AN ANALYSIS OF THE ROLE OF THE TISSUE ENVIRONMENT IN THE REGIONAL DIFFERENTIATION OF THE CENTRAL NERVOUS SYSTEM IN THE AMPHIBIAN, AMBYSTOMA GRACILE (Baird)

by

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ABSTRACT

This investigation considered the differentiation tendencies of specific anterior-posterior regions of the neural plate in an attempt to establish the regional differentiation capacity for Ambystoma gracile neuroepithelium. Presumptive neural tissues (hindbrain and trunk spinal-cord) were isolated from the embryo at the time of primary induction (stage 11) to post-neurulation (stage 19) and cultured in vitro alone or with combinations of axial mesoderm (notochord and somite).

Prior to primary induction (stage 11), the isolated presumptive neuroepithelium formed only atypical epidermis. Immediately subsequent to this induction (stage 11) both regions (hindbrain and trunk cord) demonstrated unorganized neural histogenesis, while the formation of organized neural tissue appeared later (stage 12-14 isolates). By stage 15-16, the histogenesis of the isolated hindbrain resembled that of the control, whereas the isolated trunk cord only formed a neural tube. The presence of somite tissue enhanced hindbrain differentiation considerably; notochord was effective to a limited extent. The combined effect of both tissues on neurogenesis was greater than with somite alone.

The addition of notochord to trunk spinal-cord enhanced histogenesis to a greater extent than either somite alone or the combination of notochord and somite. The trunk neural tissue, whether alone or in combination with mesoderm, never demonstrated normal spinal-cord morphology and seemed to develop independently of the tissue environment during the late neurula stages (16-19). The presence of inherent differentiation tendencies within the hindbrain and the trunk spinal-cord, as well as the possible role of the mesodermal tissue in conditioning the neural tissue microenvironment with metabolic precursors, is discussed.

PAGE

ERRATUM

Acknowledgements:

"Canadian government" should read, the Government of Canada.

Page 4: insert in line 9 after, "gastrulation and neurulation," in *Ambystoma gracile*.

Page 32: line three should read: "...the embryo: 1) directly beneath the presumptive hindbrain region and 2) beneath the presumptive trunk neural region."

Page 62: omit the two sentences beginning on line 7, "No cases of nephric ..." to "...*i.e.* myogeneisis."

Page 81: in number 4 insert after, "isolated" the word, presumptive.

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ACKNOWLEDGEMENTS

To Dr. Cyril V. Finnegan, my sincere appreciation for his advice, guidance, and friendship that does not end with this investigation.

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INTRODUCTION

> "... in the development of the central nervous system the initial inductive stimuli are followed by a chain of secondary tissue interactions which lead to the final segregation of the tissue" (Saxen and Toivonen, 1962, p. 221).

Experimental analysis of the "initial stimuli" revealed that the dorsal lip tissue of the amphibian gastrula evoked the formation of an embryonic axis (Spemann, 1938). Spemann observed that the dorsal lip tissue, while itself differentiating into notochord, induced the formation of a neural axis in the overlying epithelium and brought about condensation of mesoderm to form somites. As the mesodermal mantle (chorda-mesoderm) comes to underly the presumptive neuroepithelium in the normal embryo, differentiation tendencies occur in this tissue leading to the regionalization of the nervous system (i.e., formation of forebrain, hindbrain and trunk spinal-cord). Regional differentiation of the central nervous system (C.N.S.) is believed to be the result of regional inductive capacities of the mesodermal mantle of the late gastrula and neurula (see reviews by Holtfreter and Hamburger, 1955; Dalcq, 1960; Saxen and Toivonen, 1962).

Extensive investigation of the initial interactions of neural induction have lead to several theories. Yamada (1950) discussed regionalization as a result of two interacting morphogenetic gradients, dorso-ventral and cephalo-caudal. Nieuwkoop et al., (1952) observed the activation of neuroepithelium toward forebrain differentiation and the subsequent transformation of this activated epithelium towards more posterior differentiations (eg., hindbrain and trunk spinal-cord). Toivonen (1958), on the other hand, indicated that two separate factors, one neuralizing and the other mesodermalizing can operate independently in neural induction, and the combined effect was reported (Saxen and Toivonen, 1961) to result in regional differentiation of the nervous system. It should be emphasized that the latter authors employed heterogenous indicators, i.e., guinea-pig tissues and HeLa cells, whereas Nieuwkoop et al. (1952) (see also Eyal-Giladi, 1954; Sala, 1955) used both live and dead amphibian organizer tissue in their investigations. The above has established, however, that the underlying mesoderm, during gastrulation, initiates events leading to the regional differentiation of the overlying neuroepithelium.

