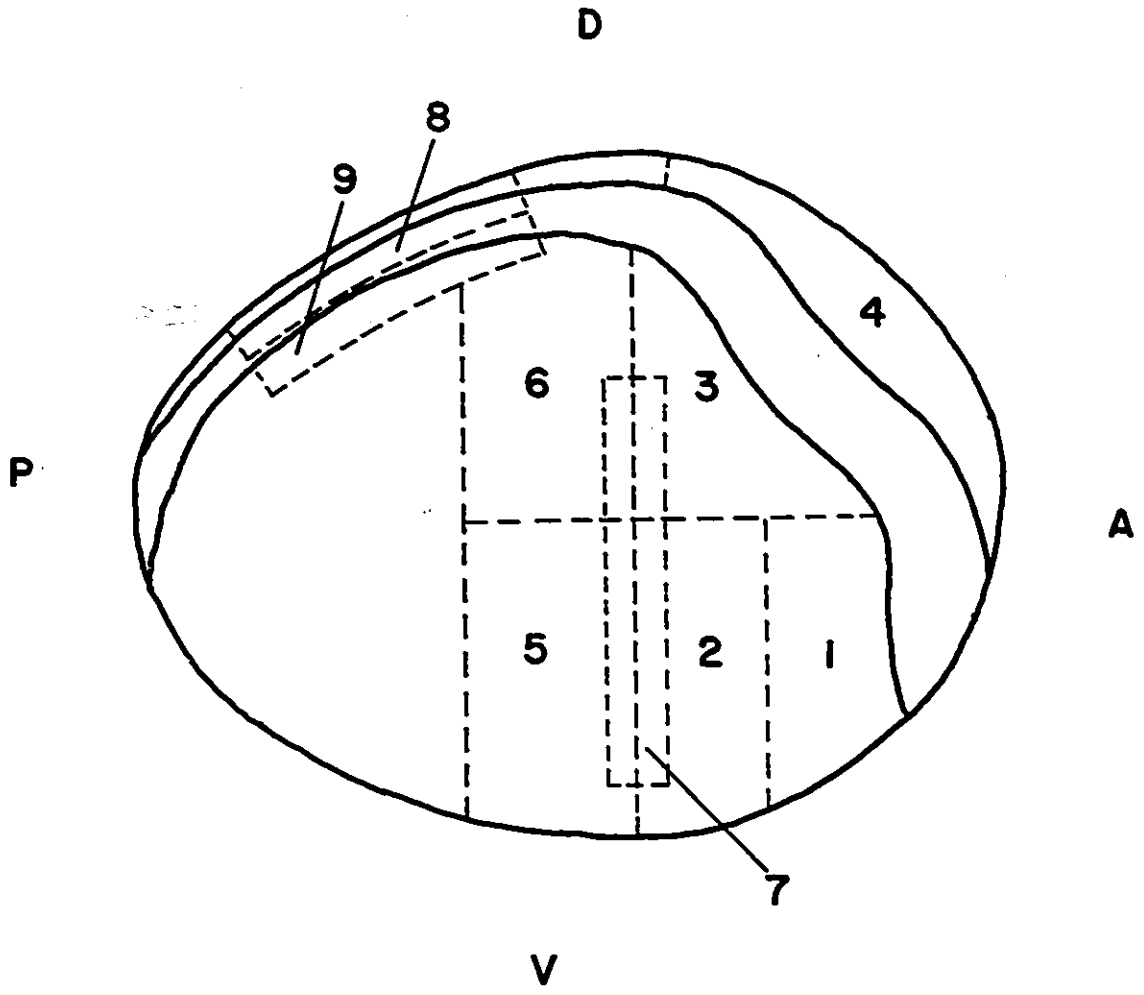
In the previous experiments, the pieces of endoderm had a surface area of approximately 600 μm^2. When the size of the inductor in the cultures was reduced to approximately 40 μm^2, cartilage was still formed. The amount of tissue required for induction to occur is therefore quite small. These small pieces of tissue were difficult to handle, and it was impossible to tell whether endoderm was indeed incorporated in the explants after they had healed. Therefore, the average size of the inductive tissue used in all of the following experiments was a more manageable 80 μm^2.

3-5. Mapping of inductive endoderm:

The inductive ability may be a property of all endoderm, or it may be restricted to specific areas. Furthermore, there may be gradations of inductive strengths with endoderm from different regions. These possibilities were tested by culturing endoderm from different regions of embryos, usually neurulae, with cranial neural folds from neurulae.

The different areas of endoderm which were used are depicted in Figure 3.1. As often as possible, natural boundaries within the embryo were used in determining the limits of the particular regions. This was more reliable

Figure 3.1: Lateral view of a stage 16 embryo, depicting the areas of endoderm which were tested for inductive activity. Regions 1, 2, 3, 4, and 7 line the pharyngeal cavity of the embryo. Region 7 is the floor of the archenteron, and is shown edge-on. Regions 5 and 6 overlie the mass of yolky endoderm. Region 8 is trunk notochord. Region 9 is the endoderm lining the midgut. A: anterior; P: posterior; D: dorsal; V: ventral.


D
8
9
4
6
3
P
A
5
2
1
7
V

than actual measurements since it ensured that comparable regions were removed, even from embryos of different sizes. The junction between the pharyngeal wall and the mass of yolky endoderm divided regions 2 and 3 from regions 5 and 6, whereas the neural folds defined one side of each of regions 4, 1, and 3. Region 4 consisted of all the endoderm underlying the cranial neural plate, excluding that under the neural folds and the neural groove (notoplate and developing notochord). For regions 5, 6 and 7, the tissue removed was the surface layer of endoderm, which contained smaller, more cohesive cells than those of the underlying yolky mass endoderm. Region 7 was the archenteron floor, which formed the anterior portion of the yolky mass. Regions 8 and 9 consisted of trunk notochord and trunk midgut endoderm, respectively.

All cultures tested the inductive abilities of these tissues on the cartilage differentiation of neural crest from neurulae. Although both tissues were usually taken from the same embryo, some cultures contained region 8 or 9 tissue from older embryos. All explants were cultured for the usual 14 days.

The results are shown in Table 3.4. There do not appear to be different levels of inductive ability; for each region of endoderm, cartilage was seen in either almost all or almost none of the cultures. Furthermore, there appears to be

TABLE 3.4

Mapping of Inductive Endoderm.[1]

Region of Endoderm[2]	Total no. of cultures	No. of cultures containing cartilage	Percent positive
1	28	24	85.7[a]
2	29	22	75.9[a]
3	26	22	84.6[a]
4	20	17	85.0[a]
5	62	4	6.5[b]
6	23	1	4.3[b]
7	13	0	0.0[b]
8[3]	18	1	5.5[b]
9[4]	23	4	17.4[b]
8+9	3	0	0.0

[1] Unless otherwise specified, the neural fold and endoderm were removed from the same embryo. Embryos were between stages 14 and 18.

[2] Regions of endoderm are as depicted in Figure 3.1.

[3] 3 cultures contained notochord from stage 25 (one of which yielded the single positive case), 3 contained this tissue from stage 30, and 2 from stage 35.

[4] 3 cultures contained midgut endoderm from stage 25 embryos, none of which produced cartilage.

[a] Not significantly different ($P_{.05}$).

[b] Not significantly different ($P_{.05}$).

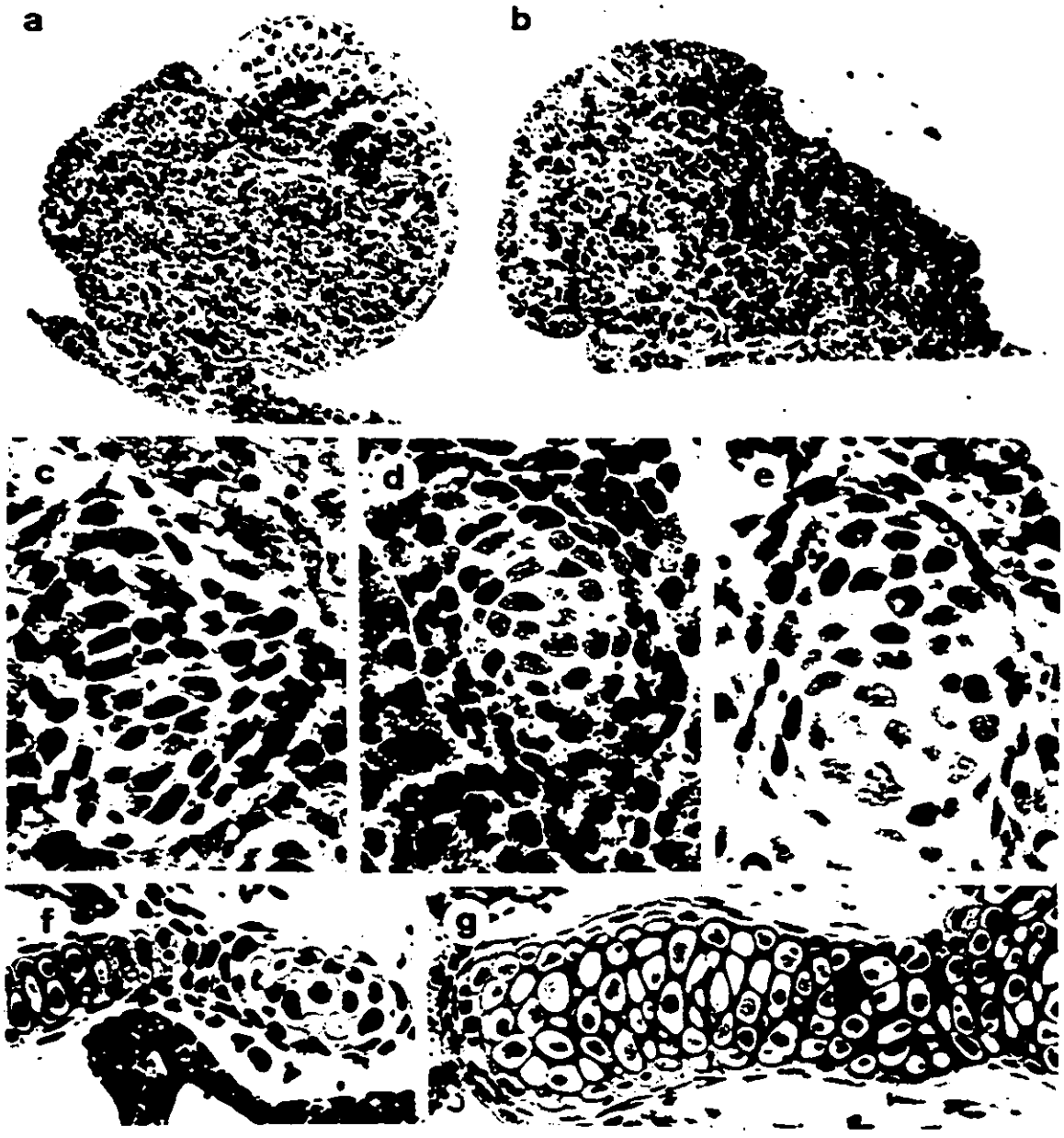
a sharp boundary between the inductive and the non-inductive endoderm. This boundary occurs at the junction between the pharyngeal wall and the floor of the archenteron, which corresponds to the division between the head and trunk of the embryo. All endoderm anterior to this line (*i.e.* regions 1, 2, 3, and 4) is inductive, whereas all endoderm which is posterior to this line, including the archenteron floor itself (regions 5, 6, 7, 8), is not.

3-6. *Time course of in vitro cartilage formation:*

Stage 17 cranial neural folds were placed in explant culture with either inductive (regions 1 and 2 of Fig. 3.1) or non-inductive (regions 5 and 6 of Fig. 3.1) endoderm. Over the course of two weeks, samples of each type were fixed daily and processed for histological examination, in order to determine the time course for cartilage differentiation *in vitro*. The two types of cultures were compared with one another, and with the appearance of the developing branchial arch cartilage *in vivo*.

Figure 3.2 contains photomicrographs of sections of these cultures at different time points. For the first 6 days of culture, no differences were seen between the cultures containing the inductive and the non-inductive endoderm (Fig. 3.2a and b). The cultures containing the non-inductive endoderm retained this appearance for the the

Figure 3.2: Time course of cartilage formation *in vitro*. Chondrogenesis, depicted in the photomicrographs by cell shape and spacing changes, was even more evident in the actual slides by the metachromatic (pink) staining of the extracellular matrix seen between the blue-stained chondrocytes. a, b: Day 6; no differences can be seen between cultures with noninductive (a) and inductive (b) endoderm. X 120. c: Day 7; the first patches of cartilage have appeared: the cells are condensed and are surrounded by a pale, metachromatically-stained matrix. d, e: Days 8 and 9; the patches have become more distinct, and the matrix more deeply stained. c-e: X330. f,g: Days 10 and 14; mature cartilage nodules have formed. X190.


a
b
c
d
e
f
g

remainder of the 14 day culture period. By day 7, however, the cultures of neural crest and inductive endoderm had small patches of cells with pale, extracellular metachromatic staining (Fig. 3.2c). Over the course of the next three days, these patches increased in size and number of cells. The matrix staining also darkened during this time (Fig. 3.2d and e). By day 10, these cultures contained mature cartilage nodules (Fig. 3.2f). These contained compact, regularly-shaped chondrocytes which were isolated in lacunae. The matrix appeared quite dense and darkly stained. The boundaries of the nodules were frequently delineated by elongated perichondrial cells, aligned parallel to the edge of the nodule. The size of the nodules increased with an additional 4 days of culture, but their appearance was not drastically affected (Fig. 3.2g).

The time course for cartilage differentiation from cranial neural crest *in vitro* appears to be quite similar to that found *in vivo*. Pale metachromatic staining of cartilage matrix could first be seen in embryos at about stage 39, which is approximately the seventh day after neurulation (see Appendix I). Chondrocyte differentiation occurred in much the same manner as *in vitro*, with respect to matrix staining, cell shape changes, and the appearance of perichondrial cells. By stage 42 (approximately one day after hatching), the cartilage could be considered fully differentiated; the

compact chondrocytes were isolated in lacunae within a dense, darkly metachromatic matrix, the whole being surrounded by perichondrial cells. Thus, on the tenth day post-neurulation, the cartilage nodules seen in the embryos were remarkably similar to the day 10 induced cultures described above.

CHAPTER IV

THE PREMATURE DEATH MUTANT

As described in Chapter I, the premature death mutatation, when present in the homozygous state, leads to a wide variety of abnormalities in the embryo. The underlying cause was suspected to be endoderm defective in both inductive and differentiative abilities (Trottier and Armstrong, 1977; Mes-Hartree and Armstrong, 1980).

If the anterior endoderm was indeed defective for so many of its normal functions, then it seemed probable that its ability to induce cartilage formation from the cranial neural crest would also be impaired. It should be noted that homozygous *p* embryos begin to disintegrate well before the normal appearance of cartilage in wild-type embryos (see Figs. 1.1b and 4.8b). Therefore, the lack of cartilage in mutant embryos could be due to the death of the embryos, rather than actual defects in the tissues involved.

In order to use this mutant to study cartilage induction, it had to be shown that cartilage could not be induced from mutant tissues, and that this lack of cartilage was not simply due to the premature death of the tissues.

