Developmental Biology 445 (2019) 115–144

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Commentary

Commentary on Le Douarin, 1973 and 1974

Developmental Biology has a long, rich history of publishing papers that have influenced the field for decades. As 2019 marks the 80th anniversary of the Society for Developmental Biology, I thought it would be useful to republish a sampling of these classic papers both to highlight their impact and also to give a sense of how the field has advanced.

To begin this series, I have chosen two papers by Nicole Le Douarin and colleagues that are personally important to me and helped to shape my thinking as a young scientist. The first is a technical note that introduces the quail-chick chimera grafting technique; the second one, published one year later, utilizes the technique to test the developmental potential of neural crest cells by grafting them to other axial levels. Published in *Developmental Biology* in 1973 and 1974, respectively, these papers were paradigm shifting in the neural crest field and established Le Douarin as one of the most influential scientists of the 20th century.

In the 1970s, the quail-chick chimera was a pivotal technology that transformed cell lineage analysis in higher vertebrates. Le Douarin recognized that quail and chick embryos are of similar sizes and develop at similar rates under the same conditions, yet quail cells can be distinguished by the condensed mass of heterochromatin in their nucleolus. In contrast, chick cells are euchromatic such that the two cell types can be recognized by staining for DNA and ultrastructural analysis (Le Douarin, 1973). To perform lineage analysis, Le Douarin and colleagues transplanted quail neural tubes in place of chick neural crest populations. First, this was used to map the normal derivatives arising from the neural crest all along the body axis. These studies represent the most complete understanding of the derivatives that arise from the neural crest to date.

The second paper extends the quail-chick chimeric technique to test the developmental potential of the neural crest from different regions of the body axis when grafted to ectopic sites (Le Douarin and Teillet, 1974). The results showed that neural crest cells have broader developmental potential than they normally expressed. For example, when trunk cells were transplanted to the vagal (neck) level, they acquired the ability to migrate into the gut and were able to differentiate into enteric ganglia. This shows that trunk neural crest cells are capable of broader differentiative ability. It also shows that these cells can follow migratory cues that are appropriate for cells arising from the location to which they were transplanted. Similarly, cranial neural crest cells transplanted to the trunk can follow normal trunk migratory pathways and give rise to derivatives appropriate for site of transplantation, like sympathetic ganglia and cells of the adrenal medulla. However, some cells also formed ectopic cartilage, which is appropriate for their site of origin but not for the site of transplantation. Thus, some neural crest cells express their intrinsic program whereas others are flexible and adopt fates to one appropriate for their new environment. Importantly, this technique enables investigators to challenge the developmental plasticity of cell populations by putting them into ectopic locations.

Today, cell lineage analysis has undergone a resurgence of interest with the advent of tracers including inducible Cre and Confetti lines, other lineage markers, and bar-coded single cell RNA-seq, yet still we use these classical papers as reference for our modern experimental techniques. To put these classical papers in context, the quail-chick chimera in its time had similar impact on the ability to study cell lineage not only for neural crest development but also in many other developmental contexts.

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https://doi.org/10.1016/j.ydbio.2018.12.011
0012-1606/

DEVELOPMENTAL BIOLOGY 30, 217–222 (1973)

A Biological Cell Labeling Technique and Its Use in Experimental Embryology

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Accepted July 21, 1972

A cell-labeling technique is proposed, based on structural differences between the interphase nucleus of two species of birds, the japanese quail (*Coturnix coturnix japonica*) and the chick (*Gallus gallus*). The quail nucleus shows large heterochromatic masses associated with the nucleolar RNA. In the chick, on the contrary, the arrangement of chromatin during the interphase fits the general pattern observed in vertebrate cells: DNA is rather uniformly distributed in the nucleoplasm, and the quantity of nucleolus-associated chromatin is not significant. It is possible to distinguish the cells of the two species in histological sections stained by the Feulgen Rossenbeck reaction, and also at the electron microscope level in sections involving the nucleolus. Thus quail cells can be used as "natural markers" to study various embryological problems such as those related to intercellular interactions and cell migration during ontogeny.

INTRODUCTION

The association of tissues from quail and chick embryos in experiments previously devised to study heterospecific intertissue reactions focused our attention on a peculiar feature of the quail interphase nucleus. With hematoxylin staining, the quail cell nucleus shows a large nucleolus in all cell types, including, for instance, mesenchymal cells which may lack such a characteristic. Therefore it seemed interesting to compare appearances of chick and quail nuclei after several specific nuclear staining procedures.

Then it appeared that cells of the two species can be identified beyond any doubt if appropriate staining techniques are employed. Thus, the quail cells can be used as "biological markers" in the study of various embryological problems, such as the course of morphogenetic movements, cell migration, and intertissue or intercellular interactions. The labeling techniques previously used in higher vertebrates were based on the incorporation of tritiated thymidine (Weston, 1963; Johnston, 1966; Rosenquist, 1966). They have the inconvenience of not being stable owing to the dilution of the nuclear marker since extensive proliferation usually occurs before the embryonic cells finally express their phenotypes. The use of quail cells as natural markers makes it possible to identify the cells at any developmental stage up to the time they have reached a fully differentiated state. Since our first observations (Le Douarin, 1969), the quail cell labeling technique has already been used to study the evolution of neural crest cells (Le Douarin and Le Lièvre, 1970; Teillet and Le Douarin, 1970; Le Douarin and Teillet, 1971a,b; Teillet, 1971; Le Lièvre, 1971), the morphogenetic movements of gastrulation (Vakaët, 1971) and other problems (Martin, 1971; Saxod, 1971). It is the purpose of this brief note to bring the method to the attention of developmental biologists.

MATERIAL AND METHODS

Various embryonic and adult tissues of the White Leghorn strain of the chick (*Gallus gallus*) and japanese quail (*Coturnix coturnix japonica*) were observed.

Light microscopy. Tissues were fixed in Zenker's fluid and 5 μ paraffin-embedded sections were stained by the Feulgen and Rossenbeck's (1924) technique for the se-

lective staining of DNA.

Some fragments were fixed in Carnoy's fluid in order to localize nucleolar RNA. The Unna-Pappenheim staining method was combined with ribonuclease digestion according to Brachet (1940).

Electron microscopy. For electron microscopic studies, tissues were fixed in a 6% glutaraldehyde solution in 0.15 *M* Sörensen-phosphate buffer at pH 7.4 for 20 min, washed, and then postfixed for 1 hr in 1% OsO_4 in the same buffer. After alcohol dehydration, the specimens were embedded in Epon, according to the usual procedure. Sections were cut with a Reichert ultramicrotome, mounted on uncoated copper grids and stained with uranyl acetate followed by lead citrate according to Reynolds (1963).

Experimental procedures. Tissues of chick and quail embryos were associated either *in vitro* in organ culture according to the Wolff and Haffen technique (1952) or in grafts *in ovo*. After various association periods, tissues were observed by light or electron microscopy according to the techniques previously described.

RESULTS AND DISCUSSION

I. Comparative Study of Chick and Quail Interphase Nucleus

1. *DNA Arrangement as Evidenced by Feulgen-Rossenbeck's Technique*

In the quail, a considerable amount of the chromatin in the interphase nucleus is usually condensed in a large central mass, strongly Feulgen-positive. Apart from this central mass, the nucleoplasm seems to be relatively poor in heterochromatic DNA and therefore stains weakly with Schiff's reagent. Occasionally, some nuclei show 2 or 3 chromatin condensations. In young embryos (2–3 days of incubation) cells from all three germ layers show this pattern. In most embryonic and adult tissues whether from organ rudiments of from fully differentiated organs, the same arrangement is observed. Such is the case, for instance, in the spinal cord, the encephalic nervous tissue, the sensory ganglia and various differentiated nervous derivatives. The mesonephros, metanephros, suprarenal glands, lungs, digestive epithelium, thyroid, and parathyroid glands all show the same pattern of interphase nuclear chromatin. In muscle cells and hepatocytes, the nucleus usually contains two or three and sometimes more chromatin masses.

The same tissues in the chick show a strikingly different disposition of the chromatin material. It is fairly evenly distributed in the nucleoplasm, and forms a network with small dispersed chromocentres (Fig. 1).

2. *Cytochemical Localization of the Nucleolar RNA in the Quail Nucleus*

In adult or embryonic quail tissues, the nucleolar RNA, stained red by pyronine in the Unna-Pappenheim technique, is closely associated with the methyl-green staining the DNA masses. In hepatocytes, the nucleolar RNA is often located between two masses of DNA. Therefore the central body of the quail nucleus may be considered as a nucleolus with especially large amount of associated DNA. In the chick embryonic and adult hepatocyte nuclei, the chromatin material stained by methyl-green is scattered in the nucleoplasm. The nucleolus shows a ring of green staining DNA surrounding a pyroninophilic RNA center.

FIG. 1. Embryonic mesencephalic neuroblasts of quail (on the right) and chick (on the left) at 7 days of incubation after the isotopic and isochronic graft of the right mesencephalic quail neural primordium in a 10-somite chick embryo host. Feulgen staining. × 1150.

FIG. 2. Adrenomedullary cell of a 12-day-old chick embryo. Glutaraldehyde osmium fixation. Uranyl acetate–lead citrate staining. × 7700.

FIG. 3. Adrenomedullary cell of a 12-day-old quail embryo. Same treatment as in Fig. 2. Large nucleolus

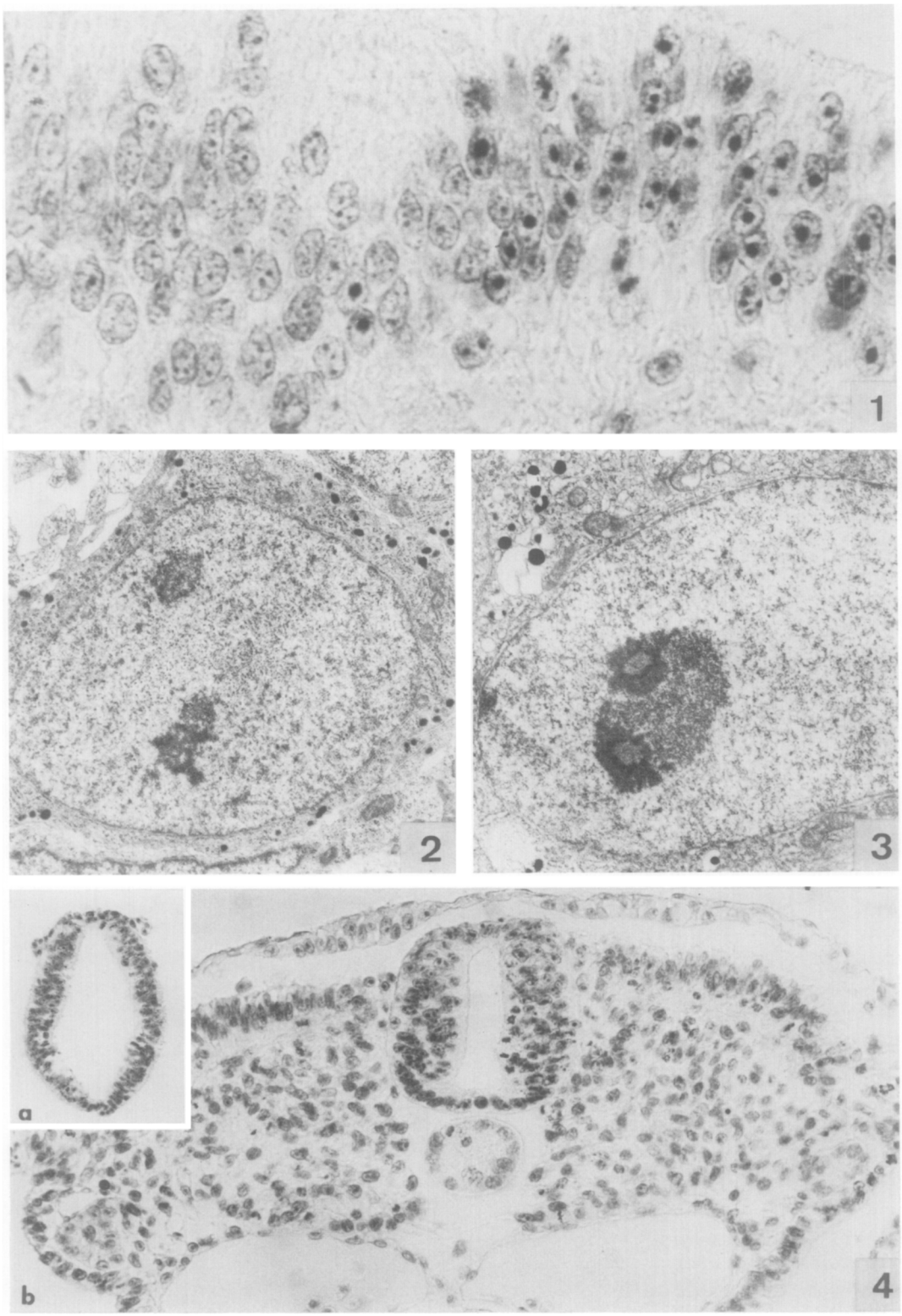

FIG. 4. Graft of a quail neural tube in a chick embryo. Feulgen and Rossenbeck staining. (a) Quail neural tube and associated neural crests before the graft. The neural anlage has been separated from the quail embryo by trypsinization and is completely devoid of mesenchymal cell contamination. × 230. (b) Transverse section of the chick embryo host 24 hr after the graft of the quail neural tube. The latter is normally incorporated in the host axial structures. Feulgen and Rossenbeck staining. × 300.