Limited attention has been given to subsequent tissue interactions. Takaya (1955; 1956a, b) studied the

neural-mesodermal interactions during regionalization of the nervous system. In explants of presumptive stage 16 trunk spinal-cord, subsequent neural differentiations were shown to be dependent upon the presence or absence of mesoderm. In the complete absence of mesoderm the differentiating neural mass demonstrated forebrain characteristics. By varying the amount of mesoderm tissue, hindbrain or spinal-cord could be obtained in these isolates (viz., loose mesenchyme and notochord → hindbrain; somite and notochord → spinal-cord). These results seem to confirm the previous observations of Nieuwkoop et al. (1952) that the intrinsic differentiation tendency of the neural tissue is toward forebrain structures and the presence of mesoderm transforms the tissue toward more caudal structures. However, Gallera (1958, 1959, 1960) presented evidence that the intrinsic differentiation tendency of isolated rhombencephalon (stage 11) is toward neural crest derivatives with the archencephalic influence of the mesoderm appearing later in time.

The question of the control of regional differentiation in the developing central nervous system, in particular the contribution of both intrinsic factors and extrinsic influences, has not been answered fully. The role

of the mesoderm tissue in differentiating systems has been demonstrated (salivary gland, Grobstein, 1953; kidney, Grobstein and Dalton, 1957; feather and skin, Rawles, 1955; limb, Zwilling, 1961, nose, eye, and ear, Jacobson, 1963a, b, c; 1966) and this tissue should be considered as a possible factor in the development of the neuroepithelium.

It was the purpose of this investigation to analyze the differentiation capacity of the neural tissue during late gastrulation and neurulation with respect to the intrinsic nature of the induced tissue and the possible modification of differentiation during these stages by the underlying mesoderm. In order to ensure a completely neutral environment for the developing tissues, the ectodermal sandwich or vesicle was employed. This system provides a favorable environment for the differentiation of tissues and allows the experimenter to control the components of the environment by adding known tissues (see also Yamada, 1959; Landesman and Dalton, 1964).