4-1. Cartilage formation from p/p tissues:

Explant cultures of cranial neural folds and pharyngeal endoderm were made from stage 17-19 neurulae. Each explant contained tissues from a single embryo. These embryos were from spawnings between heterozygous adults. Following a 14-day culture period, the explants were examined for the presence of cartilage. The results are summarized in Table 4.1.

Mature cartilage nodules were seen in only 75.5% of the cultures, compared with a cartilage-forming frequency of 93% for similar cultures containing only wild-type tissues. Neither cartilage nor procartilage was seen in the remaining 24.5% of the cultures. This corresponds quite well with the expected 25% *p/p* embryos, and led to the conclusion that *p/p* embryos do not possess the ability to form cartilage from cranial neural crest.

The cultures which did not contain cartilage appeared to be alive and healthy, resembling wild-type cultures of cranial neural folds with noninductive endoderm. These results therefore confirm that *p* is not a cell-lethal mutation. Some *p/p* cell types are capable of better survival in explant culture than *in situ*, even in the absence of wild-type tissues.

TABLE 4.1

Ability of *p/p* Tissues to Form Cartilage.[1]

Stage of Development	Total no. of cultures	No. of cultures containing cartilage	Percent positive
17	35	26	74.3*
18	9	7	77.8*
19	5	4	80.0*
Total	49	37	75.5

[1] Embryos from *p/+* X *p/+* spawnings were used. Cranial neural folds and inductive endoderm were removed from the same embryos and cultured together for 14 days. The results from two separate spawnings have been pooled.

* Not significantly different ($P_{.05}$).

4-2. Determination of defective tissue:

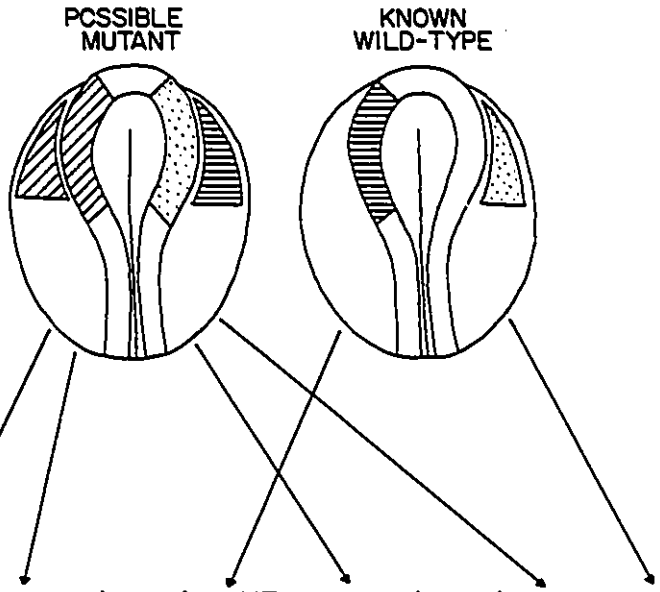
In the preceding series of cultures, both components of each explant were taken from the same embryo. The lack of cartilage formation in the suspected *p/p* cultures may have been due to defective cranial neural crest, defective pharyngeal endoderm, or both. The two following experiments were devised to distinguish between these possibilities.

In the first experiment, tissues were removed from a pair of embryos, and combined to make three explants, each containing a cranial neural fold and pharyngeal endoderm (Fig. 4.1). One member of each pair was from a spawning of two heterozygous *p* individuals, and the other member of each pair was from a spawning of animals known to be wild-type for *p*.

A cranial neural fold and a piece of pharyngeal endoderm were removed from the possible *p* embryo, and combined to form the first explant (series A). The lack of cartilage in this culture after 14 days indicated that the embryo was *p/p*.

Another piece of pharyngeal endoderm was removed from the same possible *p* embryo, and combined with a cranial neural fold from the wild-type embryo (series B). The other cranial neural fold from this possible *p* embryo was combined with wild-type pharyngeal endoderm (series C). When *p/p* tissues were in fact used (as determined by the results of the A series), the lack of cartilage in the corresponding

Figure 4.1: Diagram indicating the 3 explants made from each pair of stage 17 embryos. The 3 explants from each pair formed 1 set. The result of each "A" culture indicated whether that set contained *p/p* tissues; explants from *p/p* embryos should not contain cartilage. In this way, the lack of cartilage in the corresponding "B" and/or "C" cultures should indicate which tissue(s) were defective.



A: (p? NF + p? Endo)	B: (w.t. NF + p? Endo)	C: (p? NF + w.t. Endo)	No. of Sets
+	+	+	24
−	+	−	6
−	+	+	2
		Total	32

cultures indicated which of the *p/p* tissue(s) was defective. The results are summarized in Figure 4.1.

Eight (8) of the 32 cultures (25%) in series A were devoid of cartilage. As in the preceding experiment, this corresponds to the expected 25% *p/p* embryos.

Most of the series C cultures containing neural folds from the same S did not contain cartilage either. This seems to indicate that *p/p* cranial neural crest is not competent to respond to the normal signals of the wild-type endoderm to produce cartilage.

The results of the series B cultures are unequivocal; all contained mature cartilage, although 25% of the cultures apparently contained pharyngeal endoderm taken from *p/p* embryos.

The surprising result that *p/p* endoderm was apparently normal, although the neural crest was not, was verified by the following experiment. Explant cultures of cranial neural fold and pharyngeal endoderm were made using neurulae from spawnings of heterozygous *p* animals. Only one tissue was removed from each embryo. The embryos were allowed to heal and develop in order to unequivocally identify the phenotype. The results are shown in Table 4.2.

It is obvious that when *p/p* endoderm was combined with wild-type neural crest, cartilage formation ensued almost as frequently as when both tissues were of wild-type origin.

TABLE 4.2

Determination of Defective Tissue in *p/p* Embryos.[1]

Source of tissues	Experiment[2] I	II	III	IV	Total[2] (%)
wt NF + wt E	17/21[a]	15/15[a]	6/6[a]	23/25[a]	61/67 (91.0)
wt NF + *p/p* E	5/8[b]	3/3[b]	3/4[b]	-	11/15 (73.3)
p/p NF + wt E	-	0/6[c]	2/11[c]	0/11[c]	2/28 (7.1)
p/p NF + *p/p* E	-	0/1	0/1	-	0/2 (0.0)

[1] For experiments II and III, embryos from *p/+* X *p/+* spawnings were used. For experiment I, embryos from a *+/+* X *+/+* spawning were the source of the neural folds, whereas embryos from a *p/+* X *p/+* spawning were the source of the endoderm. For experiment IV, embryos from a *p/+* X *p/+* spawning were the source of the neural folds, whereas embryos from a *+/+* X *+/+* spawning were the source of the endoderm. Cranial neural folds and inductive endoderm were removed from different embryos and cultured together for 14 days. The donor embryos were allowed to heal and continue developing to identify the phenotype.

[2] Number of cases containing cartilage / total number of cases.

[a] Not significantly different ($P_{.05}$).

[b] Not significantly different ($P_{.05}$).

[c] Not significantly different ($P_{.05}$).

NF: neural fold; E: endoderm; wt: wild-type; *p/p*: homozygous mutant.

This indicates that the inductive capacity of the mutant endoderm is normal.

Cranial neural crest from *p/p* embryos, on the other hand, has a lower probability of forming cartilage. Although the two positive cases contained mature, though small, cartilage nodules, the negative cultures, which appeared healthy, contained neither mature cartilage nor procartilage.

Wild-type cultures of this type contained procartilage by day 7, and mature cartilage by day 10, as described in Chapter III. When five explants of *p/p* NF + wt endoderm were cultured for 17-18 days, rather than the usual 14 days, there was still no evidence of cartilage formation. Therefore, the results described above could not be due to a delayed response time of the *p/p* neural crest. Rather, there appears to be an absolute inability to form cartilage.

4-3. Heart inductions

Another *in vitro* induction system for which the tissues and timing are well known in the axolotl is the induction of precardiac mesoderm by pharyngeal endoderm. It has been shown that the induction of heart-forming mesoderm is completed by the end of neurulation (stage 20; Smith and Armstrong, 1989). Although *p/p* embryos do have beating hearts, the beating tends to be rather weak. It is not known whether this is an actual defect of the heart-forming and/or

-inducing tissues, or a consequence of the presence of abnormal or degenerating tissues in the embryo. Therefore, the ability of heart-forming mesoderm to differentiate in explant culture was tested. These explants also directly tested the capability of the pharyngeal endoderm to induce a tissue other than cranial neural crest.

The embryos used in this experiment were from a spawning of heterozygous *p* animals. Precardiac mesoderm from one side of stage 20 embryos was placed in hanging drops of 100% Steinberg's solution. The embryos were allowed to heal, and kept for phenotype identification.

Twenty-one (21) cultures were made, of which 6 were found to be from *p/p* embryos. The mutant mesoderm formed beating tissue as often as wild-type mesoderm (Table 4.3). Beating continued in all positive cultures, wild-type or *p/p*, for the entire week of observation.

4-4. Axial level:

Only specific axial levels of neural crest normally give rise to skeletogenic derivatives. In order to test the possibility that the mutation causes a shift in anterior/posterior determination, neural folds from regions anterior and posterior to these levels were tested for their cartilage-forming capacity. The embryos used in this study were from a spawning of heterozygous *p* animals.

TABLE 4.3

Heart Differerntiation in *p/p* Embryos.[1]

Donor embryo	Total no. of cultures	No. of cultures beating	Percent beating
p/p	6	5	83.3[a]
+/?	15	12	80.0[a]

[1] Embryos from *p/+* X *p/+* spawnings were used. Heart-forming mesoderm was explanted from the right side of each embryo, and the donors were allowed to heal and develop for phenotype identification. Cultures were examined periodically over the course of 7 days.

[a] Not significantly different ($P_{.05}$).

Eighteen (18) cultures were made of anterior neural fold (Fig. 4.2a) and pharyngeal endoderm. After 14 days of culture, none showed evidence of cartilage differentiation. Three (3) of these cultures contained *p/p* tissues. Explants of cranial neural fold and pharyngeal endoderm were taken from the same embryo, and cultured for identification of the donor embryo.

Trunk neural folds (Fig. 4.2b) and pharyngeal endoderm were taken from another 18 embryos. Again, no cartilage was present at the end of the culture period.

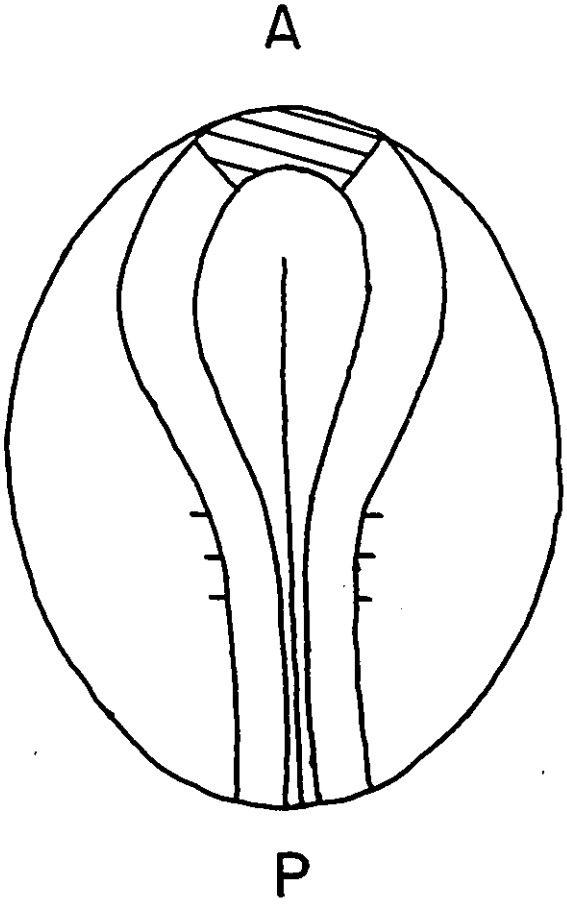
It would appear that only the neural crest which normally differentiates into cartilage *in vivo* has the ability to do so, even when in the presence of known inductive tissues. Furthermore, the *p* mutation does not seem to produce a shift such that cartilage-producing neural crest is found at abnormal axial levels.

4-5. Migration patterns of cranial neural crest:

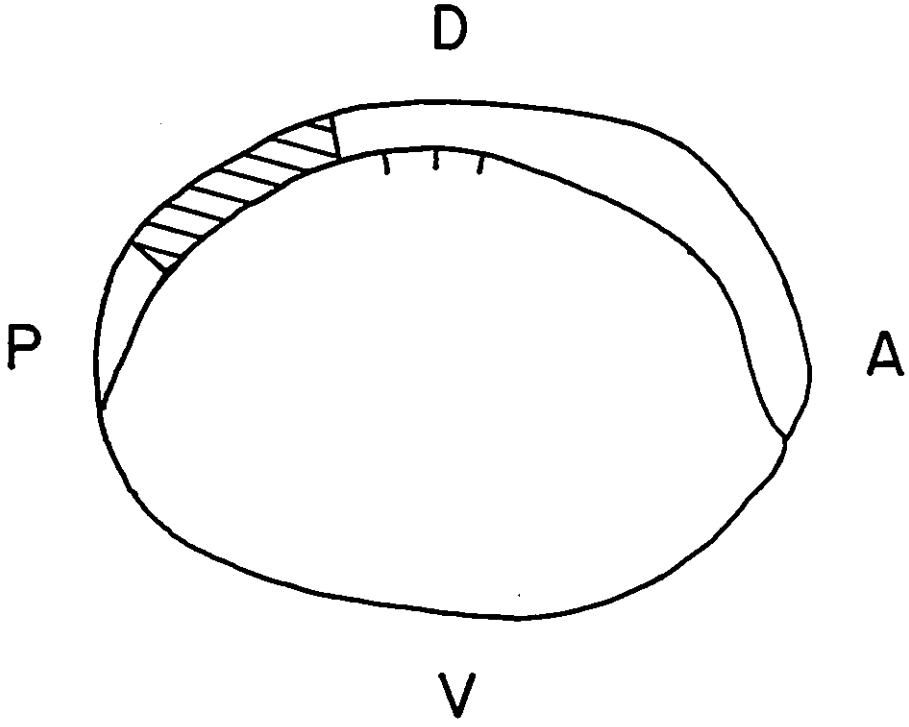
The differentiation of neural crest cells is preceded by their migration along definite pathways. Cranial neural crest cells from *p/p* embryos may have abnormal spatial and temporal patterns of migration. This possibility was tested by taking advantage of the availability of embryos from albino parents, whose cells contain no maternally-derived pigment. Unilateral homotopic transplantations were made of

Figure 4.2: Dorsal (a) and right lateral (b) views of a stage 17 embryo. The hatched areas depict the axial levels of neural folds taken for testing (a) anterior and (b) posterior cartilage-forming abilities in *p/p* and +/? embryos. Eighteen stage 17 embryos were used for the anterior fold cultures (a), whereas 12 stage 17 and 6 stage 19 embryos were used for the posterior fold (b) cultures. Each neural fold was cultured with a piece of inductive endoderm taken from the same embryo. A: anterior; P: posterior.

a



b



possible *p/p* cranial neural folds to unpigmented embryos. Both donor and host embryos were at stage 17, and the transplanted folds were of the levels depicted in Figure 4.3. The donor embryos were kept for phenotype identification.