3. *Electron Microscopy*

Numerous embryonic and adult quail tissues have been observed in electron microscopy after the double glutaraldehyde-osmium fixation and uranyl acetate-lead citrate staining and compared to the homologous tissues of the chick. In all cell types of the quail the nucleus shows one or sometimes several large nucleoli. As it has been demonstrated elsewhere (Le Douarin, 1971) with the preferential RNA staining technique with EDTA (Bernhard, 1968) quail nucleolar RNA is associated with a large heterochromatic mass of DNA. The important amount of nucleolus-associated DNA is responsible for the large nucleolar size in this species. Therefore it is possible to distinguish quail and chick cells at the electron microscope level by the size of their nucleolus in many tissues. Such is the case for instance of adrenomedullary and calcitonin secreting cells (Le Douarin et Teillet, 1971a; Le Douarin, 1970). In the chick, these cells contain one or two small nucleoli the size of which varies from 0.5 to 1.9 μ, the most frequent size being 1 μ (Fig. 2). In the corresponding cells of the quail, the nucleolus is usually single and measures 2–4 μ when cut in its largest diameter (Fig. 3). Of course, this criterion can be applied only to sections of the cell containing the central nucleolar region, allowing species identification of only a certain percentage of cells in a tissue. This percentage can be estimated at about 50–60% of the cells. Moreover, the structure of the quail nucleolus as it appears at the electron microscope level is characteristic: beside the main central mass of DNA, some sections of the nucleolus show one, two, or sometimes three peripheral RNA containing structures with different electron densities (Fig. 3). This nucleolar organization is found in many other cell types of the quail, such as connective tissue cells, various endocrine cells, muscle cells, and distal kidney tubule epithelium. In certain kind of quail cells such as hepatocytes, young blastomeres, epithelium of small intestine and proximal kidney tubule cells, some structural variations are observed; in these nucleolar DNA substructures are more closely interwoven.

Our observations have shown that the arrangement of the nucleolar components and the size of the nucleolus are different in homologous cell types of the quail and chick. To be able to distinguish with certainty quail and chick cells at the electron microscope level, a careful comparative study of every cell type must be done in the two species. This study has to be done not only by the routine electron microscopic technique but also using the preferential staining procedure for RNA with EDTA (Bernhard, 1968) which evidences the pattern of DNA and RNA nucleolar containing structures (Le Douarin, 1970).

II. DISTINCTION BETWEEN CHICK AND QUAIL CELLS IN EXPERIMENTAL ASSOCIATION

Since the chromatin pattern is different in quail and chick interphase nuclei, it appeared that quail cells could be used as "natural markers" in the study of embryological problems. Whether associated *in vitro* or *in vivo*, the cells of the two species retain their characteristics during the process of differentiation as well as in fully differentiated organs, and can be easily recognized by applying the Feulgen-Rossenbeck's technique.

1. *Association of Chick and Quail Rudiments in Culture*

In vitro associations were mainly concerned with mesenchymal and epithelial primordia of certain endomesodermal organs such as liver and lung.

Heterospecific association of the endodermic and mesodermic components of the liver. Two combinations are possible: quail mesenchyme with chick endoderm or vice versa. The hepatic mesenchyme, isolated according to a method previously described (Le Douarin, 1964), is taken from

6-day-old embryos. The hepatic endoderm is dissociated by trypsinization from the floor of the foregut of 22- to 25-somite embryos and the two primordia are associated together on a semisolid culture medium for 3 or 4 days. As previously observed (Le Douarin, 1964) in homospecific association of mesodermal and endodermal liver rudiments of the chick, the endodermal cells multiply actively and invade the hepatic mesenchyme, giving rise to a small piece of liver tissue. In quail mesenchyme + chick endoderm associations, quail endothelial cells, form a network between the chick hepatocytes. In the reverse association, the hepatocytes originate from quail endoderm as shown by the two or three chromatin condensations in their nuclei while the endothelial cells are derived from chick mesenchyme.

Heterospecific association of endodermal and mesodermal components of the lung. The two components of the lung primordium of 5-day-old embryos are dissociated by trypsinization and reassociated heterospecifically on the culture medium. In some cases, the explants are grafted onto the chorioallantois of a chick or quail embryo in order to prolong their survival. The endoderm, undergoes its differentiating process in contact with the mesenchyme of the other species and the derivatives of both endoderm and mesoderm can be recognized by their nuclear characteristics in most cells of the tissue whatever the duration of the culture (Le Douarin, 1969).

2. *Association of Chick and Quail Tissues in Ovo*

The quail-chick association technique has been applied to the problem of neural crest cell migrations (Le Douarin et Le Lièvre, 1970; Le Douarin et Teillet, 1970, 1971a,b; Le Lièvre, 1971). It is well known that they migrate extensively in the developing organism, and give rise to a striking diversity of differentiated cells and tissues, some of which are not yet identified.

The experimental procedure devised for this study consists in removing part of the neural anlage of a chick embryo in a well defined region of the neural axis, the somites, notocord and endoderm not being involved in the operation. In a second step, the same fragment of the neural tube is isolated from a quail embryo and grafted orthotopically in the chick host. The quail neural tube is isolated by trypsinization of the adequate transverse section of the embryo, so that the grafted tissue is perfectly devoid of mesenchymal cell contamination (Fig. 4a). The grafted neural tube is soon incorporated into the axial structures of the host embryo some hours after the graft. It is rapidly covered by the chick ectoderm and undergoes normal histogenesis in the host (Fig. 4b). The quail neural crest cells migrate in the host embryo and can be distinguished from chick cells wherever they go to and whichever the phenotype they finally express thanks to their nuclear characteristics. Histological sections of the host embryo are made at various developmental stages until the end of the incubation period or after hatching. It is thus possible to follow the crest cells until they have reached a completely differentiated state. Despite the fact that the technique involves interspecies combinations no developmental abnormalities have been observed in the host embryos, when the graft has been properly done, in the experiments concerning the neural crest cells migration from the rhombencephalic and medullary neural anlage (Le Douarin et Teillet, 1970, 1971a,b; Teillet, 1971; Le Lièvre, 1971). In most experimental embryos the axial structures induced by the grafted neural tube are normal and the cutaneous cicatrisation makes rapidly the level of the intervention not apparent. We can underline in this regard that the developmental rates of the two species are rather close during the first half of the incubation period.

These observations point out structural

differences between quail and chick interphase nuclei. The heterochromatic DNA associated with the nucleolus of the quail is found in all embryonic and adult cellular types yet observed, and is stable. It is maintained in the organ culture conditions and in xenoplastic grafts of embryonic quail rudiments to the chick embryo. In the chick nucleus such a prominent mass of heterochromatic DNA does not exist. Due to this different arrangement of the chromatin material, it is possible to distinguish cells belonging to each species, which have been artificially associated *in vitro* or *in vivo*. Therefore quail cells are good "biological markers" for experimental embryology.

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DEVELOPMENTAL BIOLOGY 41, 162–184 (1974)

Experimental Analysis of the Migration and Differentiation of Neuroblasts of the Autonomic Nervous System and of Neurectodermal Mesenchymal Derivatives, using a Biological Cell Marking Technique

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Accepted July 23, 1974

By isotopic and isochronic transplantations of fragments of quail neural tube into chick, it has been previously shown that enteric ganglion cells arise from the "vagal" (somites 1–7) and the "lumbo-sacral" (behind somite 28) levels of the neural crest, while the trunk region (somites 8–28) gives rise to orthosympathetic ganglion chain and adrenomedullary cells. The latter originate precisely from the neural crest corresponding to somites 18–24 (i.e., "adrenomedullary" level of the crest). Heterotopic transplantations of fragments of quail neural tube into chick have been carried out in the present work. When the "adrenomedullary" level of the quail neural tube is grafted into the "vagal" region of a chick, the crest cells colonize the gut and differentiate into enteric ganglia of Auerbach's and Meissner's plexi. If quail cephalic neural crest is transplanted in the "adrenomedullary" level of a chick, quail cells migrate into the suprarenal glands and differentiate into adrenomedullary cells. Mesectodermal cells migrate laterally, and differentiate into cartilage, dermis and connective tissues. Thus it appears that preferential pathways located at precise levels of the embryo lead crest cells to their definitive sites. On the other hand the differentiation of the autonomic neuroblasts is controlled by the environment in which crest cells are localized at the end of their migration. On the contrary, mesenchymal derivatives of the cephalic neural crest appear to be early determined since they differentiate according to their presumptive fate when transplanted into the trunk.

INTRODUCTION

The neural crest is an interesting model for the study of cell differentiation since from this apparently homogeneous transitory structure a variety of physiologically and morphologically differentiated cell types are derived. These differentiations occur after an early phase of migration of undifferentiated cells from the neural anlage to a precise site of the developing body. Thus, in the evolution of the neural crest system, several problems about the processes which control morphogenesis, histogenesis and cell differentiation in embryonic development arise.

One of the striking properties of the neural crest is the extensive and well-defined migration that its cells achieve in the embryo. How are the initiation and the arrest of migration triggered and by what mechanisms is the migration of the cells oriented? It can be supposed that, at various embryonic levels, preferential pathways guide the cells towards their definitive locations. However, it can as well be imagined that migration begins as a random event and that crest cells are thereafter specifically attracted by certain tissues to which they are selectively sensitive. It is also important to know at what time crest cells are determined to differentiate into the various cell types that they are able to turn into. Indeed if crest cells are already determined when they leave the neural primordium, they might also have specific properties resulting in selective attraction by a precise embryonic tissue. On the contrary, if they remain pluripotent until they have reached their definitive

location, in order to explain the precise and constant pattern of distribution of crest derivatives, it must be admitted that they spread in the organism through preferential pathways. The problem is difficult to solve, due to the multiplicity of factors involved in the successive steps of neural crest evolution and to the probable heterogeneity of the initial crest cell population.

It has previously been demonstrated that, at least during the early stages, trunk crest cells migrate in two well defined streams: a dorsolateral one, closely associated with the ectoderm, and a ventral one (Rawles, 1947; Weston, 1963; Weston and Butler, 1966). In addition, various experiments suggest that the metameric distribution of sensory and sympathetic ganglia is imposed on the crest cells by the segmentation of somitic mesenchyme: crest cells migrate uniformly adjacent to the neural tube (Detwiler, 1937) but secondarily, the somitic environment enhances migration while the intersomitic environment limits it; metameric sensory ganglia would then condense from this discontinuous population of cells (Weston, 1963). On the other hand, the neural tube seems to play an important role in the initial orientation of the neural crest migration (Cohen and Hay, 1971), the collagenous basal lamina rich in acid mucopolysaccharide (Cohen and Hay, personal communication) which lines the neuroepithelium, seeming to be responsible for this effect. Even when the tube is experimentally turned upside-down, the migration proceeds tangentially to its surface (Weston, 1963; Weston and Butler, 1966) and a sort of "contact-guidance" by the substrate (Weiss, 1958) could perhaps be suggested to account for this phenomenon. On the other hand, it is striking that in all cases, even in experimentally produced abnormal conditions, the net direction of early crest cells migration is away from their source, whatever their relationships with the environment may be (Twitty and Niu, 1948 and 1964; Twitty, 1949; Hörstadius, 1950).