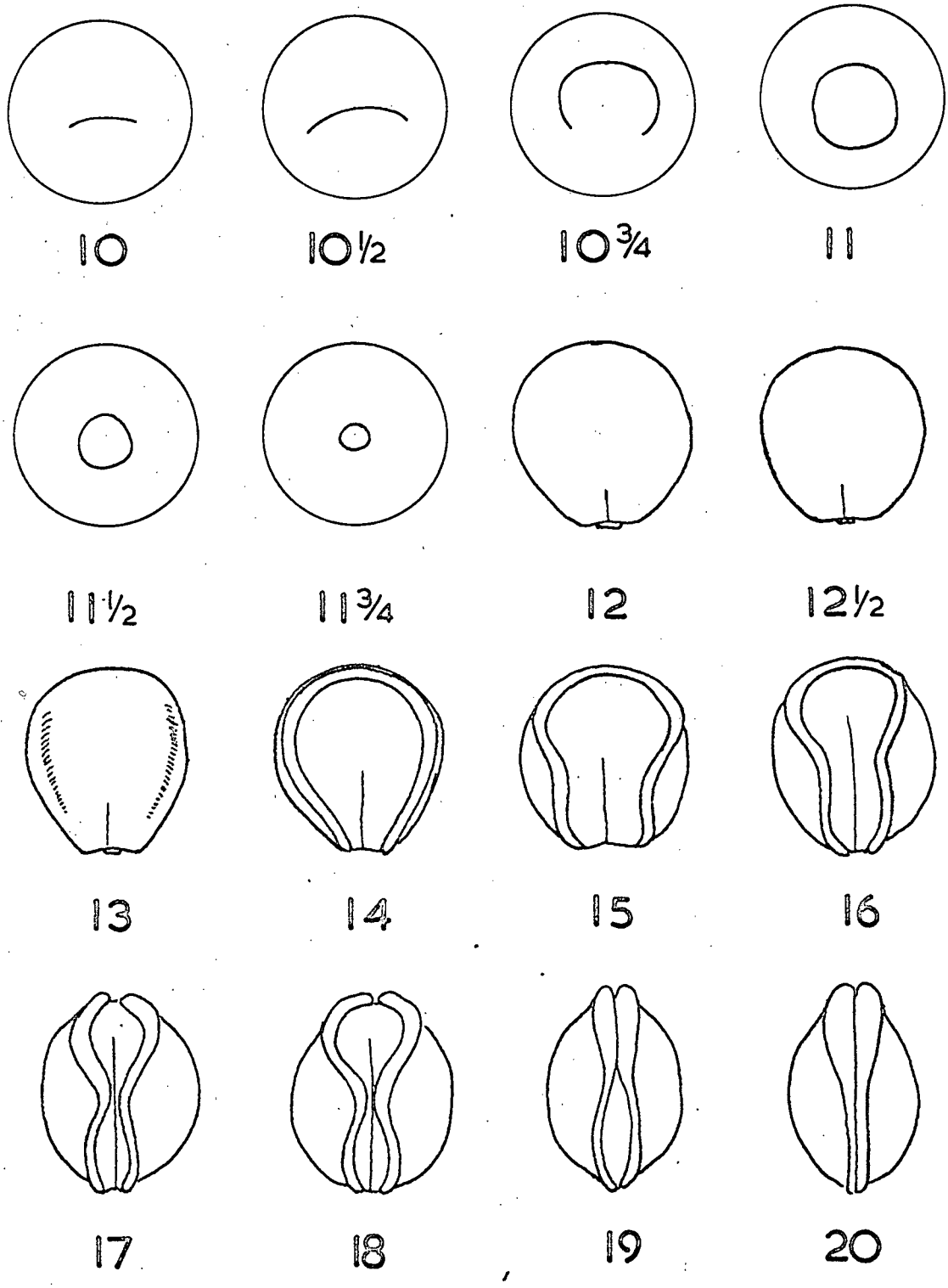
MATERIALS AND METHODS

The amphibian embryos used in this investigation (Ambystoma gracile (Baird)) were collected locally. The decapsulated eggs, still in their vitelline membranes, were washed several times in sterile pond water and then maintained at 7°C in sterile pond water. Antibiotic (15 mg/l of streptomycin sulfate) was added to all solutions used.

The developmental stages of the A. gracile embryos could be determined grossly by comparing them with the published staging schemes for A. punctatum and Taricha torosa (Rugh, 1962). However, sufficient differences exist between the development of these species and that of early A. gracile to warrant the preparation of a separate staging scheme (see Fig. 1). The latter system was used in staging the embryos for this investigation.

To determine the relative positions during gastrulation of invaginating chorda-mesoderm and prospective neuroepithelium, as well as the patterns of migration of superficial cells in A. gracile, the following procedures were used. A series of embryos viz., stages 10-19, were fixed in a 10% amphibian Ringer's solution, without carbonate, containing 1.3% sulfuric acid (Legname, 1964). This procedure permitted gross dissection of embryos without distortion of

Figure 1. The normal stages in the early development of Ambystoma gracile (Baird).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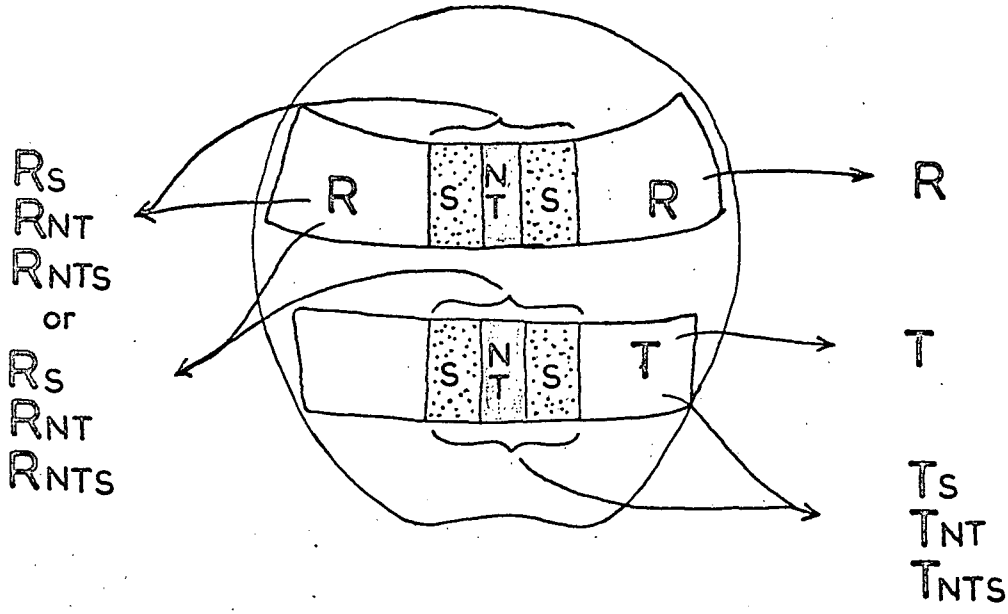
the tissue layers. In order to observe the migration of regions within the prospective neuroepithelium during gastrulation, a series of vital staining experiments were performed. A small piece of cellophane, previously intensely stained with nile blue sulfate (1%), was placed on the surface of a decapsulated embryo and secured for 30 minutes with a glass bridge (Rugh, 1962) so that the vital stain was transferred through the vitelline membrane to the underlying cells. The stain did not appear to diffuse appreciably either into the surrounding medium or to cells not in direct contact with the cellophane. The marked embryos were then observed daily and the pattern of migration of the stained neuroepithelial region(s) was recorded diagrammatically.