The migrating tongues of pigmented neural crest could clearly be seen through the albino ectoderm, as shown in Figure 4.4. The timing and pattern of the migration appeared identical for neural crest from both the *p/p* embryos and their phenotypically wild-type siblings. The majority of cranial neural crest cells, at least, are capable of normal migration.

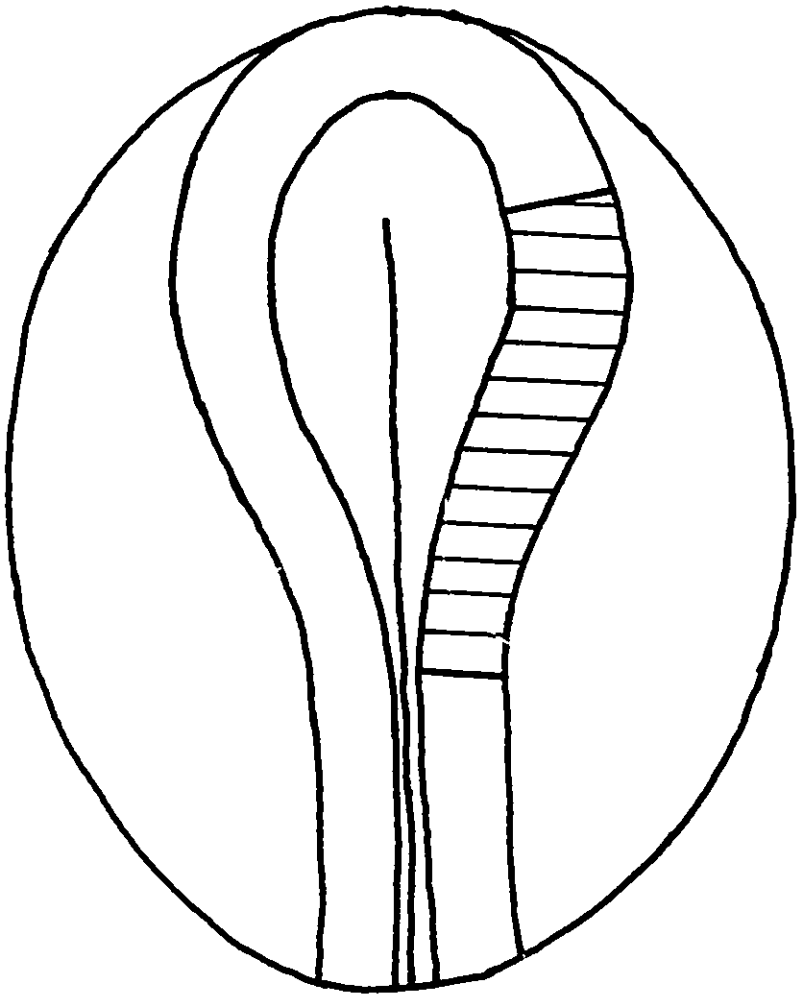
4-6. Neural induction:

Secondary neural plates can be induced by transplanting or implanting dorsal lip material into an ectopic location on a host an appropriate stage (see Chapter V). The axial types of neurectoderm structures which are formed depend on the axial type of chordamesoderm used. For example, presumptive head mesoderm tends to yield brain and head structures. While the processes involved in neural crest specification and regionalization are not known, they probably occur either concomitantly with, or immediately subsequent to, neural induction.

Therefore, a series of experiments was designed to test whether *p/p* chordamesoderm could induce secondary neural

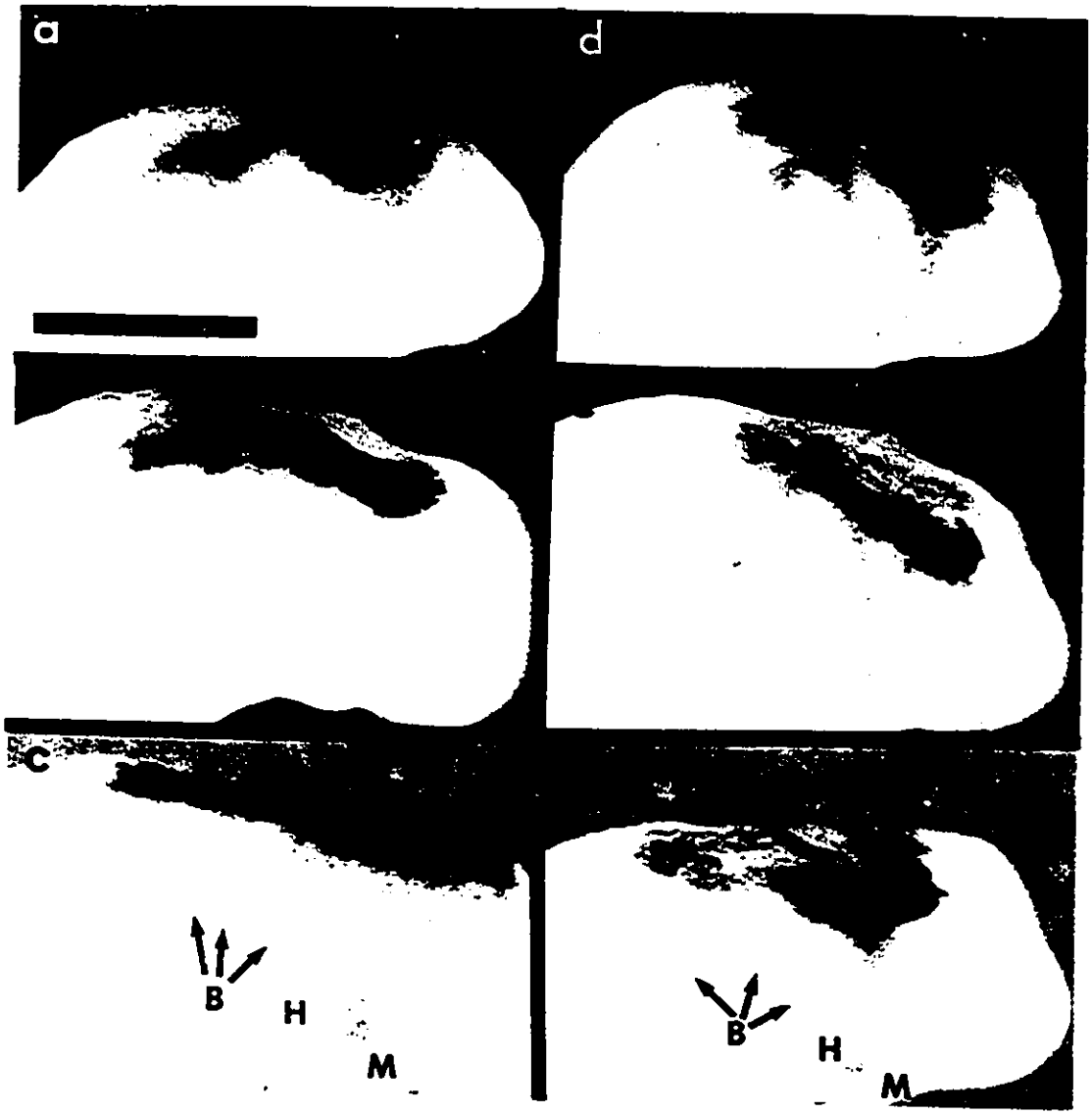
Figure 4.3: Dorsal view of a stage 17 embryo. The hatched area depicts the area of neural fold homotopically transplanted from (*p*/+ X *p*/+) embryos to albino hosts. Both donors and hosts were at stage 17. A: anterior; P: posterior.

A



P

Figure 4.4: Migration patterns of wild-type (a,b,c) and *p/p* (d,e,f) cranial neural crest. Homotopic transplantations of cranial neural folds of the region depicted in Fig. 4.3 were performed, using albino hosts and possible *p/p* donors. (a) and (d): early stages of migration; (b) and (e): prior to medial migration; (c) and (f): subsequent to medial mirgation. M: mandibular arch; H: hyoid arch; B: branchial arches. Anterior is at right and dorsal is at the top of all frames. Bar = 1 mm.


a
d
c
B
H
M
B
H
M

plates and folds from wild-type gastrula ectoderm, and also whether the cells in these secondary neural folds had cartilage-forming ability. As well, ectoderm from *p/p* gastrulae was tested for its ability to respond to the signals given by wild-type chordamesoderm. Again, the feature being studied was the chondrogenic potential of the secondary neural crest.

The technical feasibility of such studies had to be established, particularly in view of the large number of cases required, since the operations were necessarily performed prior to the identification of the mutant embryos. *Ambystoma* embryos are not recommended for secondary plate inductions (Hamburger, 1960), since these embryos readily exogastrulate and thus lack all neural structures, primary or secondary. Axolotl embryos, in particular, are even considered by some workers to be the material of choice for exogastrulation studies, due to the high incidence of exogastrulation which can be provoked simply through the removal of their jelly coats and vitelline membranes (Hamburger, 1988).

The production of exogastrulae was found to be quite variable. In approximately 1/3 of the spawnings, almost all embryos exogastrulated following the operation; all embryos from such spawnings were discarded. All remaining spawnings produced a lower (though variable) proportion of

exogastrulae; in a total of 229 attempts, the average proportion of exogastrulae was 20%, and ranged from 0% to 53% for each of 11 spawnings. Usually, almost all non-exogastrulae produced secondary structures; however, only those with recognizable neural folds were taken for explantation.

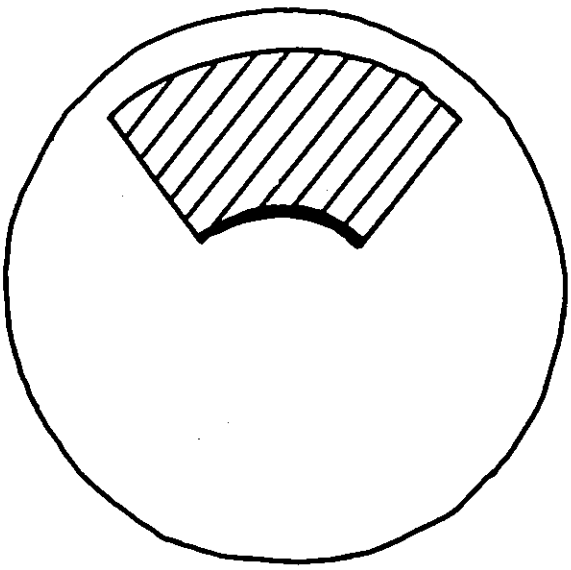
The cartilage-forming ability of the cells in the secondary folds was tested, under the same *in vitro* conditions used in the previous studies. Wild-type dorsal lip material of the area depicted in Figure 4.5a was removed from embryos of stages 10 to 10-1/2, and implanted into the blastocoeles of wild-type embryos of stages 9-10.

When neurulae stages were reached, 9 host embryos had secondary structures resembling neurectoderm. The structures which resembled secondary neural folds were explanted with pharyngeal endoderm taken from unoperated wild-type embryos for the usual 14 day culture period. Of the 7 surviving cultures, 6 contained mature cartilage. All live cultures appeared healthy, and all contained melanocytes, indicating that neural crest was indeed present.

When embryos from a *p/+* X *p/+* spawning were used as donors, and wild-type embryos used as hosts, 9 secondary plates were formed. All 9 secondary folds formed cartilage when cultured with inductive endoderm for 2 weeks. At least 2 of these cultures should have contained neural crest

Figure 4.5: Vegetal pole views of stage 10-1/2 embryos. Hatched areas were removed from the donors, and implanted into the blastocoeles of the hosts. The tissues removed included the material already invaginated, and included most of the material between the dorsal lip and the equator. Tissue was removed bilaterally (a) when the donor embryos were no longer required, or unilaterally (b) when they were kept for phenotype identification.

a



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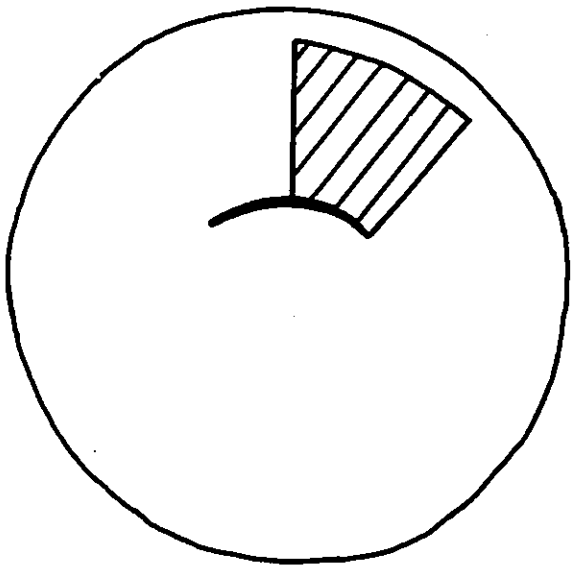


arising from wt ectoderm which had been induced by *p/p* chordamesoderm, assuming that *p/p* dorsal lip material is as competent as its wild-type counterpart in producing the secondary plates and folds.

This assumption, and the results, were verified using a slight modification of the technique. Instead of removing all of the dorsal lip material, only the right half was removed (Fig. 4.5b). According to Bautzmann (as referred to by Hamburger, 1988), half a dorsal lip will regulate to induce a bilateral secondary embryo. It was hoped that the embryo which had had half its dorsal lip removed would survive, and develop long enough for the phenotype to be identified.

Eight (8) sets of secondary folds were induced in wild-type hosts by dorsal lips from known *p/p* donors. These were cultured for 2 weeks with endoderm taken from unoperated neurulae. When examined, all 8 explants were found to contain mature cartilage. Therefore, *p/p* dorsal lip material is normal with respect to its ability to induce neural plate formation from competent ectoderm. Furthermore, it is clearly able to set into motion those processes which are required for the specification of neural crest with chondrogenic potential.

The reverse experiment was also performed, to test the ability of ectoderm from *p/p* gastrulae to respond to normal

signals from chordamesoderm and eventually yield cartilage-forming cranial neural crest. The area shown in Figure 4.5a was removed from the donors. The recipients were embryos from a *p/+* X *p/+* spawning. Following removal of the secondary neural folds, the host embryos were allowed to heal and develop for phenotype identification. Of the 24 host embryos, 5 were *p/p*. None of the secondary neural folds which had been induced to form in these embryos developed cartilage, although they had been in the presence of inductive endoderm for 14 days. Therefore, it appears that *p/p* ectoderm cannot respond to the signals for neural crest specification.

4-7. Neural fold transplantations:

Since *p/p* neural crest is defective in at least one capacity, the presence of normal neural crest might correct at least some of the defects of mutant embryos. Conversely, the presence of mutant neural crest in a normal embryo should give a partial phenocopy of the mutant.

Homotopic reciprocal transplantations were performed between neurulae from a spawning of two heterozygous *p* animals. Transplantations of long pieces of neural folds were rarely successful. Therefore, short lengths of neural folds from several axial levels were used. These are shown in Figure 4.6. Since the operations were performed

unilaterally, the unoperated sides were used to identify the phenotype.