The most accurate techniques used up to now for following the various steps of cell migration are isotopic labelling of crest cells nuclei by tritiated thymidine (Weston, 1963 and 1970; Johnston, 1966; Chibon, 1966). Because of dilution of the label through intensive proliferation of embryonic cells, whatever the advantages of this technique (Weston, 1967 and 1970; Le Douarin, 1974), it could provide precise data only about early morphogenetic cell movements, so that its usefulness for the study of the whole migration process is limited.

The cell marking technique devised by one of us (Le Douarin, 1969, 1971, 1973) and based on structural differences of the interphase nucleus in two species of birds closely related taxonomically, the Japanese quail (*Coturnix coturnix japonica*) and the chick (*Gallus gallus*), has the advantage of being stable. Quail and chick cells, experimentally associated, definitively retain their species characteristics in the chimaera. By applying this technique to the problem of neural crest cell migration and differentiation it is possible to follow the migration process through the whole embryonic development, until cells have reached their definitive location and are fully differentiated. As it stands presently, our work contributes new data to the following points:

1. The mechanisms of migration and differentiation of the neuroblasts of the autonomic nervous system.

2. The determination and the differentiating capabilities of neural crest mesenchymal derivatives.

In a previous study, isotopic and isochronic grafts of quail neural primordium into the chick embryo were made; the precise level of the neural axis from which the enteric ganglia (Le Douarin and Teillet, 1971a, 1973) and the adrenomedullary cells arise (Le Douarin and Teillet, 1971b; Teillet and Le Douarin, 1974) were determined, points which were controversial (see Andrew, 1971; Le Douarin and Teillet,

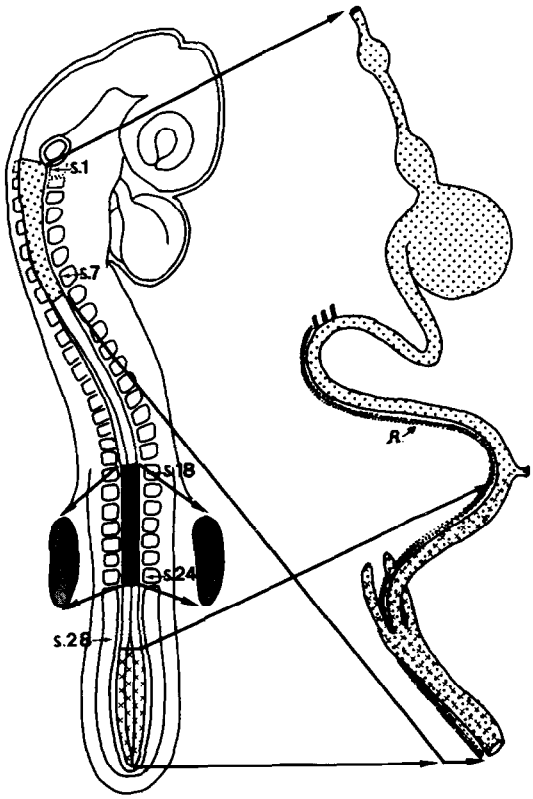


FIG. 1. Diagram showing the anterior and posterior levels of the embryonic neural axis from which the enteric ganglion cells originate as demonstrated by isotopic and isochronic transplantations of quail neural tube into chick embryo (Le Douarin and Teillet, 1973). The neuroblasts arising from the anterior level (between the levels of somites 1 and 7) colonize the whole gut. Those which come from the posterior level located behind somite 28 contribute only to the formation of the ganglia of the postumbilical gut. The neural crest of the cervical and dorsal region (from 8 to 28 somites) does not participate in the formation of enteric ganglia but gives rise to adrenergic orthosympathetic neurones and to adrenomedullary cells which come from the precise level of somites 18 to 24. R.: nerve of Remak originating from the lumbo-sacral level of the neural axis (behind somite 28) (Le Douarin and Teillet, 1971).

1973; Le Douarin, 1974 for bibliography). The enteric ganglia derive exclusively from two different parts of the neural axis: the "vagal" level (from somite 1–7) and the lumbo-sacral level (behind the level of somite 28) (Fig. 1), while the cervico-dorsal neural crest, corresponding to somites 8–28, do not participate in the histogenesis of the cholinergic enteric ganglia. The cervico-dorsal neural crest only contributes to the formation of orthosympathetic chains and plexuses and to the adrenal medulla, the cells of which derive from an area of the trunk neural crest located between somites 18–24 ("adrenomedullary" area of the neural crest).

It must be emphasized that, though dorsal crest cells which give rise to the adrenergic aortic plexuses, migrate ventrad very close to the dorsal mesentery, they are never attracted by splanchnic structures: in no case are quail cells found inside the digestive tract when a quail neural anlage is grafted orthotopically at the level of somites 8–28 (Le Douarin and Teillet, 1971a, 1973). However the problem arises whether these cells are able to provide enteric ganglia if they are transplanted at an early stage into the vagal region (level of somites 1–7). In this case, into which type of neurones, adrenergic or cholinergic, would the neuroblasts differentiate. To answer this question, the "adrenomedullary" area level of the crest was selected for transplantation. The developmental capabilities of the neural crest in the abnormal environmental conditions provided by the splanchnic mesoderm have also been investigated by *in vitro* and *in vivo* tissue culture methods.

Finally to find out whether a preferential pathway leads the neural crest cells towards the suprarenal glands in the dorsal region corresponding to somites 18–24, quail cephalic neural primordia have been transplanted into the "adrenomedullary" area of a chick embryo.

Moreover as several mesenchymal derivatives arise from the cephalic neural crest (Hammond and Yntema, 1964; Johnston, 1966; Le Lievre, 1971a and b, 1974; Le Lievre and Le Douarin, 1973 and 1974), and whereas the trunk crest is completely devoid of this developmental capability in higher vertebrates, it was interesting to see

whether cephalic mesectodermal crest cells were able to migrate and differentiate after being transplanted into the trunk region.

MATERIAL AND METHODS

White Leghorn and Japanese quail eggs incubated at 38° ± 1°C were used throughout this investigation. The stages of development of the embryos were determined according to the commonly employed criteria of Hamburger and Hamilton (1951). At the early development period the stages have been referred to the number of somites.

A. *Surgical Procedures*

Heterotopic and heterochronic transplantations of fragments of the quail neural primordium into chick embryos are carried out as outlined in Fig. 2.

In the first step, part of the neural tube and associated neural folds are excised from a chick embryo. In the second, an equal length of a quail embryo neural rudiment is isolated by trypsinization (0.1% trypsin DIFCO in Ca^{2+}, Mg^{2+} free Tyrode solution (Moscona and Moscona, 1952)) and grafted into the chick in the groove previously made by removal of the neural tube. This procedure makes it possible to obtain nervous rudiments perfectly devoid of mesenchymal cell contamination as revealed by histological examination (Le Douarin, 1973).

Two kinds of experiments are performed:

1. The graft of the "adrenomedullary"

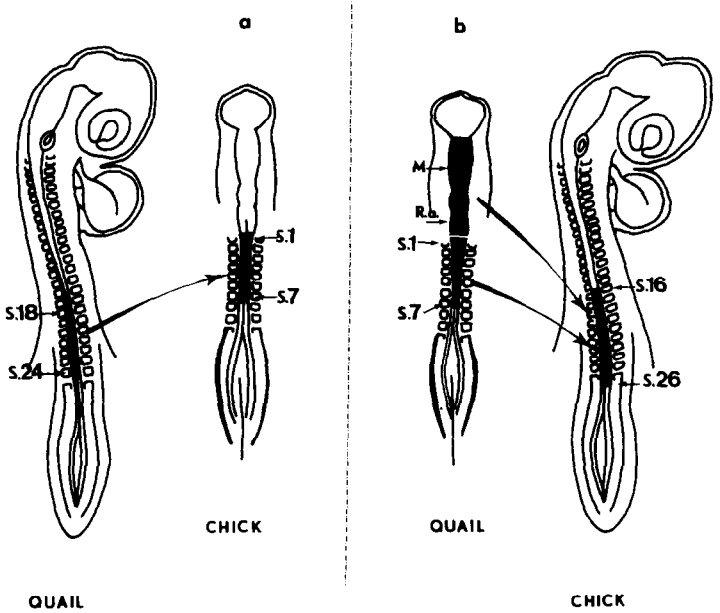


FIG. 2. Heterotopic and heterochronic transplantations of quail neural fragments into chick embryo. (a) Transplantation of the neural tube and neural crest of a 24-somite quail into a 9-somite chick. The neural primordium of the chick is removed at the level of somites 1–7 corresponding to the "vagal" region of the neural crest from which enteric ganglia originate. The neural region of the quail corresponding to the level of somites 18–24 is isolated by trypsinization and grafted in the vagal area of the chick. The so called "adrenomedullary" region of the neural crest corresponding to somites 18–24 gives rise in normal development to orthosympathetic neurones and adrenomedulla, but does not participate in enteric ganglion formation (Experiment 1). (b) Graft of mesencephalon (M) + anterior rhombencephalon (R.a.) of quail into the "adrenomedullary" region of the chick neural axis. In this case, the donor embryo is at the 4- to 9-somite stage (upper arrow). A graft of the "vagal" neural primordium of a 7- to 12-somite quail into the "adrenomedullary" region of a chick is also indicated (lower arrow) (Experiment 3).

region (somites 18–24) of 24- to 26-somite quail embryos into the "vagal" region (somites 1–7) of 7- to 12-somite chick embryos (Fig. 2a).

2. The graft of the cephalic neural primordium of 4- to 12-somite quail embryos into the "adrenomedullary" region (somites 18–24) of 24- to 26-somite chicks. In this case, the graft consisted either of mesencephalon and anterior rhombencephalon (down to the level of somite 1) of 4- to 9-somite quail embryos, or of the "vagal" region (somites 1–7) from 7- to 12-somites embryos (Fig. 2b). In this experimental series, the length of the grafted neural tube is larger than the "adrenomedullary" area, and the excision of the host neural tube is performed from somite 16 to 26.

Donor and host embryos must necessarily be at different developmental stages, in order that crest cells have not begun migrating at the time of the operation in either the host or the graft at the concerned level. It is well known that neural crest cell migration begins in the head and successively progresses towards the tail shortly after closure of the neural tube. Isotopic and isochronic transplantations of neural tube give rise to normally developed axial structures. In contrast, heterotopic transplantations of cephalic regions into the trunk, disturb embryonic morphogenesis at the level of the graft because of discrepancies in growth and morphogenetic capacities of trunk and cephalic neural anlage. After transplantation of the "vagal" region of a quail into the "adrenomedullary" area, the abnormalities of the axial structure are less important. In some cases skin heals completely and the external gross aspect of the host is normal.

A transverse strip of quail-like pigmented feathers is observed in operated embryos after isotopic transplantations, as previously described (Teillet and Le Douarin, 1970; Teillet, 1971).

The embryos are sacrificed from the 7th to the 21st days of incubation. The suprarenal glands and the different levels of the digestive tract (Fig. 3) from the embryos sacrificed at the 11th day onwards are fixed in various ways according to the experimental series. Prior to this stage the whole embryo is cut in serial transverse sections.

B. *In Vitro and in Vivo Culture Experiments*

These experiments have been carried out to study the developmental capabilities of the trunk neural crest cells in the environment of the wall of the digestive tube.