Most wild-type embryos which received a *p/p* section of neural fold appeared to develop normally, regardless of the axial level of mutant graft. Several recipients of transplants of *p/p* anterior cranial neural folds (region A; Fig. 4.6) exhibited underdeveloped eyes on the operated sides. However, this may have been an effect of surgical damage rather than a phenocopy effect.

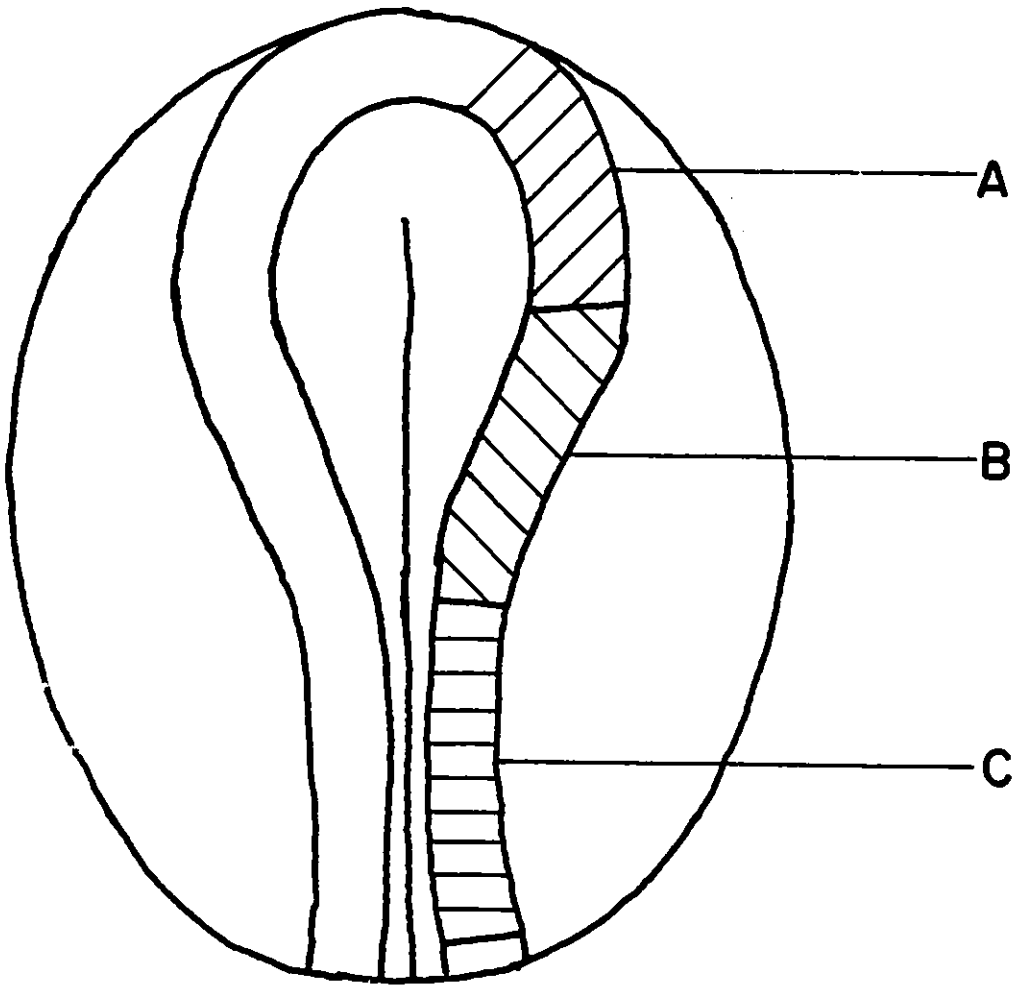
The presence of a normal trunk neural fold (region C) had no obvious effects on the development of mutant embryos. These embryos displayed the same mutant characteristics as unoperated *p/p* controls, and the disintegration appeared to occur in the same general pattern and at the same time.

Wild-type anterior neural fold (region A) transplants led to more advanced eye development on the operated side of the mutant embryo. This was the only external morphological difference in these embryos. Again, there was no increase in the survival of these embryos compared with unoperated sibling mutants.

The most dramatic effect was seen when posterior cranial neural folds (region B) were transplanted, although there was still no difference in the survival of the mutant embryos. The primary filaments on the operated side of the mutants grew noticeably longer than those of the unoperated side

Figure 4.6: Dorsal view of a stage 17 embryo. The different hatching patterns indicate the various axial levels of neural folds used in reciprocal homotopic transplants between (*p*/+ X *p*/+) embryos. All operations were performed unilaterally, on the right side, thereby allowing the unoperated, left sides to be used for identifying the host phenotype. Anterior cranial (A), posterior cranial (B), and trunk (C) regions were taken. A: anterior; P: posterior.

A



P

(Fig. 4.7a). Furthermore, the bulb-like structures at the distal ends of the primary filaments, which are a distinguishing feature of the mutant gills, were not usually present on these longer filaments. The gills were still abnormal, however, in that secondary filaments never formed. When these embryos were fixed and processed for histological examination, approximately 1 week after their phenotype was identified, large quantities of mature cartilage were found in the branchial region of the operated side (Fig. 4.8a). It should be noted that these embryos already had epidermal blisters, indicating degeneration had begun.

4-8. Extirpation of neural folds:

In order to determine whether the characteristics of the *p* mutant could be ascribed to defective neural crest, another attempt was made to produce phenocopies in genetically wild-type embryos. The neural folds of stage 20-21 embryos have begun to fuse in all but the most anterior and most posterior regions of the embryos, although neural crest cell migration away from the neural tube has not begun. The dorsal and lateral portions of the developing neural tube were removed along the length of the embryo, as indicated in Figure 4.9. Therefore, the neural crest cell population from all of the head, and all but the most posterior portion of the embryo was removed. The neural plate of this unoperated posterior

Figure 4.7: a: Dorso-lateral view of *p* mutant embryo containing wild-type transplant from Region "B" (see Fig. 4.5) on the right side. This embryo was a sibling of, and was the same age as, the embryos shown in Figure 1.2. The primary filaments (which are separate) are much longer on the operated (lower) side, and do not have bulb-like structures at their distal ends.

b: Dorso-lateral view of wild-type embryo with the neural tissue excised as described in Figure 4.8. The embryo was fixed approximately 9 days after the initiation of heartbeat. The neural crest was removed (as determined by the lack of a dorsal fin) to the level of the left edge of the photomicrograph, although melanocytes (m) can now be seen at more anterior levels. The embryo shown was one of the most highly developed of the series, and maximal development of branchial nodules is evident (curved arrow). Bars = 1 mm.

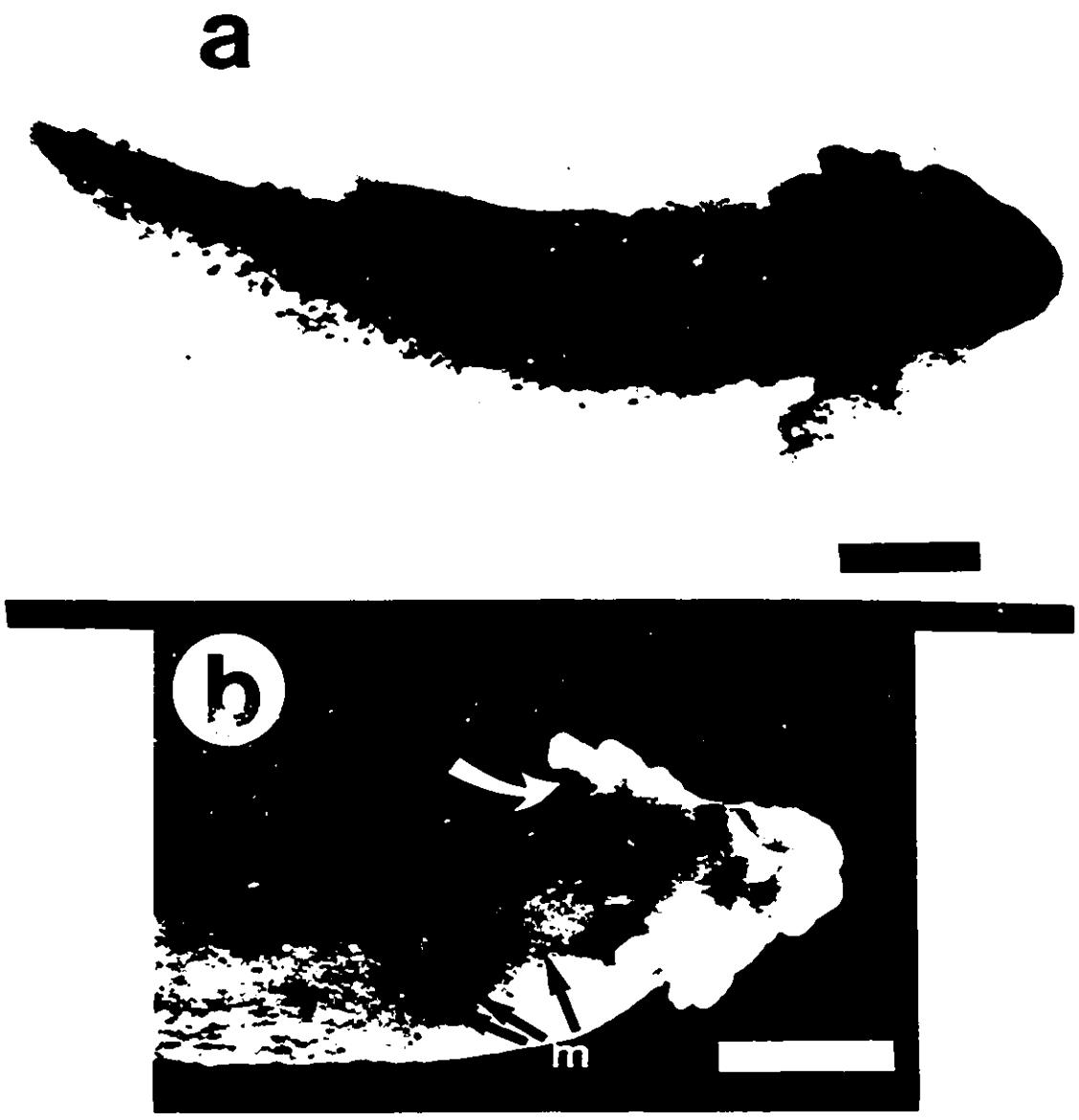

a
b
m

Figure 4.8: Frontal sections at the level of the gills of three embryos of similar chronological age. Anterior is at the top of each photomicrograph. p: pharynx; c: cartilage; v: aortic vessels. Bars = 0.5 mm.

a: Homozygous *p* embryo with wild-type neural crest transplanted into region "B" (as in Fig. 4.6a). Operated side is shown on the left. Note the relatively normal appearance and placement of the cartilage in the branchial arches of the operated side. The unoperated side occasionally contained cartilage, although always in abnormal locations and in smaller amounts. Abundant mesenchyme was always present.

b: Unoperated *p/p* embryo, with characteristic bulb-like structures on the distal ends of primary gill filaments (arrow). Cartilage was never seen in these embryos, although there was an abundance of mesenchyme. Disintegration had begun in this embryo, as evidenced by epidermal blisters (not shown), round and loose epidermal cells, and edema. The aortic vessels, though distended, are present.

c: Normal wild-type embryo. The development is much more advanced than in (a) and (b). The branchial arch cartilages are similar, however, to those seen in the operated side of (a).

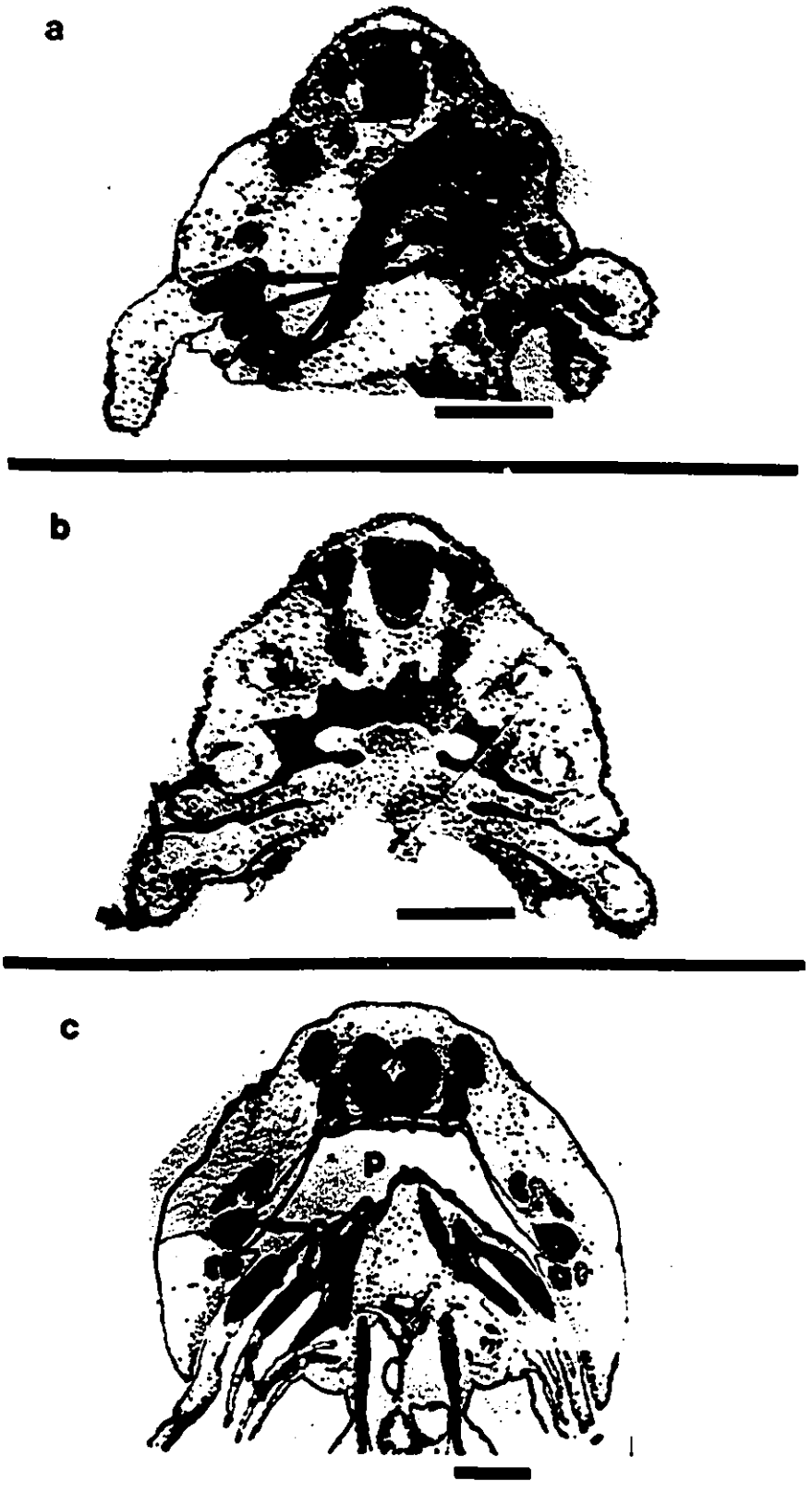
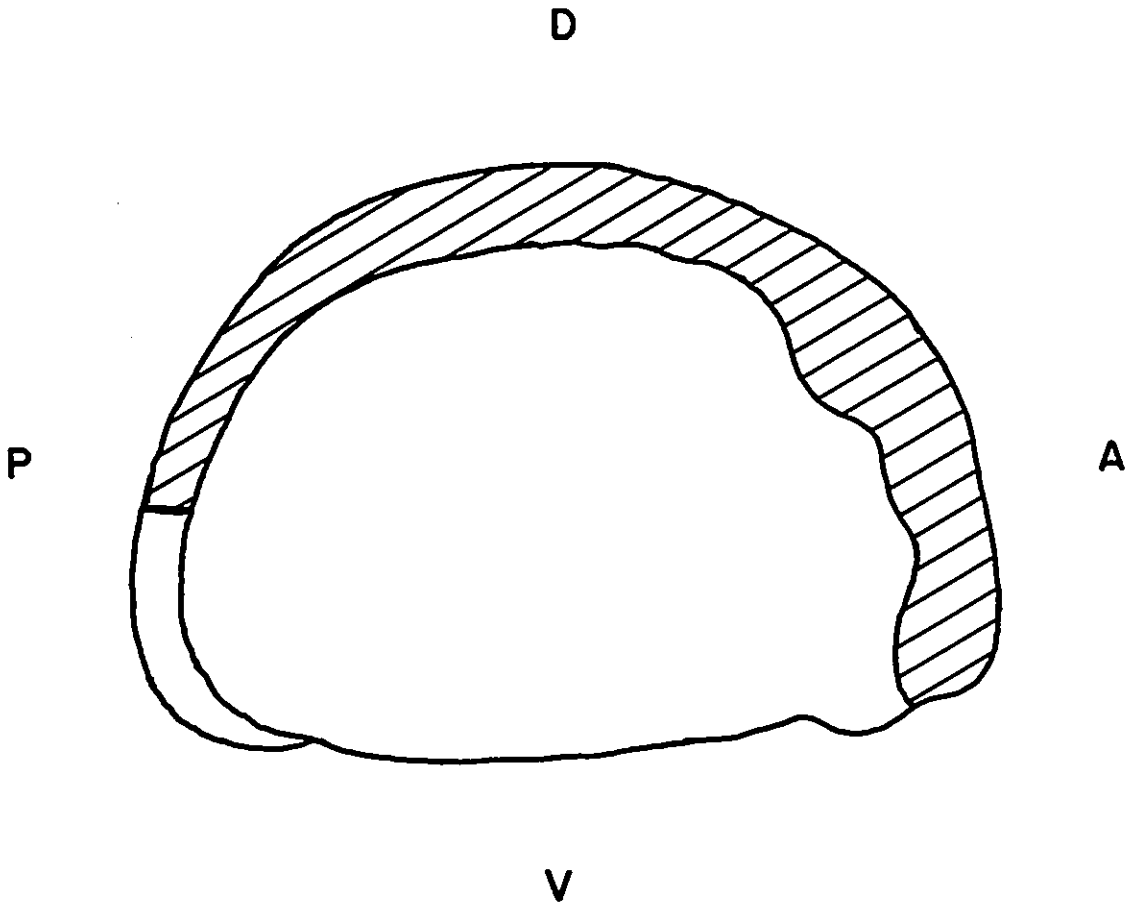