Fragments of neural primordium from the 13–25 somite levels of 13- to 26-somite quail embryos have been associated with the rectum taken from 5-day old chick embryos in organotypic culture according

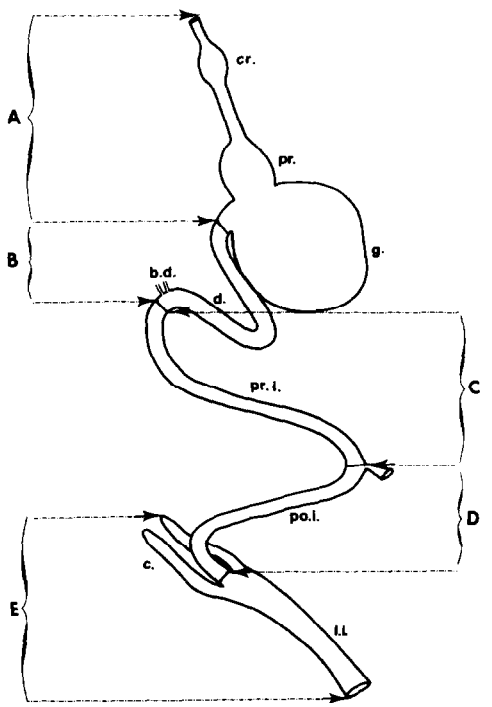


FIG. 3. Diagram showing the different parts of the gut observed. (A) cr., Crop; pr., proventriculus; g., gizzard. (B) d., Duodenum; b.d., biliary ducts. (C) pr.i., Preumbilical ileum. (D) po. i., postumbilical ileum. (E) c., caeca; l.i., large intestine.

to the technique of Wolff and Haffen (1952).

Previous results (Le Douarin and Teillet, 1973) have shown that enteric ganglion cells coming from both the vagal and lumbosacral levels do not appear in the rectum before the 7th day of incubation. For this reason, it seemed especially convenient to choose this part of the digestive tract from 5-day old chick embryos for this experiment. The quail neural primordium was inserted into a longitudinal slit made in the wall of the gut (Fig. 4), in order to ensure a close contact between the neural crest and the splanchnic mesoderm. The *in vitro* culture lasts 12 hr; the whole explant is then transplanted on the chorioallantoic membrane (CAM) of a 6-day old chick for 3 to 6 days. Thereafter the grafted tissue is fixed for histochemical detection of catecholamines (formol vapor induced fluorescence (FIF) technique) according to Falck (1962) or silver staining procedure according to Tinel (1947).

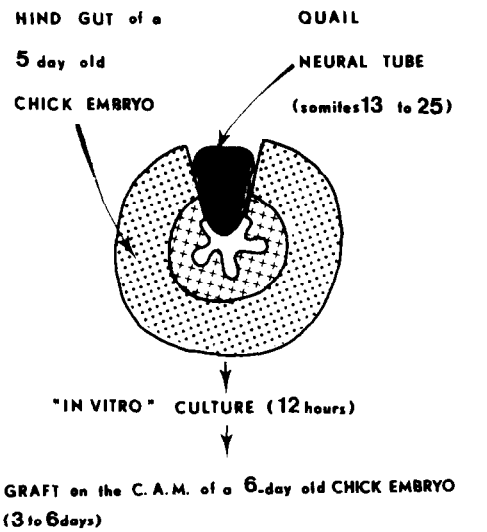


FIG. 4. Association of hind gut mesoderm from 5-day chick embryo with fragments of trunk neural primordium of 13- to 26-somite quail embryos. The neural tube and crest is inserted in a slit made in the gut wall. The two tissues are cultivated *in vitro* 12 hr, become closely associated, and then are grafted to the CAM for 3–6 days (Experiment 2).

C. *Histological and Histochemical Techniques*

Three kinds of histochemical techniques have been applied to the tissues:

1. *Specific staining procedure for DNA according to Feulgen–Rossenbeck* (1924) which makes it possible to distinguish quail and chick cells by the structure of the interphase nucleus (Le Douarin, 1969, 1973): quail nuclei of all cell types show one or several large heterochromatic nucleolar condensations. In the chick, as in most other animal species, the chromatin is evenly dispersed in a fine network through the nucleoplasm. The different disposition of the chromatin makes it easy to distinguish experimentally associated quail and chick cells (Fig. 5).

The Feulgen–Rossenbeck reaction can be applied to tissues after fixation by Zenker's fluid and embedding in wax. In some experimental series, the tissues are treated by the FIF procedure and embedded in epon 812. The sections made with a Pyramitome LKB type, are first observed in UV light and postfixed overnight in Zenker's fluid, and then stained by the Feulgen–Rossenbeck technique. By this procedure it is possible to study precisely correlations between fluorescence and quail cells. Prior to postfixation, the embedding resin is removed by treatment with sodium methoxyde, sequential rinsing in benzene–ethanol and acetone, and washing in water for a few minutes, according to Mayor, Hampton, and Rosario (1961).

2. *Silver impregnation technique according to Tinel (1947)* for detection of parasympathetic or sympathetic neurones. The tissues are fixed in Bouin's liquid or prepared according to the FIF technique. When silver impregnations are to be carried out, serial sections of tissue are alternately placed on two different slides. One is stained by Tinel's method, the other one is postfixed in Zenker's fluid, and stained

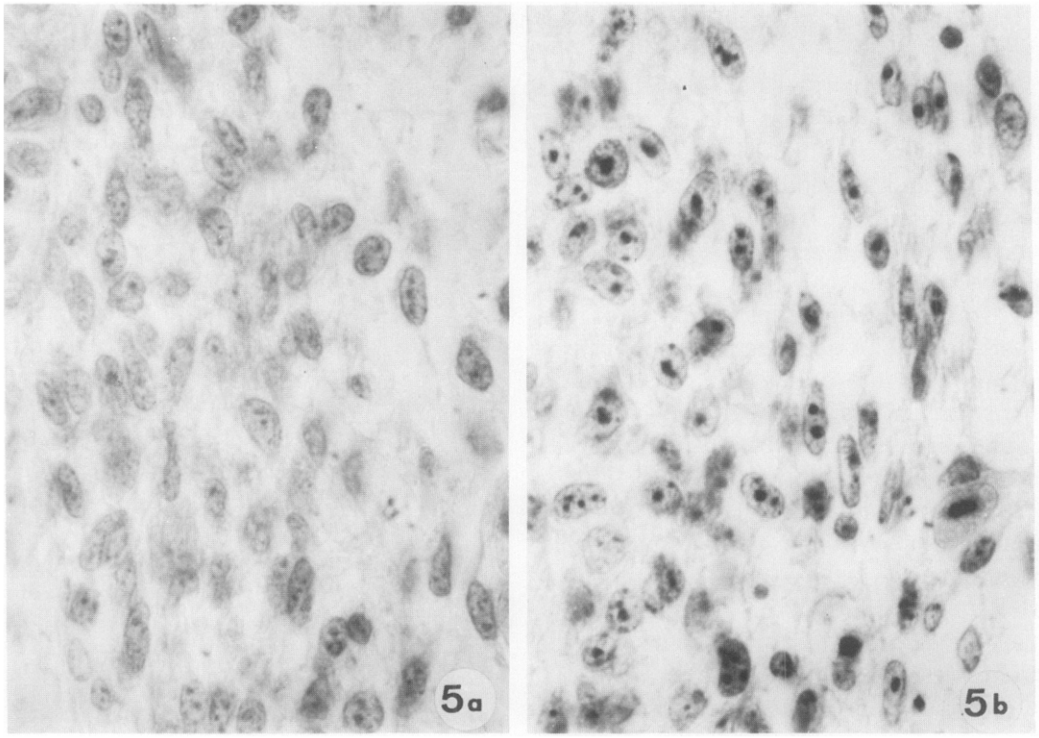


FIG. 5. Chick (a) and quail (b) cells stained by Feulgen-Rossenbeck reaction showing in the quail one or two large clumps of nucleolar heterochromatin, and small dispersed chromocenters in the chick. Dermis of 10-day old embryos. × 720.

by the Feulgen-Rossenbeck technique. This procedure makes it possible to learn two different facts about a group of cells: whether it is ganglionic and whether it is of quail or chick origin.

3. *The FIF procedure (Falck, 1962)* to detect fluorogenic amine content in ganglionic or paraganglionic neural crest derivatives (adrenomedullary cells) has been used. In certain series, the ability of adrenergic neurones to take up L-Dopa and decarboxylate it into dopamine has been taken advantage of in order to enhance the fluorescence of differentiating cells. One hundred miligrams per kilogram of L-Dopa (L-3,4-dihydroxyphenylalanine; crystalline, Sigma Chemical Company) is injected 1 hr prior to fixation in a chorio-allantoic vessel of 14- to 19-day-old chick host embryos.

The FIF treatment is applied as follows:

The blocks are frozen-dried in a thermoelectric tissue dryer at −40°C for 18 hr. They are then exposed to formaldehyde vapor at 80°C for 2 hr and directly embedded *in vacuo* in Epon-Araldite. In each case, part of the frozen-dried material is embedded without formaldehyde vapor treatment. Those samples form the control series. Serial sections from all blocks are cut at 5 μm, placed in a drop of distilled water on glass slides, attached through rapid water evaporation, and observed directly without a coverslip. All sections are examined by fluorescence microscopy using a Leitz Orthoplan microscope fitted with an HBO 200W mercury arc lamp. Filters used are BG 12/5mm, BG 12/3mm, BG 12/1.5mm for excitation with a K 510 barrier filter. Photomicrographs were taken on TRI-X, Pan film or Rayoscope film.

4. *Electron microscopy*. Suprarenal glands of control and grafted embryos are

fixed in 6% glutaraldehyde in 0.1 *M* phosphate buffer, at pH 7.4 for 20 min at 4°C, and postfixed in 1% osmium tetroxide in phosphate buffer for 1 hr. Blocks are embedded in Epon; sections, stained by lead citrate and uranyl acetate, are observed in an Hitachi HS8—electron microscope.

RESULTS

1. EXPERIMENT 1—GRAFT OF THE "ADRENOMEDULLARY" NEURAL CREST OF THE QUAIL INTO THE "VAGAL" REGION OF THE CHICK EMBRYO (FIG. 2a)

The digestive tract of the chick embryo hosts ranging from 7 to 18 days of incubation is examined on Feulgen-Rossenbeck stained serial sections (Table 1). In 13 of 16 cases, the ganglia of Auerbach's and Meissner's plexuses of the anteumbilical digestive tract are partially or completely made up of quail cells; these plexi are normally developed, as shown by silver impregnation (Fig. 6).

Caudad to the umbilicus, some quail cells are found in the wall of the gut but they are exclusively pigment cells and never participate in ganglion formation. Such melanocytes do not appear in the gut following isotopic and isochronic transplantation of quail vagal primordium into the chick, nor in the gut of the normal quail embryo. These pigment cells are always found in two regions: one lining the inner surface of the circular muscle layer, that is in the *submucosa* where Meissner's plexus cells are also located and the other in contact with the outer surface of the circular muscle layer (Fig. 7). It must be emphasized that after the isotopic and isochronic graft of a quail neural tube into chick, at the level of somites 1–7, quail cells were found to participate in the formation of the enteric ganglia in the whole postumbilical gut (Fig. 1). Thus it seems that the migrating capabilities of the "vagal" neuroblasts inside the developing gut environment are more extensive than those of dorsal neuroblasts transplanted into the vagal region.

TABLE 1

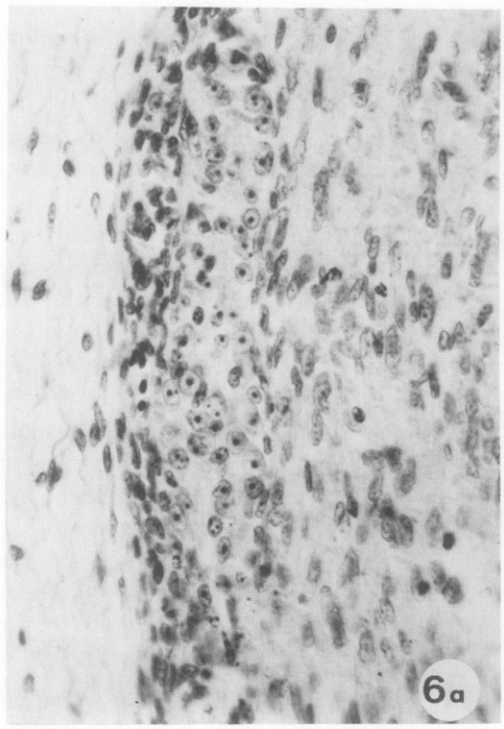
GRAFT OF THE "ADRENOMEDULLARY" NEURAL CREST OF A QUAIL EMBRYO INTO THE "VAGAL" REGION OF A CHICK

Age of the embryos at the time of sacrifice	Presence of quail ganglion cells in Auerbach's and Meissner's plexuses in the different parts of the digestive tract of the host				
	A[a]	B	C	D	E
7–9 days	5/7	5/7	4/7	0/7	0/7
13–18 days	8/9	8/9	4/9	0/9	0/9

[a] A, B, C, D, E correspond to the different parts of the digestive tract as represented in Fig. 3.