a
b
c
P

Figure 4.9: Right lateral view of a stage 20 embryo. Hatched area indicates the axial levels of the excised neural tissue. The neural crest and all but the medio-ventral portion of the neural tube was removed, as well as the overlying ectoderm. A: anterior; P: posterior; D: dorsal; V: ventral.


D
P
A
V

region was very thin and narrow; the folds were only slightly elevated and had not fused. Removal of these folds led to the exposure of the notochord. These embryos healed very poorly, and usually died.

Although not normal in appearance, some of the operated embryos survived, in a few cases for two weeks. The days immediately following the initiation of heartbeat were of greater interest, with respect to this study. The hearts began beating at the same time in both operated and unoperated embryos. As in unoperated stage 35 embryos, the initial heartbeats were weak, and no circulation was seen. One day later, both operated and unoperated embryos had strong heartbeats. However, circulation was only seen in the unoperated embryos; red blood cells remained in the blood islands in the operated embryos.

By the following day, these embryos still had no circulation. Their siblings had reached stage 38, when secondary filament formation was indicated by the presence of small nodules. It should be noted that these nodules were not present on the gills of the operated embryos.

By the third day following the initiation of heartbeat, some movement of the blood did occur in the operated embryos. Circulation was not definitely established, however, since the blood tended to pool in the gills. These gills appeared swollen, except for the distal tips, and were rather short.

Furthermore, there was still no evidence of secondary filament formation, although those of the control embryos were quite long.

Subsequent development of these embryos was variable. Secondary filaments did develop eventually, in some cases. Not all filaments, primary or secondary, developed at the same rate, even within one embryo. Even the most well-developed gills were not completely normal, however; they had a rather twisted appearance, and the secondary filaments were irregularly spaced and fewer in number than normal.

Circulation was usually established in the survivors, although in some cases it bypassed some of the primary gill filaments of the embryo. These bypassed gills tended to be those which did not develop secondary filaments, and which retained the pooled blood.

Some regulation was evident in the operated embryos. Pigment cells initially appeared only in the most posterior regions of the embryo. However, the pigment cells gradually colonized more anterior levels of the embryos. The operated embryos which survived the longest, and developed the best, tended to be those whose pigmentation was greatest and extended the furthest anteriorly.

CHAPTER V

DISCUSSION

This study was undertaken to examine various aspects of the tissue interactions involved during cartilage differentiation from cranial neural crest cells in *Ambystoma mexicanum*.

5-1. Cartilage differentiation from normal tissues:

As described in Chapter I, there have been conflicting reports in the literature concerning the stage by which cranial neural crest cells are determined for cartilage formatior in the axolotl. In this study, when *A. mexicanum* cranial neural folds were cultured *in vitro*, neither cartilage nor procartilage developed. This indicates that cranial neural crest cells are not determined for cartilage formation prior to the beginning of migration. When the self-differentiating capacity of the neural crest from older embryos was tested, cartilage did form in a small number of cases. The neural crest may have been determined for cartilage formation in these positive cases, although it is impossible to completely rule out the possibility that the neural crest had been contaminated with other tissues during explantation. Since such cases were relatively rare, and

their frequency did not increase with increased donor age, it is obvious that the determination events have, at best, only begun. Therefore, at the oldest stage which could be tested (stage 28), the cranial neural crest cells were still incapable of chondrocyte self-differentiation. Once the cells had begun the medial migration into the branchial arches, the neural crest cells could not be removed without contamination from the mesodermal cells with which they were in intimate contact. Therefore, the stage at which the neural crest cells finally, if ever, become independent of the inductors could not be established.

Pharyngeal endoderm was proposed as an inductor for cartilage differentiation in *Ambystoma mexicanum* following a series of transplantation experiments where ectopic cartilage was found when pieces of pharyngeal endoderm were co-transplanted with cranial neural folds to the host flank (Hörstadius, 1950). As described above, however, *in vitro* studies would probably yield more reliable results, since specific tissues could be tested to the exclusion of all others. Such studies, performed with other urodeles, have shown that pharyngeal endoderm, either alone or in combination with other tissues, is an inductor for cartilage differentiation.

Cartilage developed in explant cultures of *Ambystoma mexicanum* cranial neural folds and pharyngeal endoderm,

supporting the conclusions made by Hörstadius (1950) that pharyngeal endoderm is an inductor of chondrogenesis in the axolotl. When compared with similar *in vitro* studies, these results agree best with those for *Triturus alpestris* (Seno and Nieuwkoop, 1958; Drews *et al.*, 1972; Epperlein and Lehmann, 1975) and for *Ambystoma tigrinum* (Holtfreter, 1968); pharyngeal endoderm is the only inductor required for cartilage differentiation from neurula stage neural crest.

The cartilage which was produced in these cultures was clearly mature; the chondrocytes were isolated in lacunae within a dense, metachromatically-staining matrix, and the nodules were delineated by perichondrial cells. This contrasts with the results of other studies, where the presence of pharyngeal endoderm induced only procartilage formation. Another *Ambystoma* species, *A. maculatum*, required stomodeal ectoderm (Wilde, 1955), and *Pleurodeles waltl* required dorsal mesoderm (Corsin, 1975) for complete differentiation into cartilage.

It is unknown whether these differences are actual species differences, or if they are due to the slight differences in technique and culture conditions used in the various studies. For example, both Wilde's (1955) and Corsin's (1975) culture media contained serum, but neither Epperlein and Lehmann's (1975) nor mine did.

The conclusion, therefore, is that pharyngeal endoderm

is the only inductor required for full chondrogenesis from cranial neural crest cells, from the earliest stage that the neural folds are discernible (stage 14). This requirement is still present when the cells begin their medial migration into the branchial arches (stage 28). This is much later than for the only other species in which this was examined, *Pleurodeles waltl*, where the neural crest cells no longer required pharyngeal endoderm after mid-neurulation (Corsin, 1975). The time course for the differentiation of cartilage from cranial neural crest in the presence of pharyngeal endoderm *in vitro* is essentially the same as that which occurs *in situ*, which indicates that the *in vitro* system contains all the essential components and mimics the processes occuring during chondrogenesis *in vivo*.

It is possible that ectoderm is also an inductor, since this tissue was included in all the explants. Neural crest cells isolated from early tailbud embryos rarely survived the culture period unless enveloped by ectoderm. However, the area from which this ectoderm was removed did not affect results, unlike the situation in *A. punctatum* (Wilde, 1955). Therefore, it is more likely that the ectoderm has a non-specific, supportive role.

A question which remains unanswered, however, is the timing of the induction itself. This must occur while both the inductor and the responding tissues are active, although

it need not require this entire length of time. If the induction were to begin prior to stage 28, then the process would have to be diffusion-mediated, since this is the stage of the initial contact between the two tissues. The *in vitro* system used in this study did not allow the mode of signal transmission to be examined; the tissues were in close contact for the entire culture period. Furthermore, the few previous studies which examined this issue report conflicting results. In *Triturus alpestris* (Epperlein and Lehmann, 1975), the interaction between the two tissues was not diffusion-mediated, whereas in *Pleurodeles waltl* (Corsin, 1975), the endodermal signal must have been diffusible, since the interaction was finished well before direct contact was made between the tissues. If the situation in *A. mexicanum* is similar to that in *Triturus*, as the tissue studies would suggest, then the induction could only begin after stage 28. If, on the other hand, the signal is diffusible, the induction could begin prior to stage 28, although, unlike the case in *Pleurodeles*, at least part of the interaction must occur after stage 28, since endoderm is still required at or after this stage.

Using the *in vitro* system, tissues of different stages were combined. For induction to occur, the endoderm must be inductive at the same time as the neural crest cells are responsive. Tissues of different stages were combined *in*

vitro, in an attempt to determine the active periods of the tissues involved. Cartilage was formed even when the tissues were taken from embryos which were developmentally at least a day apart, indicating that one, or both, of the tissues has an active period lasting at least one day. Unfortunately, neither earlier nor later stages can be used as tissue donors; different culture systems would be required to continue these investigations. Various attempts to establish cell-free systems with inductive ability, which could test endoderm from specific stages, or filter-separation techniques, which could allow the addition or removal of tissues, were unsuccessful.

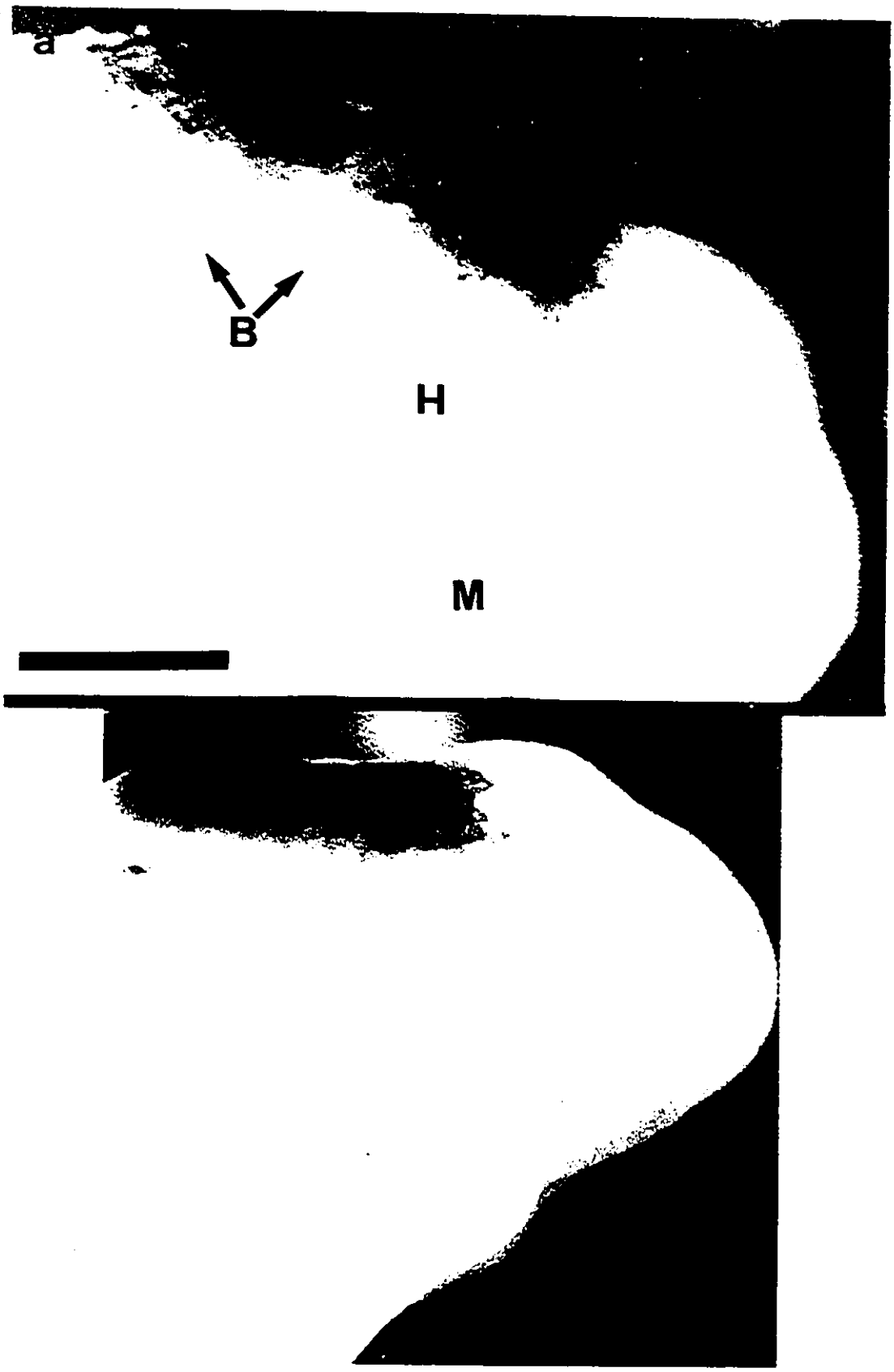
Various studies have shown that neither the anterior-most regions of the cranial neural folds (0° - 30° of Chibon's, 1966, system of co-ordinates) nor the trunk neural folds contain neural crest cells which normally contribute to the urodele skeleton (Chibon, 1966). Transplantation of trunk neural folds to cranial levels does not promote chondrogenesis of the transplanted neural crest (Chibon, 1966). The conclusions reached by this worker was that the trunk neural crest cells were incapable of responding to the normal signals which were present in the head of the embryo. However, an alternate possibility is that the transplanted cells were never properly exposed to the inductive tissues, perhaps because the trunk neural crest cells did not migrate

normally in the head. When pigmented trunk neural folds were transplanted to cranial levels of albino neurulae, the migration of the transplanted neural crest cells was not normal; most of the cells did not appear to migrate laterally at all, particularly not in the "tongue" pattern usually seen with cranial neural crest cell migration (Fig. 5.1). Again, explantation of the tissues would probably give more reliable results than transplantation, since the test tissues could be placed in direct contact. This would eliminate the possibility that the lack of induction was due to a physical barrier rather than incompetence to respond.