It appears from this experiment that presumptive orthosympathetic neuroblasts which, in normal development, do not penetrate into the splanchnic mesoderm, are able to invade the gut and to form enteric ganglia when transplanted at the hind-brain level. Thus it must be recognized that, in this precise region, a preferential migration pathway conveys crest cells towards the developing gut. The question arises at this point whether, in the splanchnic mesodermal environment, dorsal neuroblasts from the 18- to 24-somite level will differentiate according to their presumptive fate into adrenergic neurones, or into cholinergic ones as in normal enteric ganglia.

To answer this point, the FIF-Feulgen-Rossenbeck associated techniques were applied to the guts of three experimental embryos of 14–19 days of incubation. In no case have quail cells forming the Auerbach's and Meissner's plexi shown the faintest fluorescence even after an intravascular injection of L-Dopa. Thus neuroblasts originating from the dorsal neural crest, which have colonized the gut in this experimental situation do not differentiate into adrenergic neurones, as their origin would imply. On the other hand, as in normal guts, fluorescent adrenergic fibers are found in the plexus and also are dispersed epithelial cells showing the charac-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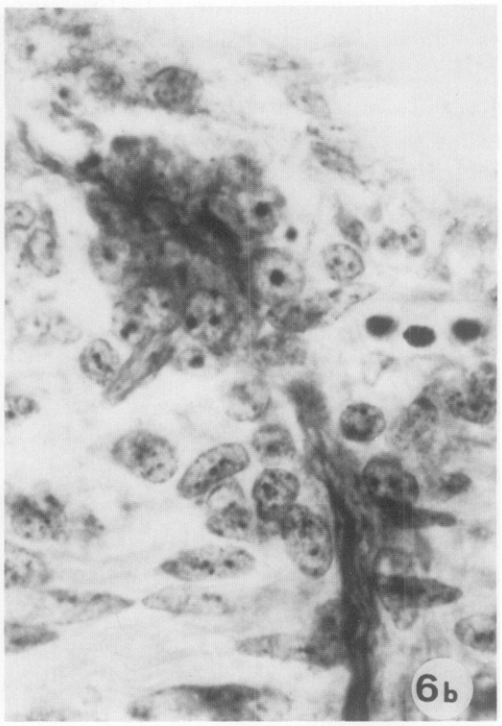
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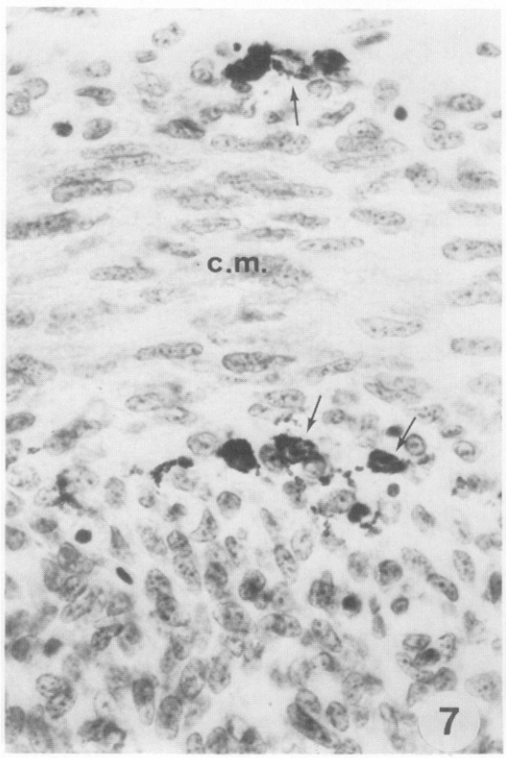
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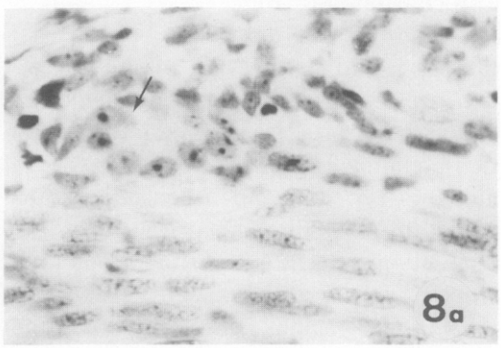
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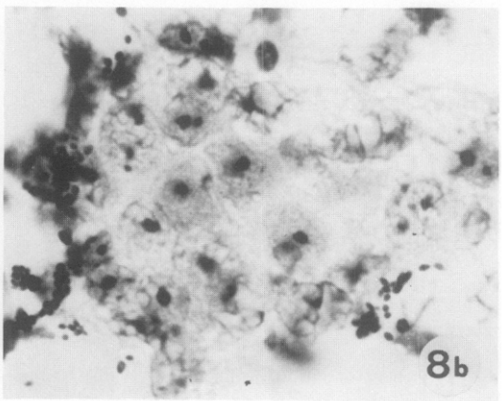
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teristic yellowish fluorescence of enterochromaffin cells.

2. EXPERIMENT 2—DIFFERENTIATING CAPABILITIES OF DORSAL NEURAL CREST CELLS IN THE HIND-GUT MESODERM

Fragments of the quail neural tube 6-somites long are taken at the level of somites 13–25. The stage of donor embryos varies according to the level chosen which always involves the most caudal segmented region. The neural rudiments are associated with 5-day-old chick rectum for 12 hr *in vitro* and then cultured on the CAM for 3–6 days (Fig. 4).

The evolution of the hind-gut in the explants is similar to that of control embryos at the corresponding developmental stages. Enteric ganglia entirely made up of quail cells are seen in Auerbach's and Meissner's plexi (Fig. 8). Tinel's staining procedure has shown that in these, quail ganglion cells have differentiated into neurones. The disposition of the ganglia is often irregular, some areas of the gut being completely devoid of quail cell aggregates. On the other hand, it must be pointed out that the ganglia of each plexus are less numerous and smaller than in either quail and chick control embryos or in chick embryos grafted orthotopically or heterotopically with a quail neural tube in the "vagal" region (Le Douarin and Teillet, 1973).

In this experimental series, the intestine that developed in the CAM in association with a dorsal quail neural tube contained numerous pigment cells distributed in two layers on each side of the circular muscle layer as in the experiment previously described (Fig. 9). However, in this series, the pigment cells are much more numerous than in the experiment described in paragraph 1. The neural tube associated with the intestine undergoes considerable growth during the time of cultivation and gives rise to a large mass of nervous tissue.

The FIF technique has been applied to explants after 6.5 days of cultivation (12 hr *in vitro* + 6 days in CAM), the total age of the intestine being 11.5 days. No fluorescent cells have been found either in the enteric ganglia or in any fiber of the explant. The enterochromaffin cells are not detectable at this stage in normal development. This observation confirms the results of the previous experiment, showing that the ganglion cells originating from the dorsal neural crest do not differentiate into adrenergic neurones when they are in the mesoderm of the gut wall.

Secondly, it appears from these results that orthosympathetic ganglia, which normally arise from the dorsal neural crest, do not differentiate under these experimental conditions, for no fluorescent cells can be seen in the explants. The presumptive melanoblasts, on the contrary, find in the

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FIG. 6. (a) Transverse section of the gizzard of a 15-day chick embryo that received a graft of the adrenomedullary area of a quail at the 8-somite stage (Experiment 1). Ganglion of Auerbach's plexus entirely made up of quail cells. × 540. (b) Same embryo. Section at the level of the duodenum. Silver impregnation technique according to TINEL showing a differentiated ganglion made up of quail cells in Auerbach's plexus. × 720.

FIG. 7. Same embryo as in Fig. 6. Behind the level of the umbilicus, no quail cells can be found in enteric ganglia, but quail pigment cells (arrows) have migrated down to the hind gut (here in the caecum) and are localized along the inner and outer surface of the circular muscle layer (c.m.) Feulgen–Rossenbeck staining. × 720.

FIG. 8. Transverse section of a chick hind gut cultivated 6.5 days (12 hr *in vitro* + 6 days on the CAM) in contact with the "adrenomedullary" (somites 18–24) part of a 25-somite quail embryo (Experiment 2). (a) Auerbach's plexus ganglion made up of quail cells. (arrow) × 720. (b) Auerbach's plexus ganglion made up of quail cells surrounded by quail pigment cells which have never been observed in normal quail gut nor after isotopic transplantation of quail neural primordia into chick. × 1350. Feulgen–Rossenbeck staining.

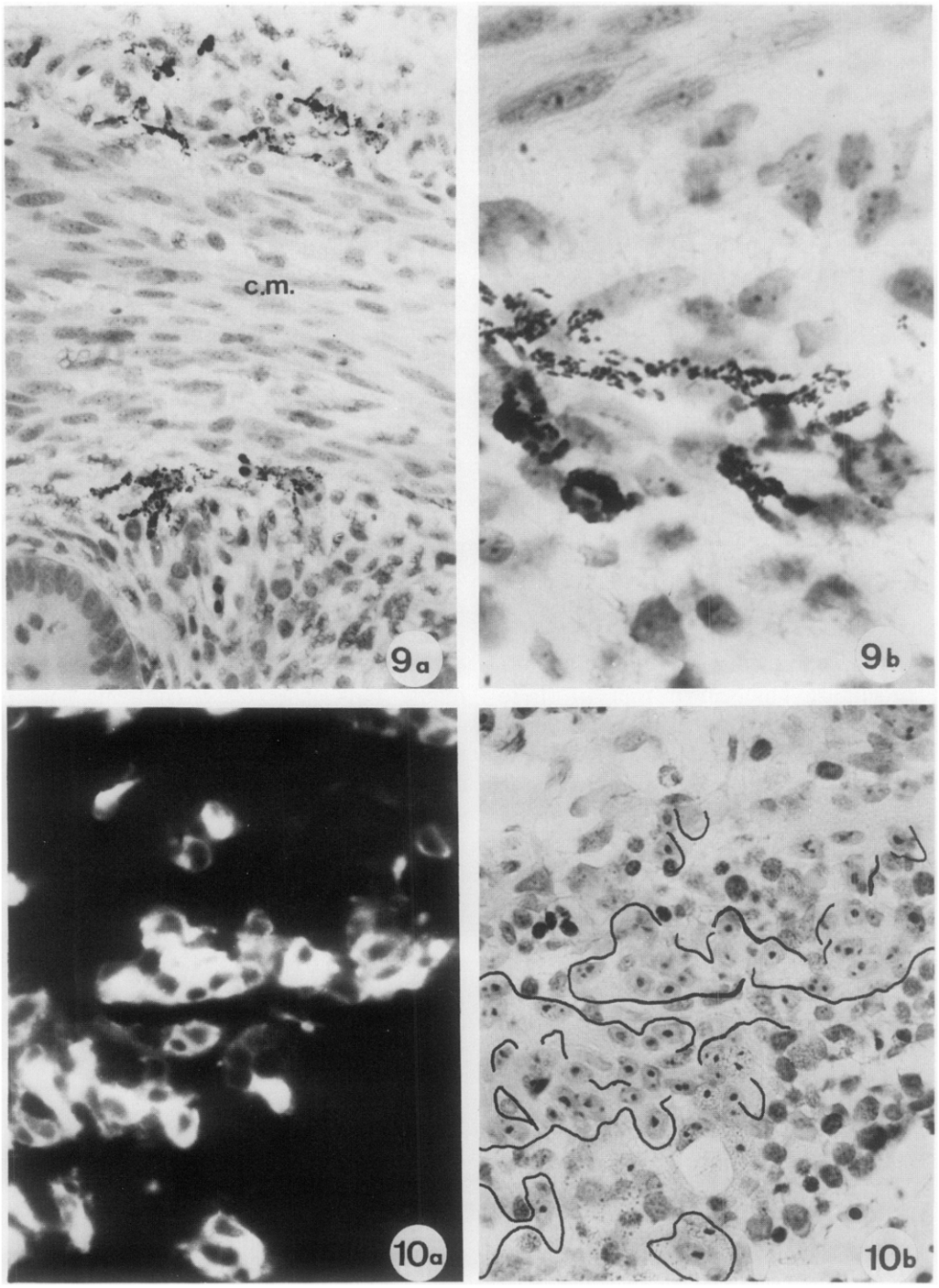


FIG. 9. Same experiment as in Fig. 8 showing the migration of melanocytes along the inner and outer surfaces of the circular muscle layer (c.m.) of the *muscularis*. (a) General view of the gut wall. × 540. (b) Detail of pigment cells in which the quail characteristic nucleus can be seen. × 1350. Feulgen–Rossenbeck staining.

FIG. 10. Suprarenal gland of a 10-day chick embryo which received at 25-somite stage the graft of the posterior rhombencephalon of a 7-somite quail in the "adrenomedullary" region (Experiment 3). (a) FIF technique according to Falck. Many cells show the characteristic greenish fluorescence of catecholamines. × 720. (b) The same section is stained by Feulgen-Rossenbeck's technique: Fluorescent cells belong to the graft. × 720.

gut good conditions to migrate, proliferate and differentiate. Their location in the wall of the gut is the same as that of the enteric ganglion cells. This fact suggests that each side of the circular layer of the *muscularis* offers an especially appropriate substrate for crest cell migration.

3. EXPERIMENT 3—GRAFT OF QUAIL MESENCEPHALIC AND RHOMBENCEPHALIC PRIMORDIA INTO THE "ADRENOMEDULLARY" REGION OF THE CHICK EMBRYO

In this series the embryos were sacrificed from 7 to 21 days of incubation. Until the 11th day, the whole trunk of the chick host was fixed and observed either after Feulgen-Rossenbeck's staining, or after treatment by FIF-Feulgen-Rossenbeck associated techniques. When the operated embryos are sacrificed after the 11th day, suprarenal glands and, in some cases, small intestine are fixed for histological observation.

A. *Observation of the Suprarenal Glands of the Host Embryos*

The results summarized in Table 2 show that in 24/27 cases, numerous quail cells were present in the host glands where they formed a network closely intermingled with chick cells. In the best cases, the aspect of the glandular tissue after Feulgen-Rossenbeck's staining is similar to what has been described in the experiments in which isotopic and isochronic transplantations of quail neural primordium corresponding to the adrenomedullary area (somites 18-24) have been carried out (Le Douarin and Teillet, 1971b; Teillet and Le Douarin, 1974). Association of the FIF and Feulgen-Rossenbeck techniques shows that most fluorescent cells in the host glands belong to the quail species (Fig. 10). However, in this experimental series, the suprarenal glands contained an abnormally large number of nerve fibers lined by quail Schwann cells and also some neurones with quail nuclear characteristics.

The electron microscopic examination of such glands has shown many quail glandular cells with the characteristic secretory granules of the adrenomedulla (Fig. 11). The quail secretory cells of these glands are often found in very close association with nerve fibers (Fig. 12).

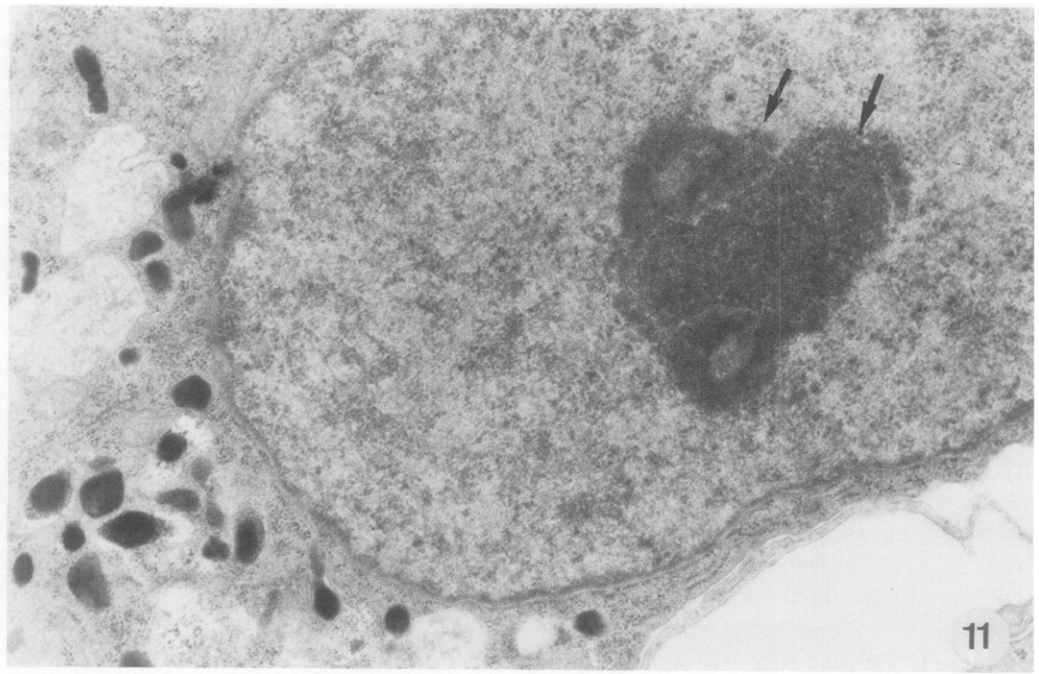
TABLE 2

PRESENCE OF QUAIL CELLS IN THE SUPRARENAL GLAND OF THE HOST AFTER THE GRAFT OF THE CEPHALIC NEURAL CREST OF QUAIL EMBRYO INTO THE "ADRENOMEDULLARY" REGION (LEVEL 18-24 SOMITES) OF THE CHICKEN NEURAL AXIS

Origin of quail neural primordium	Stage of quail donor embryo	Number of cases observed	Presence of quail cells in the suprarenal glands
Mesencephalon + anterior rhombencephalon	4- to 7-somite stage	11	10
"Vagal" neural primordium from somites 1 to 7	7- to 12-somite stage	16	14

B. *Observation of the Intestine of the Host Embryos*

In 10 cases we have fixed the fragments B, C, D, and E (Fig. 3) of the gut of host embryos that had received a graft of a quail neural primordium corresponding to somites 1-7. The stage of the embryos at the time of sacrifice was from 11 to 21 days. In all the cases observed quail cells participate in the formation of enteric ganglia at the levels of ileum and large intestine (fragments C, D, E) (Fig. 13). No quail cells have been found in the duodenum (fragments B). The most important contribution of the grafted quail cells to gut innervation is found in the postumbilical small intestine. While most of the ganglia are mixed (quail and chick) (Fig. 13), some of them are entirely made up of quail cells. No pigment cells have been found in any part of the intestine of the host. This result shows that certain vagal neuroblasts are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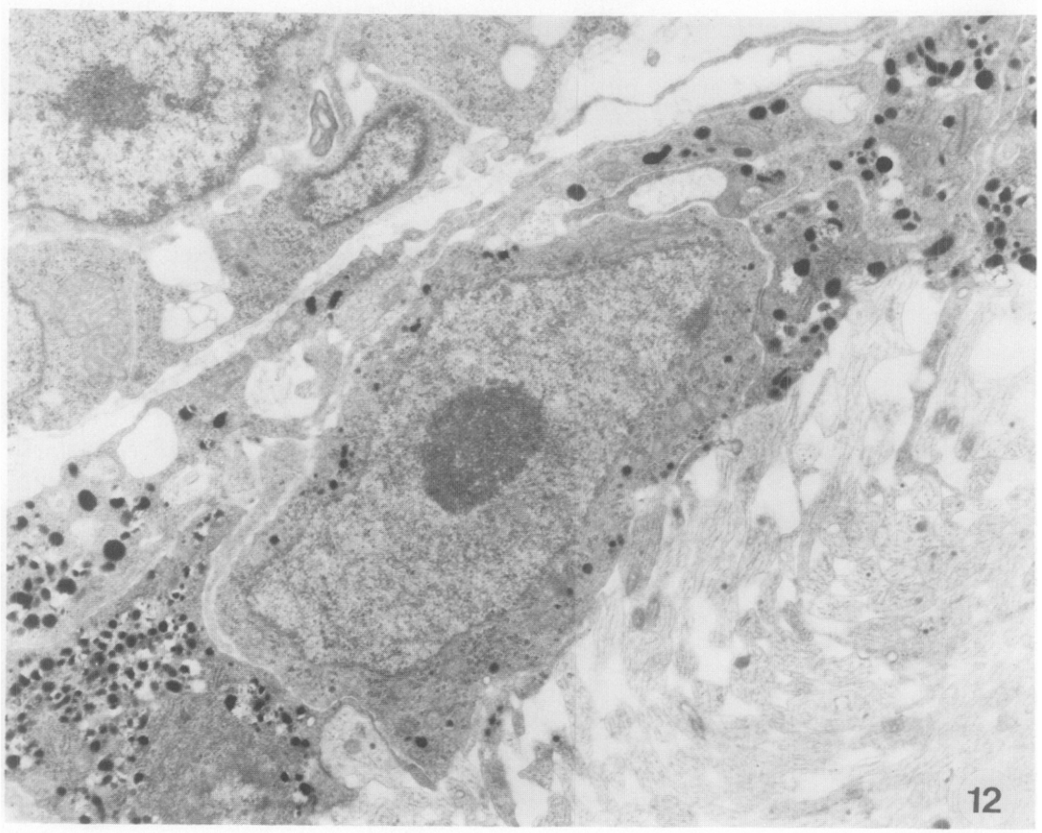
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specifically attracted by the intestine. Since in normal development, trunk crest cells never penetrate into the splanchnic mesoderm, at this level no preferential route leads the cells to the intestine. Thus a selective attractivity of vagal crest cells by intestinal structures is the only possible explanation which could account for the observed facts.

C. *Differentiation of Mesenchymal Derivatives in Heterotopic Grafts of Cephalic Quail Neural Crest into the Dorsal Region of Chick Embryos*

When the mesencephalic and anterior rhombencephalic quail neural primordia from 4 to 9 somite embryos were transplanted into the adrenomedullary region of chick embryos, mesenchymal cells originating from the graft were found in the host embryo in various locations. The cells of the implanted nervous rudiment proliferate actively and give rise to a large mass of brain tissue protruding above the skin surface. At the level of the graft, induction of cartilage in the host sclerotome occurred but the vertebral morphology was abnormal, especially on the dorsal side, because of the protrusion of the brain tissue (Fig. 14).

The mesenchymal derivatives of the grafted neurectoderm are dermis, cartilage and connective cells (Table 3).

Dermis. At the transverse level of the graft, some limited areas of the dermis are made up of quail cells (Fig. 15). They are located on the dorsal and dorsolateral sides of the operated embryo and are never found in the ventral skin. Chimeric feather buds, the ectoderm of which belongs to chick while the mesenchyme derives from the quail neural tube, undergo normal development at least up to 10th day of incubation, i.e., the oldest stage observed.

Cartilage. Differentiation of cartilage originating from the grafted neurectoderm occurs in various location in the host embryo. Nodules or elongated pieces of cartilage made up of quail cells have been encountered in four cases in the intermediate cell mass derivatives, i.e., inside the mesonephritic tissue (Fig. 16), in the vicinity of the kidney and of the Müllerian duct. In two cases, quail cells have been found to participate in the formation of vertebral cartilage though only in rather restricted areas (Fig. 17).

Connective cells. Quail cells are often encountered in the mesonephros, where they form the connective intertubular elements (Fig. 16), and in the wall of the Müllerian duct (Fig. 18).

DISCUSSION

The present investigation deals with the following aspects of the mecanisms controlling the migration and differentiation of neural crest cells: 1) the existence and the role of preferential migration pathways; 2) the determination of the neuroblasts of the autonomic nervous system and the influence of environmental factors on their differentiation; 3) the migration and differentiation capabilities of cephalic mesenchymal derivatives when they are submitted to the abnormal environmental conditions of the trunk region.

1. EVIDENCE, NATURE AND ROLE OF PREFERENTIAL MIGRATION PATHWAYS OF NEURAL CREST CELLS

Trunk neural crest cells transplanted at the vagal level give rise to enteric ganglia

FIG. 11. Quail adrenomedullary cell in the chick host suprarenal gland of a 13-day old chick embryo into which the posterior rhombencephalon of a 9-somite quail embryo has been grafted at the "adrenomedullary" level. Large DNA-rich nucleolus of quail type (arrows) and characteristic catecholamine secretory granules. × 23,000.