Neither neural folds anterior to 30° nor trunk neural folds gave rise to cartilage when cultured with inductive endoderm. These results are similar to those of Seno and Nieuwkoop (1958), who found that *Triturus alpestris* trunk neural crest would not respond to inductive signals *in vitro*. Neural crest cells from more anterior and more posterior axial levels are therefore qualitatively different from those possessing chondrogenic potential, with respect to their ability to respond to the inductive signals, as well as in their migratory behavior.

The ability to induce cartilage formation by cranial neural crest cells could be a common property of all endoderm, or could be limited to endoderm from specific regions. Furthermore, the inductive strengths of endoderm

Figure 5.1: Migration patterns of cranial (a) and trunk (b) neural crest in the branchial region. Grafts of cranial (a; as per Fig. 4.3) or trunk (b; as per Fig. 4.2b) neural folds from wild-type stage 17 embryos were transplanted to albino embryos of the same age. The host site was that shown in Fig. 4.3. The tongues of migrating neural crest cells which are evident in (a) are not seen in (b). M: mandibular arch; H: hyoid arch; B: branchial arches. Anterior is at right and dorsal is at the top of both frames. Bar = 0.5 mm.


a
B
H
M

from different areas of the embryo could differ; a weak inductor could induce less than mature cartilage and/or a lower frequency of cultures containing cartilage. Various regions of endoderm were tested for their inductive potential. There did not appear to be any gradations of inductive ability. All cultures which were scored as positive contained mature cartilage; intermediate chondrocyte phenotypes were never seen. Also, for each type of endoderm, the frequency of cartilage formation was either very high or very low. The lack of intermediate frequencies and intermediate phenotypes indicates that inductive ability is an absolute property, and is either present, or not, within the tissue.

The distribution of the active endoderm within the embryo was examined. A sharp boundary separated the inductive from the noninductive tissue. This boundary was at the junction of the pharyngeal wall and the pharyngeal floor, which coincides with the division between the head and trunk (see Fig. 3.1). Therefore, it would appear that only endoderm which would normally be encountered by responsive neural crest is inductive.

In light of the results of transplantations to the flank, however, it is disturbing that trunk tissues, particularly the gut endoderm and notochord, were not found to be inductive. These are the tissues which would have been

exposed after somites had been damaged, leading to the ectopic cartilage seen by Hörstadius (1950). It has been reported that both notochord and midgut endoderm can induce cartilage formation from cranial neural crest cells in an *Ambystoma* species (Holtfreter, 1968). However, for midgut endoderm, Holtfreter (1968) reported only 9% positive cases, which is even lower than the 17% obtained in this study. Comparing these values with those for pharyngeal endoderm (75% or more, depending on the area used), it is obvious that this tissue is only very weakly inductive, if at all.

In *Ambystoma punctatum* (Holtzer and Detwiler, 1953) and *Ambystoma tigrinum* (Holtfreter,1968), as well as in the chick (Lash and Vasan, 1977) the notochord is an inductor of cartilage formation from sclerotome cells. Holtfreter (1968) reported that notochord from neurulae induced cartilage formation from *Ambystoma* neural crest, although the success rate was not indicated. In this study, however, the notochord was not inductive, since only 5% of the cultures contained cartilage. Although notochord from embryos of several different stages was tested, it is possible that the inductive period is actually later than any of these, since chondrogenesis in the trunk occurs much later than in the head.

Another possibility which could explain the transplantation results is homoiogenetic induction, whereby

chondrocytes derived from the somites (sclerotome) would induce chondrogenesis from the competent, transplanted neural crest cells. This type of induction has been proposed for chondrogenesis within a population of cranial neural crest cells (Holtfreter, 1968). It is not known if such an induction is possible between these different source tissues, although it would appear that each of the two types can recognize the other as different. Cartilage fragments readily fused together when both were derived from the same germ layer, but would remain separated if neural crest-derived and mesoderm-derived cartilages were apposed (Chiakulas, 1957).

For either of these two possible mechanisms (homoiogenetic induction or inductors in the trunk) ectopic cartilage would form only if two conditions were met. First, nerual crest cells would require contact with either the notochord or vertebral cartilage, and second, sufficient time would have to elapse between the transplantation and final processing of the embryo. The conflicting reports in the literature could therefore be explained by variations in the timing and/or location.

5-2. The premature death mutation:

Having determined that pharyngeal endoderm was the sole inductor required for cartilage differentiation in normal axolotl embryos, attention was directed to the study of the premature death, or *p* mutation. Since this mutation was suspected of affecting the anterior endoderm (Trottier and Armstrong, 1977; Mes-Hartree and Armstrong, 1980), it was hoped that a comparative study could be performed between the mutant and wild-type systems. In this way, more information concerning the nature of the induction could be obtained.

As described in Chapter I, histological examination of *p/p* embryos had shown that the most obviously affected organs were either formed by anterior endoderm (*e.g.* liver and pharynx) or were dependent on it for induction (*e.g.* gills and heart). If the mutant endoderm is indeed defective for all these functions, then it would not be surprising if its cartilage-inducing abilities were also affected.

Embryos which are homozygous for *p* do not possess any cranial or visceral cartilages (see Fig. 4.8b; and Trottier and Armstrong, 1977). There are several possible explanations for this lack of cartilage: one (or both) of the tissues involved in the cartilage induction system could be defective, the presence of degenerating tissue in the vicinity could inhibit either the induction or

chondrogenesis, or *p* could be a cell-lethal mutation (a biochemical deficiency which affects all cells). The latter possibility can be eliminated, since some mutant tissues survive when transplanted onto wild-type hosts (Mes-Hartree and Armstrong, 1980).

Explants of cranial neural folds and pharyngeal endoderm, taken from *p/p* embryos, did not produce cartilage. They appeared quite healthy, however, and showed no evidence of degeneration. This provides additional evidence that *p* is not a cell-lethal mutation, and indicates that the deterioration of the mutant embryos is not the reason for the lack of cartilage. Therefore, the inductive system must be defective. Extending the culture period to 18 days did not change the results; the cultures were still healthy, and still showed no evidence of chondrocyte differentiation. Therefore, the mutation's effect on chondrogenesis is not simply to delay its development. There must be a lack of inductive ability in the endoderm and/or a lack of responsiveness in the neural crest cells.

Mutant and wild-type tissues were combined in order to determine which of the tissues was defective. The results were quite unexpected; cartilage differentiation could be induced in wild-type neural crest as frequently with mutant endoderm as with wild-type endoderm. Mutant cranial neural crest, however, rarely responded to wild-type inductor.

Therefore, the lack of cartilage in the initial experiments was obviously caused by a deficiency in the responsive tissue.

One possible explanation for the inability of the cranial neural crest to form cartilage would be an "axial shift", such that the chondrogenic neural crest cells are present in the mutant, but at a different normally non-chondrogenic axial level. This possibility appeared unlikely, however, since neither mutant trunk neural crest cells nor those from cranial levels 0° to 30° were capable of forming cartilage when explanted with inductive endoderm. This is identical to the results obtained with wild-type neural crest taken from these axial levels.

The possibility of an axial shift was also examined with transplantation experiments. As discussed in section 5-1, wild-type trunk neural crest cells, when transplanted to cranial levels, appear to be incapable of following normal cranial pathways. If an axial shift was present in mutant embryos, such that the neural crest in cranial levels had been specified as trunk neural crest, then their migration patterns should resemble those of transplanted trunk neural crest.

Therefore, mutant cranial neural folds were homotopically transplanted onto albino hosts. The major migration patterns and the timing of the emigration were

identical to those seen in homotopic grafts of wild-type origin. These results indicate that the cranial neural crest cells of the mutant are indeed able to migrate along the usual cranial pathways. It must be emphasized that only the major pathways (those with large numbers of cells) could be examined. There may therefore be some cells, whose progress could not be followed, which either did not migrate, or did not follow the proper pathways to their ultimate location. However, the neural crest cells under consideration (the chondrogenic cells) should have been found along the pathways which could be seen using this technique. Therefore, the *p* mutation does not appear to give rise to an axial shift in the neural crest cells, nor does it affect the migratory ability of most cranial neural crest cells.

As previously discussed, explant cultures of wild-type and *p/p* tissues had indicated that the defect in the mutant appeared to lie within the cranial neural crest. The unilateral, homotopic replacement of segments of mutant neural fold with wild-type neural fold provides corroborative evidence for the results seen with the *in vitro* cultures. When the transplantations were of the axial levels which contribute to the branchial arches, abundant cartilage was found on the operated side (see Fig. 4.8a). These nodules were distributed in a manner which appeared normal, although for a younger stage than that of the unoperated, wild-type

control embryos. Cartilage nodules were also sometimes seen in the unoperated side, although they were usually small and abnormally distributed. These were probably derived from wild-type neural crest cells from the opposite neural fold.

The presence of cartilage in these embryos confirms that, in the *p* mutant, the signals which are responsible for the induction of cartilage are normal. Furthermore, it clearly shows that, although the development of the mutant virtually halts at stage 37 and some disintegration begins almost immediately, the embryo is capable of supporting the development and differentiation of chondrogenic cells to at least stage 41.

The most important conclusion that can be drawn from these embryos, however, is that the lack of neural crest-derived cartilages cannot be the cause of all of the abnormalities in, and the death of, the mutant. Although the one definitively known defect in *p* is in the chondrogenic neural crest, other cells must also be defective, since mutant embryos containing cartilage still do not survive. It is also clear, however, that the neural crest, as a whole, cannot be defective; there are cells which originate in the neural folds, follow neural crest pathways, and which can differentiate into at least some neural crest derivatives (such as pigment cells, dorsal fin mesenchyme, and probably Rohon-Béard cells).

If the mutant characteristics are caused by a subpopulation of neural crest cells, then the replacement of mutant neural folds with wild-type neural folds should correct the defects. At least some mutant abnormalities (eye development, gill filament length, and cranio-visceral cartilages) were indeed rectified by the addition of normal neural crest cells. However, the survival of the mutants was not affected, regardless of the axial level which was replaced. Since *p* embryos have several abnormalities, it is possible that a variety of neural crest derivatives from various axial levels are affected. A full rescue of the mutant could require the replacement of longer lengths of neural fold than was technically possible.

Although the replacements were performed unilaterally (leaving intact mutant neural folds on the unoperated sides), several workers have reported that some neural crest cells migrate down the opposite side to their fold of origin (Chibon, 1967; Rollhauser-ter-Horst, 1980; Hörstadius, 1950). Mixing of the mutant and wild-type neural crest was demonstrated, in some cases, by the presence of cartilage in the unoperated side of *p/p* hosts. It is also possible that the presence of mutant neural crest interfered with some function(s) of the wild-type neural crest, and prevented full rescue.

Additional evidence that some of the mutant

abnormalities were probably caused by defective neural crest was demonstrated by extirpation of neural crest from wild-type embryos. The most striking effect of this operation was on gill development. The gills of these embryos bore a greater resemblance to those of homozygous *p* embryos than those of their unoperated sibs; the three primary filaments remained short, often possessed bulb-like structures at their distal ends, and secondary filaments were not seen until much later than usual (see Fig. 4.7b). Similar effects of such operations on gill morphology have been noted previously (see Hörstadius, 1950).

The effect of neural crest extirpation on the development of the circulatory system was also surprising. There was a definite delay in the initiation of circulation, and even then, it was initially only present in levels posterior to the gills. Circulation in the head was only seen after a further delay. In the mutant, the lack of circulation has been ascribed to the blockage in the heart, since the major blood vessels appeared to be normal (Trottier and Armstrong, 1977). However, the smaller vessels could not be examined, due to the edema in the mutants and the disintegration of many internal tissues. It is interesting to note that neural crest has been implicated in angiogenesis in the chick (Le Douarin, 1982).

A high degree of regulatory ability has been recorded in

amphibians following removal of neural crest cells (Chibon, 1966). The ablations performed in this study were more complete than those performed by Chibon, and involved all the cranial and most of the trunk neural folds of late neurulae. The regulation which occurred was not complete; the embryos never survived, and never appeared completely normal. Therefore, neural crest is necessary for survival. Some features did appear to gradually return to apparent normalcy, although after a definite delay compared with unoperated controls. The embryos which developed the best, following removal of the neural folds, were those in which pigment cells invaded more anterior regions most quickly. These may have been the best cases of regulation, or perhaps removal of the neural crest was incomplete. The earliest features of the operated embryos would therefore be the most accurate for the determination of neural crest functions. These were the stages in which the operated embryos exhibited *p*-like characteristics.

The results of the unilateral transplant and extirpation experiments support the hypothesis that *p* is a mutation which affects a subpopulation of neural crest cells. While tissues other than neural crest may also be defective in the *p/p* mutants, when simple organ systems with known components were tested *in vitro*, no defective tissues were found. Mutant pharyngeal endoderm is normal for at least two functions:

induction of beating hearts from mesoderm, and induction of cartilage from wild-type cranial neural crest. Mutant heart-forming mesoderm, when cultured as explants, formed beating tissue which beat as strongly as, and for as long as, wild-type tissues. *In vivo*, however, the heart beats are weak, and cease within days of mutant identification. This discrepancy between the *in vitro* and *in vivo* situations may indicate that some of the mutant characteristics are due to secondary effects, such as the unhealthy environment of disintegrating tissues, rather than being a direct result of the mutation. These possibilities cannot be distinguished *in vivo*; *in vitro* tests of each organ would have to be performed in order to determine which abnormalities are directly affected by the mutation.

It is possible, however, that heart formation *in vivo* may be directly affected by a neural crest defect such as *p*. Indirect evidence for this is provided by the recent finding that neural crest is required for the proper morphogenesis of anterior regions of the heart in the chick (Kirby *et al.*, 1983; Kirby and Bockman, 1984; Besson *et al.*, 1986). While it is not known whether amphibian hearts also require neural crest, recent studies involving grafts of *Xenopus borealis* neural folds onto *Xenopus laevis* hosts have indicated that neural crest cells enter the wall of the truncus arteriosus (Sadaghiani and Thiébaud, 1987).