FIG. 12. Same experiment as in Fig. 11. A large number of nerve fibers in the vicinity of a quail adrenomedullary cell. × 11,000.

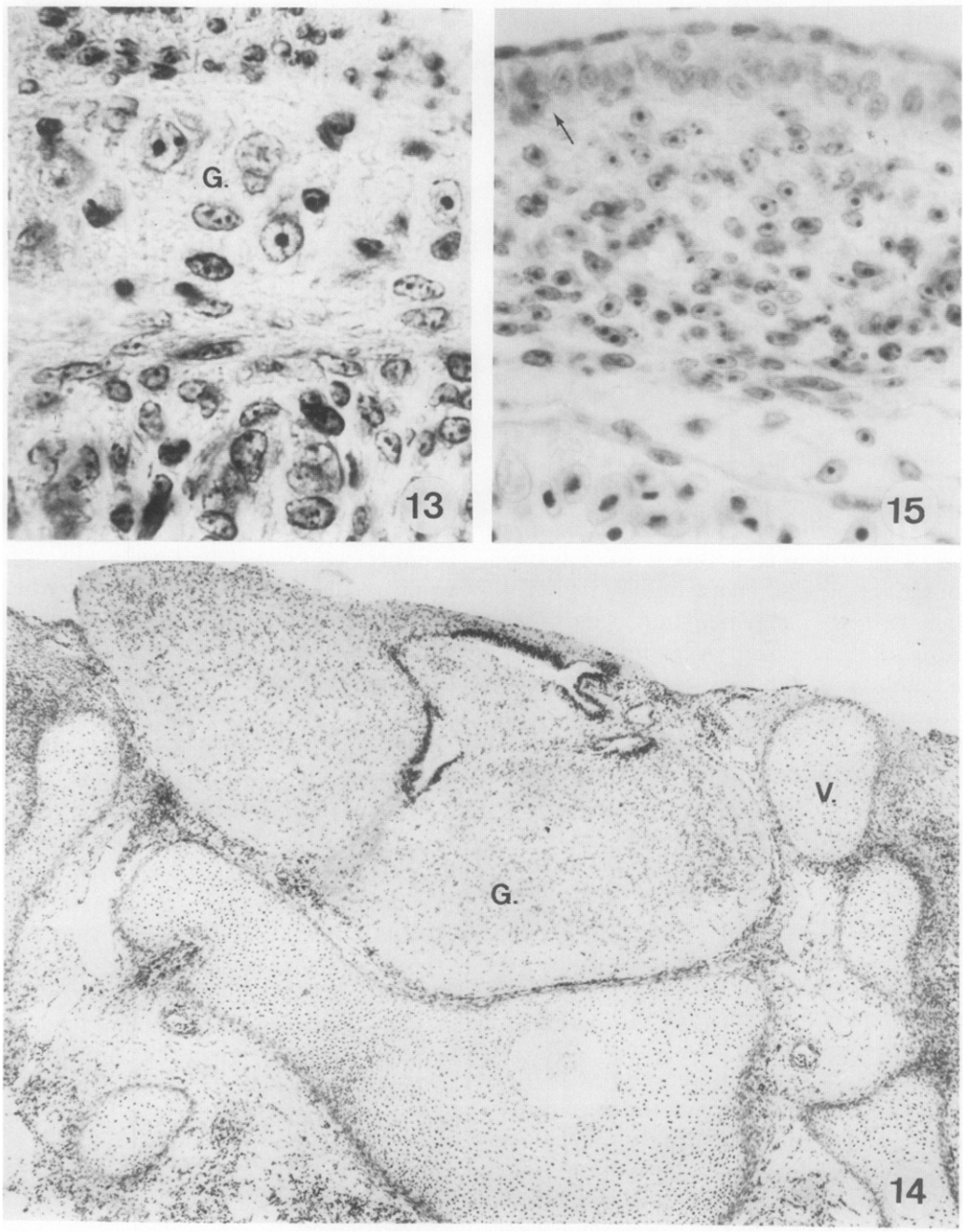


FIG. 13. Transverse section of the gut of a 21-day chick embryo that has received at 24-somite stage the graft of the "vagal" neural anlage from a 7-somite quail embryo at the "adrenomedullary" level. Enteric ganglion of Meissner's plexus (G.) made up of a mixture of quail and chick cells. Feulgen-Rossenbeck. × 1,300.

FIG. 14. Transverse section of a 10-day old chick embryo that has received at 25-somite stage a graft of mesencephalon and anterior rhombencephalon from a 6-somite quail embryo at the "adrenomedullary" level of the neural axis (Experiment 3). The chick host sclerotome has been induced by the grafted nervous tissue (G.) to differentiate into cartilage, but the morphological development of the vertebra (V.) is abnormal. Feulgen-Rossenbeck staining. × 100.

FIG. 15. Same experiment as in Fig. 14. Dorsolateral skin area of the host embryo showing that the dermis is made up of quail cells. Some quail cells—presumably melanoblasts—have penetrated into the chick epidermis (arrow). Feulgen-Rossenbeck staining. × 720.

TABLE 3

MESENCHYMAL DERIVATIVES DERIVING FROM CEPHALIC NEURAL CREST TRANSPLANTED INTO THE TRUNK

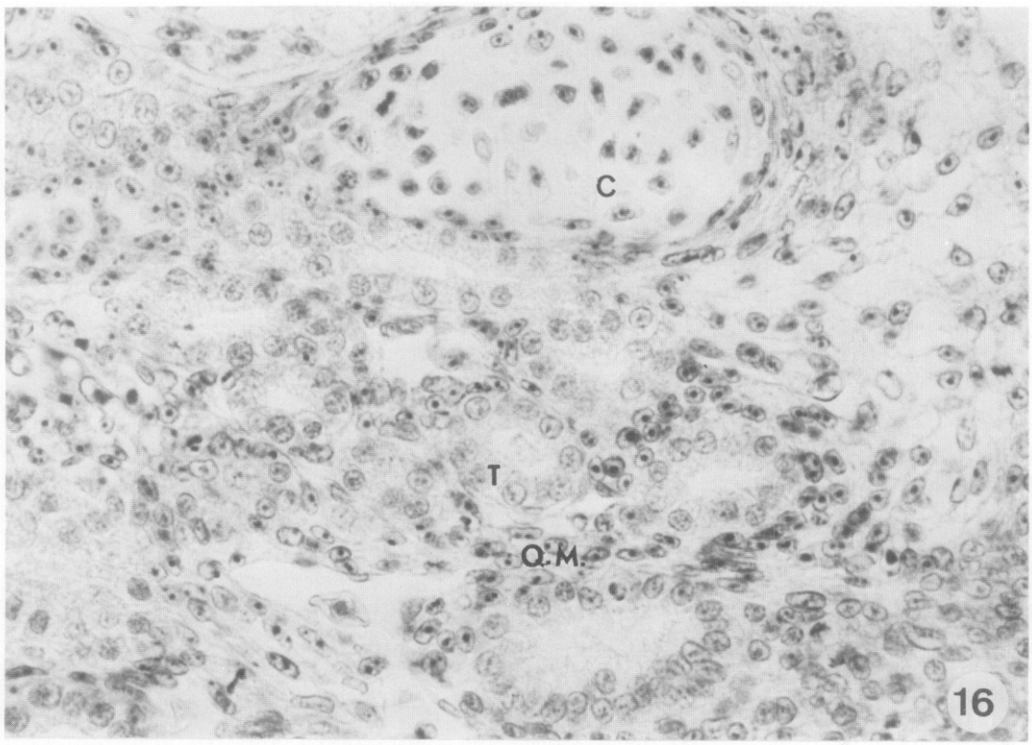
Origin of the neural primordium	Stage of quail donor embryo	Number of cases observed	Age of the embryos	Quail connective tissue		Quail cartilage		Quail dermis
				In axial region	In intermediate cell mass derivatives	In axial region	In intermediate cell mass derivatives	In dorsolateral side only
Mesencephalic + anterior rhombencephalon	4- to 7-somite stage	4	7–10 days	4	3	2	4	4
Posterior rhombencephalon from somites 1–7	7- to 12-somite stage	4	7–9 days	0	1	0	0	0

whereas in normal development they never penetrate into the splanchnic mesoderm. On the other hand, cephalic crest cells, transferred into an adequate dorsal level, migrate massively into the suprarenal gland. These results show that precise pathways guide neural crest cells from certain transversal levels of the embryo towards specific organs where their progression stops and where they proliferate and differentiate.

However, chemical attractions must also play a role in the localization of neural crest cells, as shown by the experiments concerning the formation of enteric ganglia: after orthotopic transplantation in the dorsal area (Le Douarin and Teillet, 1973), no quail cells are found in these ganglia, thus no specific pathway from the crest to the intestine is present at this level; however if "vagal" crest is transplanted to this same dorsal area, some quail cells colonize the intestine, thus demonstrating an attraction of these presumably predetermined cells. The seemingly contradicting results of these two types of experiments are best explained by a process of "double assurance," one being the existence of a specific pathway from the vagal crest to the intestine, the other being ganglionic predetermination of some of the "vagal" cells. That crest cells of the "vagal" level are selectively attracted by intestinal structures is indeed the only possible explanation which can account for the observed facts. Apart from enteric ganglia, some vagal crest cells grafted to the trunk also give rise to sensory neurones, adrenergic ganglia and adrenomedullary cells.

It has been demonstrated (Rawles, 1947; Weston, 1963) that in the trunk, crest cells begin to migrate in two main streams: a superficial one located under the ectodermal layer and a dorsoventral one between the neural tube and the somites. Trunk crest cells migrating in the ventral stream become localized in three main areas (Fig. 19). First, some crest cells settle in the somitic mesenchyme and give rise to sensory ganglia; the second area corresponds to the region where the primary sympathetic ganglion chain arises. It is located around the lateral levels of the notochord; the third, ventral in respect to the latter two is represented by the somatopleure area ventral to the aorta, where aortic plexi and suprarenal glands differentiate. Some cells also localize in the metanephric mesenchyme (Fontaine and Le Douarin, 1971).

Whether the cells are trapped in these locations by a mechanical process or are retained by a specific chemical attraction is not clear. It is now established that the cells that are in contact with the somite and the neural tube for a period of time receive an induction to which they answer by catecholamine synthesis and storage (Cohen, 1972; Norr, 1973). In normal developmental conditions, or in isotopic and isochronic transplantations of quail neural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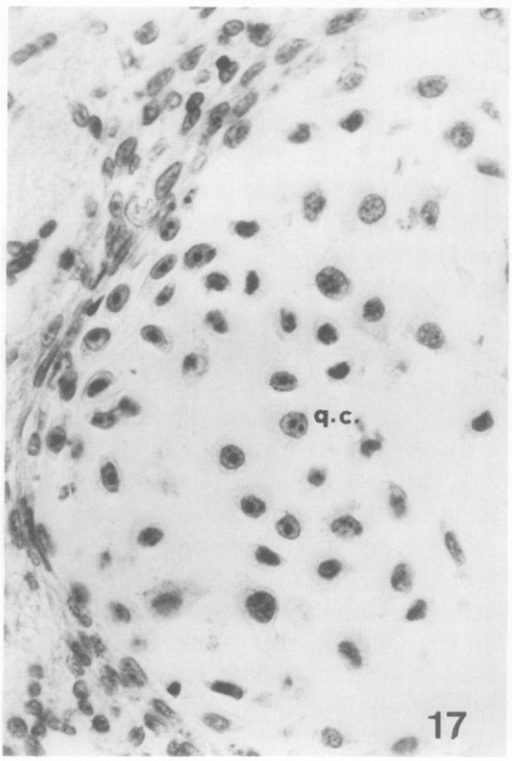
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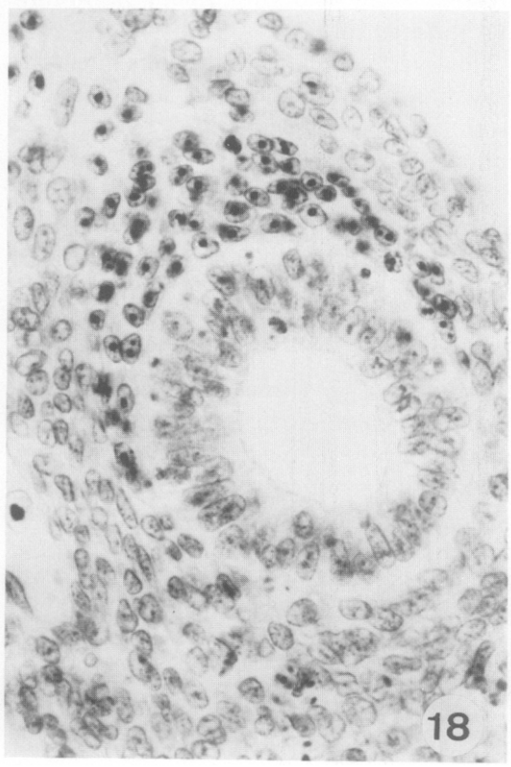
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tube into the chick, the whole population of neuroblasts originating from the "adrenomedullary" area of the neural crest becomes distributed through these three main locations. On the contrary, "vagal" crest cells transplanted to the "adrenomedullary" level are partly retained in these loci, while some of them proceed with their migration and penetrate into the mesentery and the splanchnic mesoderm. Thus it appears that the predetermined enteric ganglion cells which arise from the "vagal" neural crests are not attracted by the 3 areas that retain sensory and adrenergic neurones and also adrenomedullary cells in the trunk. On the other hand, although during their migration they are a while in contact with the axial structures (ventral neural tube and somites) that normally induce adrenergic neurone differentiation (Norr, 1973), they do not elaborate catecholamines. It can be assumed that the induction coming from the axial structures is necessary but not sufficient for catecholamine synthesis and that a "maintenance factor" present in the dorsal trunk mesoderm but absent in the gut wall is necessary for adrenergic differentiation.