From these *in vitro* and *in vivo* experiments it is clear that the premature death mutation in the axolotl affects some neural crest derivatives. It remained unclear, however, what phase of neural crest formation was specifically affected by the mutation; the *p* mutation could affect primary induction or a later step in neural crest formation.

Primary, or neural, induction has been the subject of intense study since Mangold and Spemann's discovery of the "organizer" (see Spemann, 1962, and reviews by Holtfreter and Hamburger, 1955; Saxén and Toivonen, 1962; Hamburger, 1988). In summary, this remarkable tissue is presumptive notochord and dorsal mesoderm, and is found at the dorsal lip of gastrulae. When the organizer was placed in an ectopic location in a host gastrula, a secondary axis developed. This new axis was not entirely of graft origin, however; host cells were assimilated into the structure. Furthermore, the organizer induced the formation of neural tissue (neural plate and associated structures) from the host ectoderm. The individual secondary structures which developed were usually normal, as were the spatial relationships between them.

The axial levels of the secondary structures which developed were dependent on the graft tissue. Dorsal lips from early gastrulae (fated to form head tissues) produced secondary heads, whereas those from older gastrulae (presumptive trunk notochord and mesoderm) usually produced

secondary trunks and tails. Complete embryos, of fairly normal appearance, could also be induced with large pieces of chordamesoderm which contained both future head and tail regions. In general, neural induction is thought to occur in two steps. The first involves a general induction, with archencephalic tendencies. The second involves the regionalization of the neurectoderm, such that structures of all axial levels are produced (Saxén and Toivonen, 1962; Hamburger, 1988; Nieuwkoop *et al.*, 1985; Nieuwkoop, 1985). The search for the inducer(s) and mode(s) of action, using both the natural and artificial organizers, has been quite extensive, but has yet to yield many definitive answers.

The induction and regionalization of the neural crest, however, has had very little study. Although neural crest can apparently be induced independently of neural plate, neural plate induction is almost always accompanied by the formation of neural crest (Holtfreter and Hamburger, 1955). Therefore, it would appear that neural crest induction is intimately associated with, and perhaps an inescapable consequence of, the neural induction process described above.

According to Raven and Kloos (1945), the mesoderm which underlies the neural folds is the probable inductor of neural crest, via a quantitative mechanism. The lateral portions of archenteron roof would have less "evocator", and could therefore induce only neural crest, compared with the medial

portions, which would contain more "evocator" and could induce neural plate. Mesoderm involvement in neural crest induction has also been suggested by the results of experiments using artificial inductors. Explants of gastrula ectoderm exposed to artificial archencephalic inductors differentiated into archencephalic neural structures, placodal material, and epidermis, but not neural crest (Nieuwkoop, 1963; Rollhäuser-ter-Horst, 1977a). However, neural crest derivatives differentiated when the same artificial inductors were used to produce secondary structures in gastrulae *in vivo* (Rollhäuser-ter-Horst, 1977b; 1979). The differences between the *in vitro* and *in vivo* results were attributed to the presence of underlying mesoderm in the embryo. These results have also led Nieuwkoop (1985; Nieuwkoop *et al.*, 1985) to propose that neural crest formation is the result of a second step of neural induction.

Other hypotheses for neural crest induction, however, implicate only the ectoderm. For example, Albers (1987) has proposed that the type of neural tissue, whether plate or fold, would depend on the state of competence of the host gastrula ectoderm. According to this worker, only the notochord has the ability to induce neural tissue. Neural induction would therefore begin in the notoplate, and spread medially by homoiogenetic induction through the gastrula

ectoderm. Ectoderm from early gastrulae would be the most responsive to this signal, and would respond by forming neural plate. With time, however, the competency would decrease, and the response would be the formation of neural folds, and presumably neural crest. Further loss of competence would lead to the lack of a neural response, with perhaps a period of placode formation prior to epidermis formation.

Another theory is that of Moury and Jacobson (1989), where the formation of folds is a physical response to the presence of a boundary between neural plate and epidermis. Boundaries which were artificially formed between these two tissues resulted in the formation of neural fold-like structures, and produced pigment cells and mesenchyme, which are usually considered neural crest derivatives. However, it should be noted that Niu (1954) observed pigment cell formation from neural plate, under experimental conditions, in a variety of urodele species.

It is noteworthy that none of these theories address the question of regionalization, whereby some neural crest derivatives are restricted in their differentiative potentials. This process must occur either concomitantly with, or immediately following, the induction of neural crest in axolotls, since at the very earliest stages of neural folds (stage 14), the presumptive chondrocytes are already

present and restricted to specific axial levels. All these possibilities for neural crest induction are dependent, either directly or indirectly, on the chordamesoderm. Since *p* is a neural crest defect, abnormal chordamesoderm could be the primary defect.

This was tested by implanting dorsal lip (organizer) material into the blastocoeles of host embryos, and thereby inducing the formation of secondary axes. When both donor and host were wild-type embryos, blastocoele implants induced the formation of secondary structures with varying degrees of morphological normalcy, though usually possessing recognizable neural plates surrounded by neural folds. When some of these embryos were allowed to develop past neurula stages, some of the induced structures formed secondary embryos which were remarkably normal in appearance. They possessed well developed heads and tails, indicating that the graft area used was capable of regionalizing the neural tissues that it induced. These secondary neural folds also behaved as did the primary neural folds with respect to cartilage formation *in vitro* .

Presumptive chordamesoderm from mutant gastrulae was capable of inducing secondary neural folds with chondrogenic potential from wild-type gastrula ectoderm. The converse was not the case, however. Ectoderm from mutant gastrulae could be induced by wild-type dorsal lip material to form secondary

neural plates with neural folds. (This is not surprising, as mutant neurulae are normal in appearance). These secondary neural folds did not contain chondrogenic neural crest cells. When placed with inductive endoderm *in vitro*, neither cartilage nor procartilage was formed.

Therefore, the chordamesoderm, which is ultimately responsible for the initiation of the events leading to the formation of chondrogenic (cranial) neural crest cells, is normal. It is the gastrula ectoderm which is defective in *p* mutant embryos.

In conclusion, embryos homozygous for *p* possess neural crest cells, which are apparently normal with respect to initial localization, and migratory ability. The mutation apparently does not cause an axial shift in the regional specification of the neural crest, but appears to be due to the inability of induced gastrula ectoderm to form normal neural crest. Although some of the neural crest functions are clearly affected (ability to form cranio-visceral cartilage), others (apparently) are not (ability to form pigment cells, dorsal fin mesenchyme, and probably Rohon-Béard cells). These observations suggest that neural crest specification is at least a two-part process. The first (apparently normal in the *p* mutant) is the formation of neural crest cells, and the second (which is abnormal in the mutant) subdivides the population and/or gives the

subpopulations new properties.

While the neural crest has been studied for over a century (as reviewed by Hall and Hörstadius, 1988), it is doubtful whether all of its derivatives and, especially, inductive roles are yet known, as exemplified by recent findings of its role in heart morphogenesis (see Besson *et al.*, 1986). Since the *p* mutation definitely affects several neural crest functions, such as chondrogenic potential and its role(s) in gill morphogenesis, it is very likely that other neural crest functions are also impaired. These presumably give rise to some of the abnormalities seen in the mutant.

Therefore, the *p* mutant should provide an extremely useful model system for studying the structures derived from, and induced by, the neural crest. Furthermore, this mutant may prove invaluable for examining the steps involved in the induction and specification of the neural crest, and the subsequent restriction of the developmental potentials of these cells.

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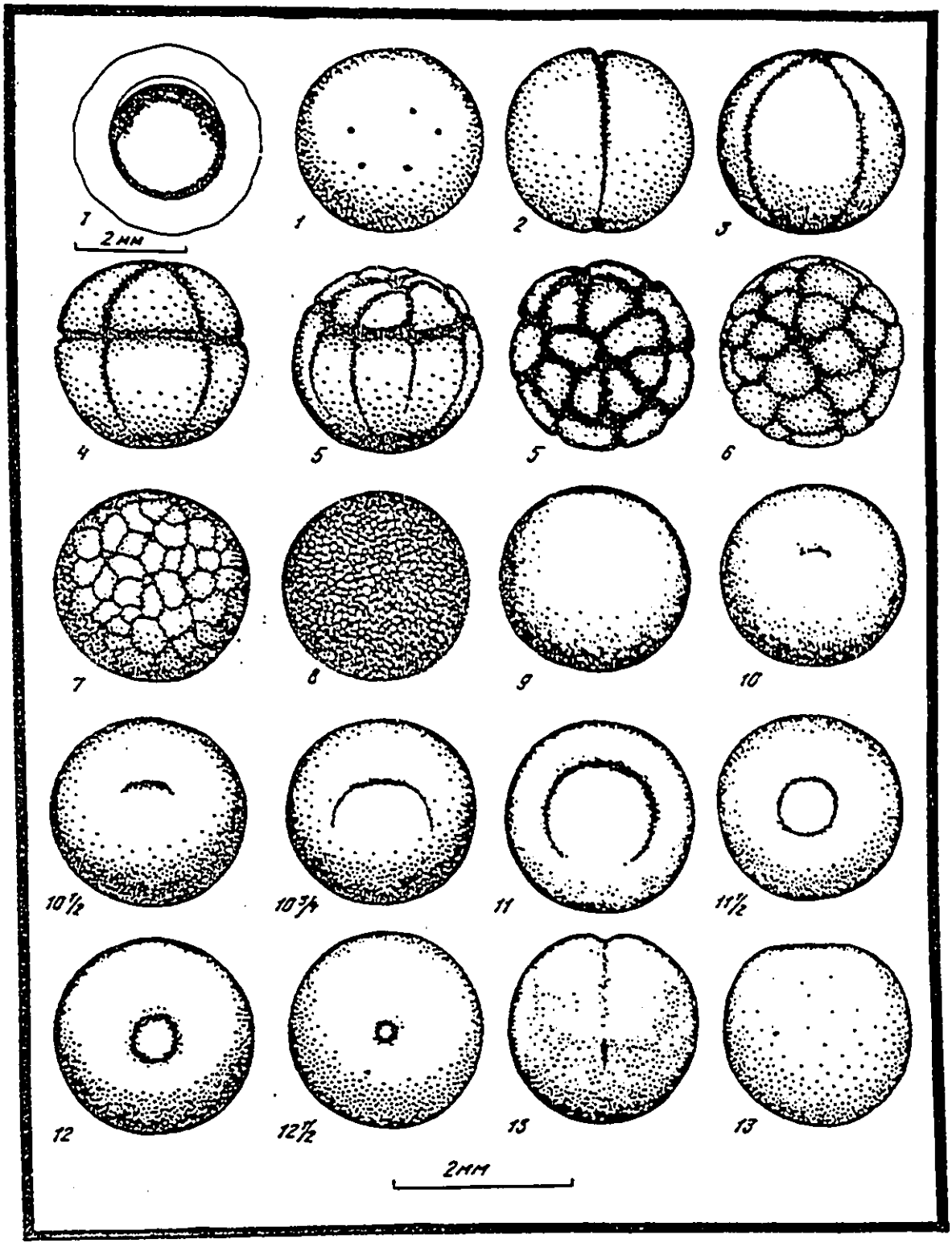
APPENDIX I

Staging table of axolotl development.