We can conclude from experiments 1 and 3 that in normal development "vagal" neural crest cells that colonize the intestine are both selectively attracted by splanchnic mesoderm and passively led to the intestine by a preferential pathway, since cells which have not a specific affinity for the gut wall nevertheless migrate into this organ when transplanted to the vagal level.

The nature of the pharyngeal pathway to gut wall. When the "adrenomedullary" neural crest is transplanted at the "vagal" level, presumptive neuroblasts of the auto-

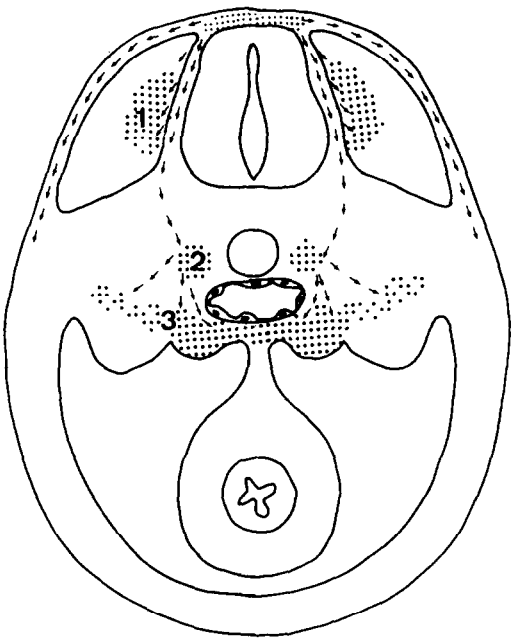

FIG. 19. Schematic drawing showing the three main regions of the trunk where the neuroblasts originating from the neural crest become localized. Crest cells migrate in two streams, one dorsolateral and one ventral. From the latter, crest cells are distributed in the somitic mesenchyme (1) where they differentiate into sensory ganglia, in the orthosympathetic chains (2) and in a more ventral area (3) involving the ventrolateral side of the aorta (aortic plexus), the suprarenal glands and the metanephric mesenchyme. The neuroblasts of the trunk do not penetrate into the dorsal mesentery.

FIG. 16. Same experiment as in Fig. 14. Transverse section of the host mesonephros containing mesenchymal derivatives of neural crest origin. T, kidney tubules belonging to the host; Q.M., mesenchyme located between the mesonephritic tubules, made up of quail cells; C., nodule of cartilage located inside the mesonephritic tissue. Feulgen-Rossenbeck staining. × 700.

FIG. 17. Same experiment as in Fig. 14. Participation of neural crest mesenchyme in the formation of vertebral cartilage in the trunk at the level of the graft. q.c., quail cartilage cells. Feulgen-Rossenbeck staining. × 720.

FIG. 18. Same experiment as in Fig. 14. Neural crest mesenchyme in the wall of the Müllerian duct of the host. Feulgen-Rossenbeck staining. × 720.

nomic nervous system, which normally differentiate into adrenergic neurones and adrenomedullary cells, massively colonize the intestine and give rise to nonadrenergic neurones. Previous studies on the contribution of cephalic neural crest to pharyngeal derivatives in the chick (Johnston, 1966; Le Douarin and Le Lievre, 1971, 1972; Le Lievre and Le Douarin, 1973, 1974; Le Douarin, 1974) have shown that crest cells originating from the mesencephalon and rhombencephalon migrate ventrad in large number and penetrate between the ectodermal and endodermal layers. Only a few cells of mesodermal origin are present in the branchial arch at this stage. Thus, the neural crest cells fill the free space between ectoderm and endoderm in the same way as the mesodermal layer does during gastrulation.

When heterotopic transplantations of quail "adrenomedullary" neural tube are made to the chick "vagal" region, the dorsal crest cells follow the same migration routes as "vagal" crest cells do, namely towards the ventral side of the embryo. They arrive in contact with the ventrolateral endoderm and then are incorporated into the developing intestinal wall. As has been suggested before (Weiss, 1961; Hay, 1968; Cohen and Hay, 1971), the basement membrane of young epithelia (here ectoderm and endoderm) might provide a substrate functioning as routes for cell migration. Such an explanation seems very likely since crest cells of various origins follow the same migration pathway and fill the empty space of the ventrolateral pharyngeal wall.

The cells that normally colonize the ventrolateral region of the pharynx have a wide range of developmental capabilities, some of them giving mesenchymal derivatives (Hammond and Yntema, 1964; Johnston, 1966; Le Lievre and Le Douarin, 1973, 1974; Le Lievre, 1974) some others differentiating into Schwann cells, carotid body cells (Le Douarin *et al.*, 1972, Pearse *et al.*, 1973) calcitonin producing cells (Le Douarin and Le Lievre, 1971, 1972; Le Douarin *et al.*, 1974), and neurones, especially enteric ganglion cells (Le Douarin and Teillet, 1973). Later on, the presumptive parasympathetic neurones of the intestine wall undergo a long migration caudad along the gut, some of them reaching the rectum.

In the present study we were interested by the eventual ability of "adrenomedullary" neural crest cells to colonize the intestine and give rise to enteric ganglia; whether some of these cells may differentiate into other structures at the vagal level has not yet been ascertained and is now under investigation. The morphogenetic capacities of dorsal crest cells to make up Auerbach's and Meissner's plexi and ganglia in normal anatomical locations is interesting to note. In *in vivo* and *in vitro* experiments, where the intestine and the trunk neural tube have been associated, crest cells that colonize the developing gut spread in two distinct layers located on both sides of the circular layer of the *muscularis externa*. Inner and outer surfaces of circular muscle layers are lined by PAS positive mucopolysaccharide—rich material; it can be assumed that this kind of structure is a favorable substrate for crest cell migration, and that it ensures their preferential localization in two layers in normal and experimental conditions. It is remarkable indeed that, in the cultures of associated neural tube and intestine, not only the neurones but also the melanocytes are distributed at the normal level of Meissner's and Auerbach's plexuses.

2. Influence of Environmental Factors on Presumptive Neuroblasts Differentiation

It has been demonstrated previously that the differentiation of orthosympathetic ganglioblasts depends on the inductive influences they are submitted to, along their migratory route towards their final site (Cohen, 1972). More precisely, it seems established from the work of Norr (1973)

that the ventral neural tube is responsible for a developmental change in somitic mesenchyme, which in turn elicits sympathoblast differentiation.

Our results show that presumptive dorsal neuroblasts which in normal development would give rise to adrenergic sympathoblasts or adrenomedullary cells differentiate into nonadrenergic neurones when submitted to the splanchnic mesoderm environment (experiment 1 and 2). Experiment 2 shows in addition that, in gut mesenchyme, presumptive sympathoblasts as well as adrenomedullary cells cannot express at all their phenotypes. Physiological evidence that they differentiate into cholinergic neurones has recently been provided by our group (Le Douarin *et al.*, 1974).

Then it appears from these experiments that the presumptive adrenergic neuroblasts have the ability to become organized into enteric ganglia. This observation confirms the previous findings of Andrew (1971) who found out that enteric ganglia could differentiate within intestinal structures developing in the CAM from dorsal level fragments of 13- to 25-somite chick embryos. Thus, the bipotentiality of dorsal crest cells to contribute either to ortho- or parasympathetic systems may be considered as established.

On the other hand, our experiments show that cephalic neural crest cells have the ability to differentiate into adrenomedullary cells when they are in contact with a particular zone of the dorsal somatopleure. In previous studies we have shown that avian metanephric mesenchyme contains during normal development small islets of adrenomedulla—like cells, which contain secretory granules of catecholamines (Le Douarin, 1968; Le Douarin and Houssaint, 1969; Fontaine and Le Douarin, 1971; Fontaine, 1974). The multiplication of these cells, which originate from the neural crest (Le Douarin, 1969), is enhanced in cultures of isolated metanephritic blastema which do not differentiate into kidney tissue. Thus we can assume that the extracellular matrix properties, necessary to promote the differentiation of catecholamine producing and storing cells from neural crest elements, overspread the precise region of the suprarenal gland and extend caudally into the metanephric mesenchyme. It must be pointed out, however, that the capacity to induce adrenomedullary cell proliferation and differentiation is greater in the suprarenal gland region than in the posterior coelomic epithelial field.

While presumptive peripheral nervous tissue needs precise conditions for differentiation, melanocytes do not appear to have such requirements. When the dorsal neural primordium (neural tube and associated folds) is combined with splanchnic mesenchyme of the rectum, melanocytes can be seen in the gut wall. The results of *in vitro* cultures of 4-day chick embryo sensory ganglia have previously suggested (Weston and Butler, 1966; Cowell and Weston, 1970) that melanocytes could derive from initially pluripotent neural crest cells, one of the factors which could promote their differentiation into melanocytes being the degree of dispersion of the cells at a decisive stage of their developmental evolution. Evidently, this kind of extrinsic influence is much less specific than those which are involved in neurone differentiation.

3. Developmental Capabilities of Ectodermal Mesenchyme in the Trunk Environment

It has appeared from previous studies (Hammond and Yntema, 1964; Johnston, 1966; Johnston and Listgarten, 1973; Le Lievre, 1971 a and b, 1974; Le Lievre and Le Douarin, 1973, 1974) that the capacity of the neural crest to give rise to connective tissue and its derivatives, is restricted to the head region. The posterior limit of this developmental capability is located ap-

proximately around the level of somite 5 (Le Douarin and Le Lievre, unpublished).

The results we have obtained in experiment 3 show that, when transplanted into the trunk, the presumptive mesectodermal crest cells can migrate and, to a certain extent, express their normal phenotypes. They give rise to dermis, cartilage and connective elements forming the wall of blood vessels and Müllerian duct or to intertubular mesonephritic mesenchyme.

Experiments performed in Amphibian embryos have shown that differentiation of cranial crest cells into skeletal structures require the influence of pharyngeal endoderm as well as an appropriate sequence of interactions with other tissues (Hörstadius, 1950; Hörstadius and Sellman, 1946; Newth, 1954; Seno and Nieuwkoop, 1958; Holtfreter, 1968): the head crest will not chondrify when cultured by itself in sandwich experiments, in cell culture, or in the flank of a host. From our results it appears that, in birds, the inductive influence of pharyngeal endoderm is not necessary to promote the chondrification process in the neurectodermal mesenchyme, since cephalic crest cells transplanted into the trunk give rise to vertebral cartilage and chondrified nodules located in various places of the somatopleural mesoderm.

The authors wish to express their grateful thanks to Dr. F. Dieterlen for her help in writing this article in English.

This work has been supported by grants of CNRS and DGRST.

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