(From Bordzilovskaya *et al.*, 1989.)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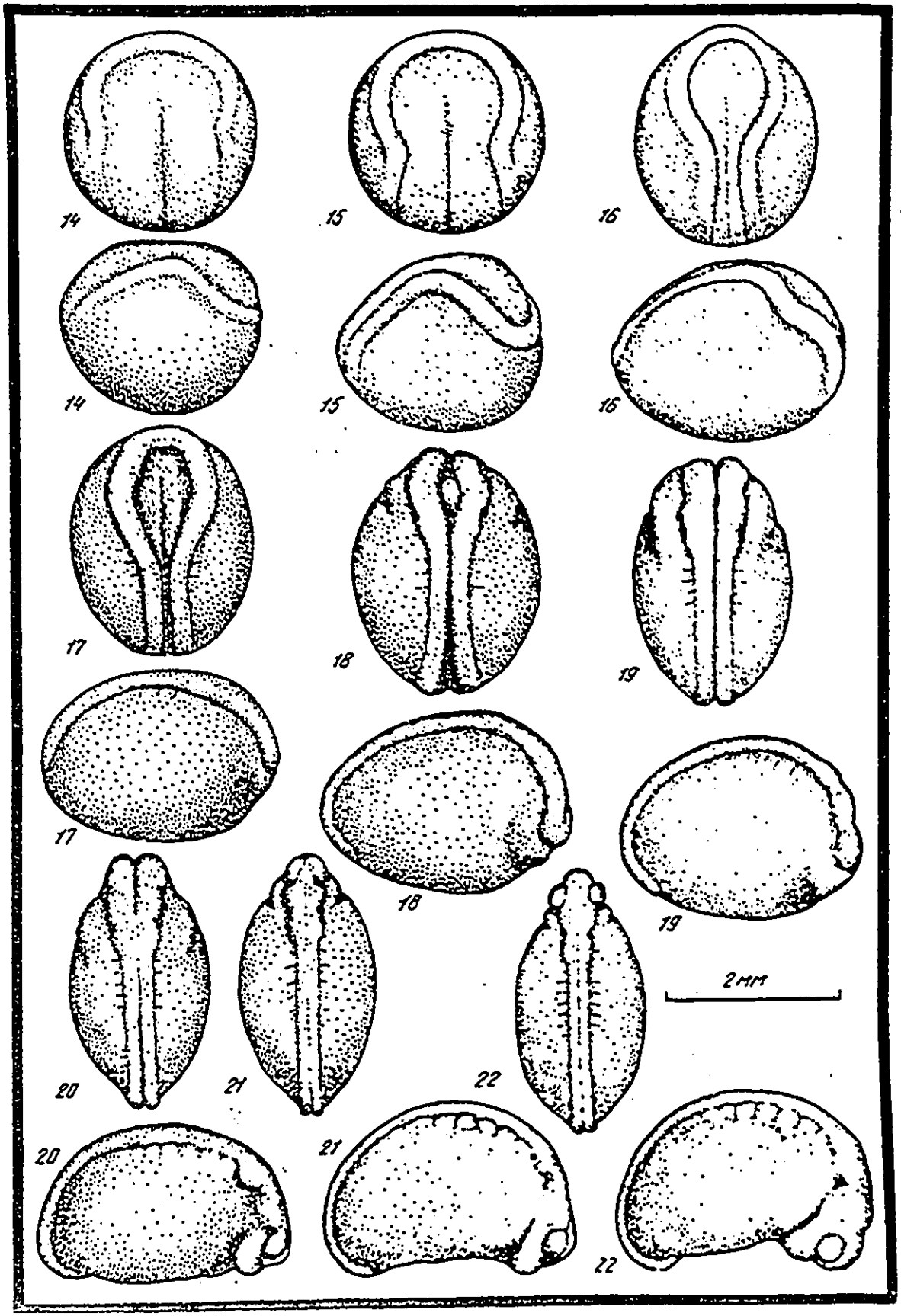
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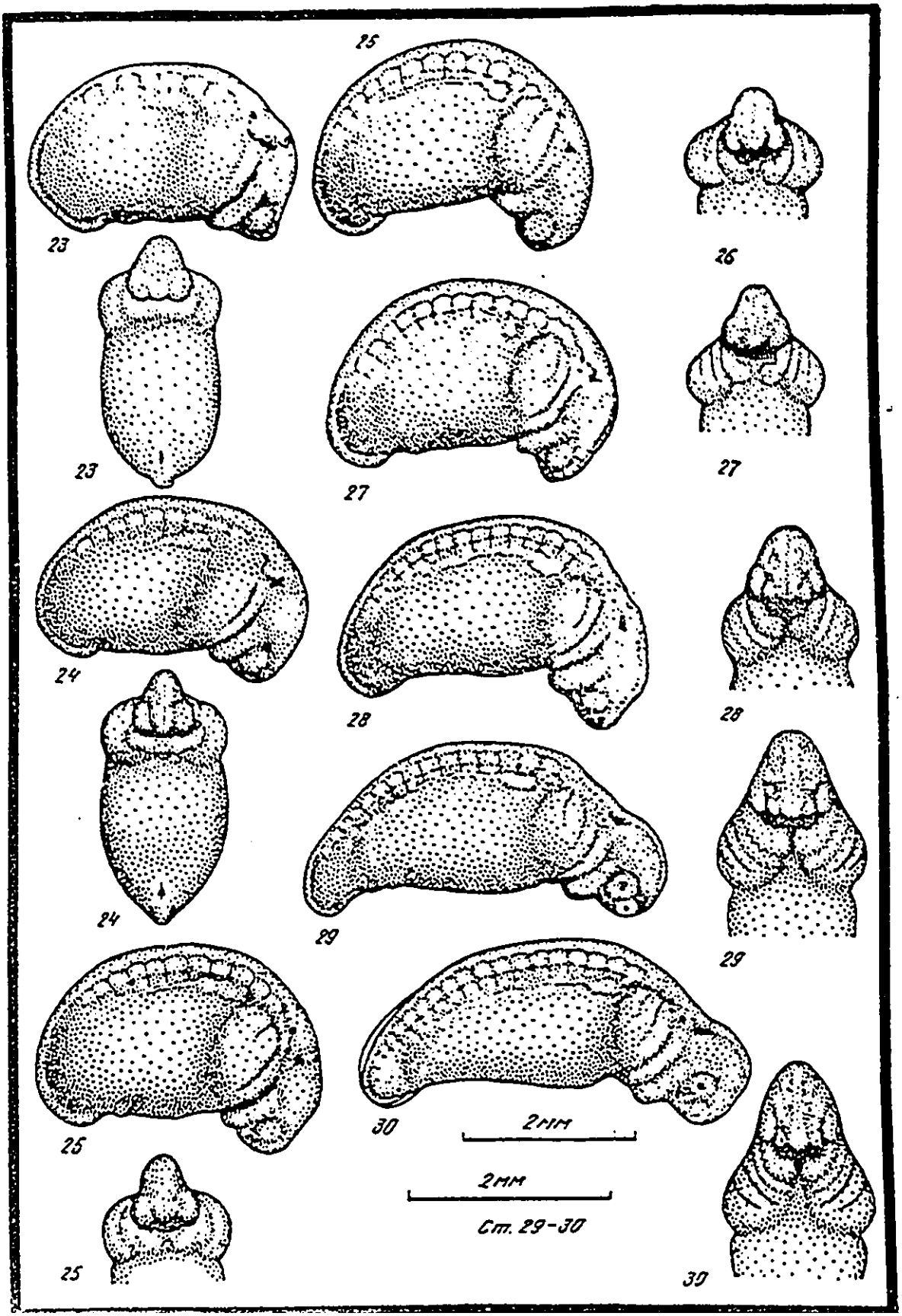
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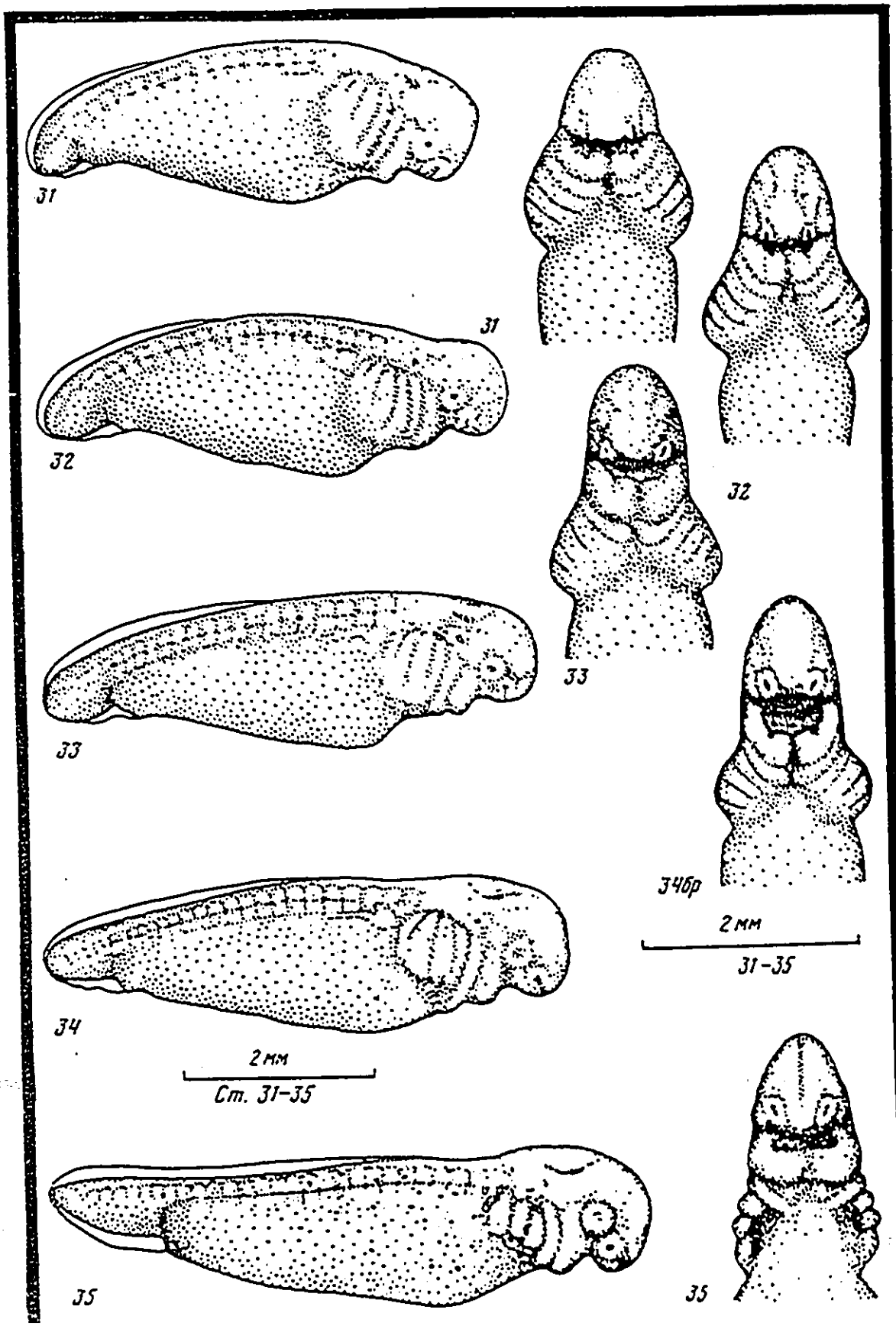
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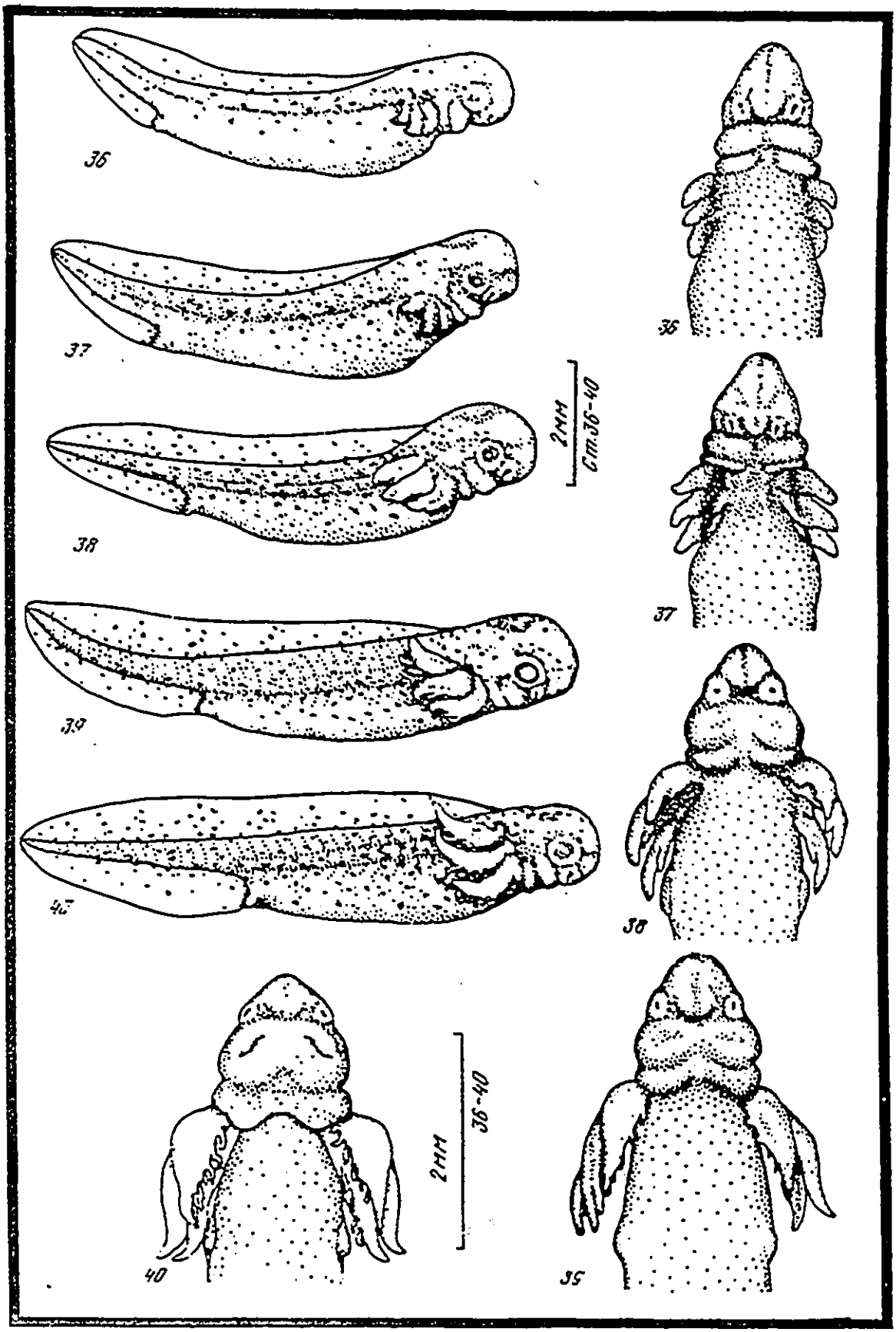
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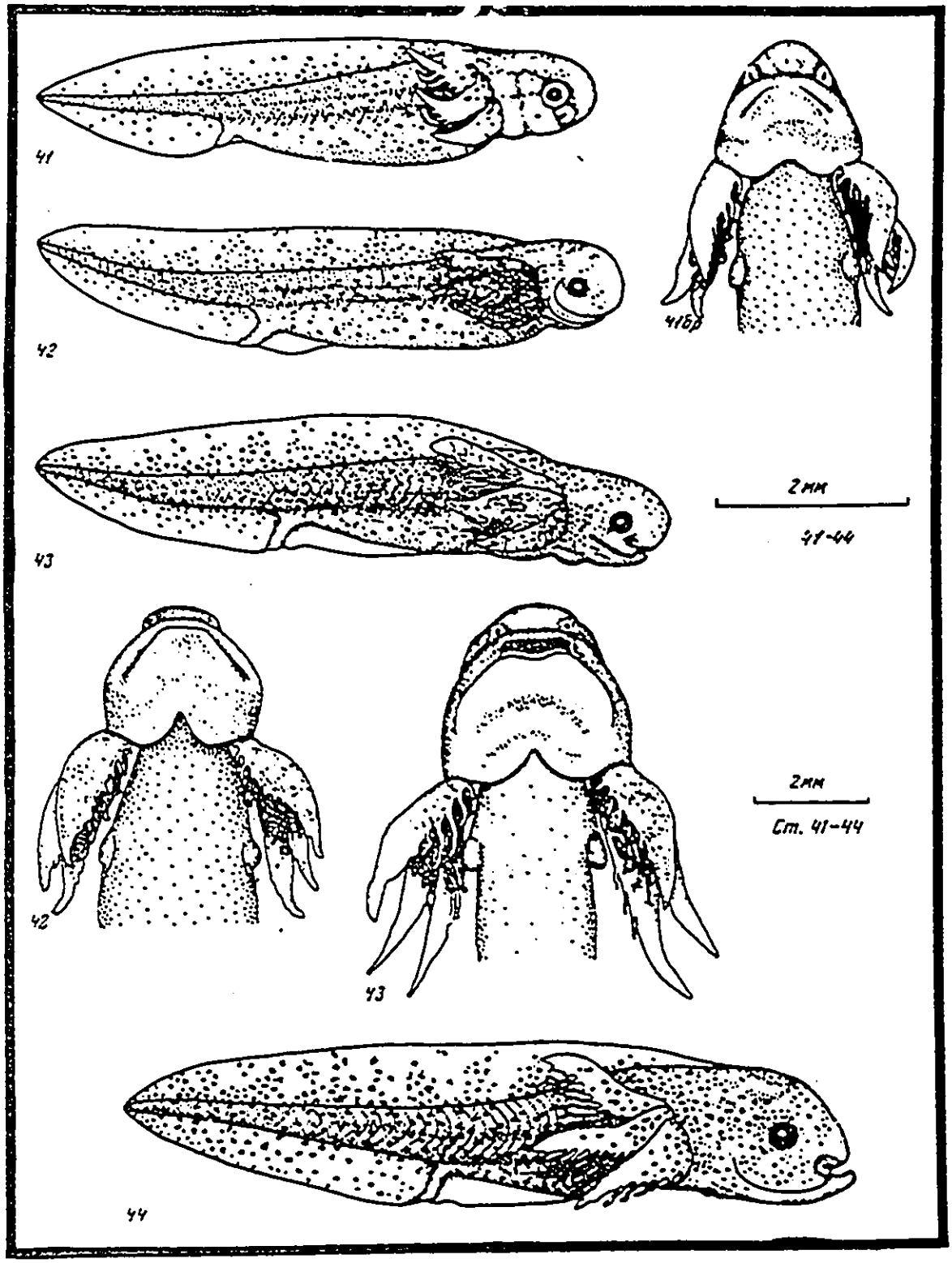
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