The operative procedure employed in preparing the various experiments involved removing regions of the presumptive central nervous system (hindbrain, and spinal-cord) with and without underlying notochord and somite mesoderm or both. Operations were performed in a operating-dish with a waxed surface containing sterile urodele operating solution (Rugh, 1962). The vitelline membranes were removed using finely sharpened watchmakers' forceps. The portions of the embryo to be isolated were incised with a sharpened steel needle and peeled from the embryo. The depth

of cut was varied by whether underlying tissue was to be included or not; however, in all cases, a visual check was made to determine which cells or cell layers were included in each isolate, and unwanted cells or layers were removed with hair loops. The following combinations were made: presumptive rhombencephalon alone (R), presumptive rhombencephalon with notochord (Rnt), presumptive rhombencephalon with somite (Rs), presumptive rhombencephalon with both notochord and somite tissue (Rnts); presumptive trunk spinal-cord alone (T), presumptive trunk cord with notochord (Tnt), presumptive trunk cord with somite (Ts), or presumptive trunk cord with both notochord and somite tissue (Tnts)(see Fig. 2). In addition to these isolates, cultures of uninduced prospective neuroepithelium were prepared. The excised neuroepithelium, alone or with underlying or added axial tissues, either was allowed to curl up, inner surfaces adhering, or a piece of ventral ectoderm was added covering exposed inner surfaces. In either case, the isolate was allowed to heal for 30 minutes and then was transferred, as an ectodermal vesicle, to a small sterile Falcon plastic Petri dish (35x10 mm) containing urodele growing solution (Rugh, 1962). In addition, a similar series of tissues was prepared and immediately fixed in Carnoy's solution, and processed for

Figure 2. Diagrammatic representation of the operative procedure used for the preparation of ectodermal vesicles.

R. presumptive rhombencephalon
T. presumptive trunk spinal-cord
nt. presumptive notochord
s. presumptive somite


R_S
R_{NT}
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or
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sectioning (see below). Controls consisted of embryos of the same developmental stage and from the same egg mass, which were maintained under similar conditions in a larger Petri dish (60x15 mm). Both embryos and isolated tissues were maintained at 13°C (± 2°C) until the control embryos reached stage 40, approximately 20 days. Stage 40 was used as an endpoint since the pattern and morphology of the C.N.S. has reached a point when subsequent development does not alter existing spatial patterns. During the culturing period, the vesicles were observed twice (about the tenth day, when they achieve maximal growth, and the last day), noting the general morphology of the vesicle for any evidence of inductions (such as the presence of a fin). More than 1200 vesicles were so prepared and over 60% remained viable throughout the entire culture period. During the preparation of ectodermal vesicles and the analysis of their subsequent differentiations, staging of early development was done to the half stage. However, the presentation of data obtained is to the nearest whole stage.

At the end of the developmental period, the vesicles and controls were fixed in Carnoy's solution (absolute ethanol: chloroform:glacial acetic acid; 6:3:1), embedded in Paraplast, and sectioned serially at 5-7 microns. The

sections were stained routinely with Chromatrope 2R (Liisberg, 1962). Sections were hand drawn with the aid of a Bausch and Lomb microprojector.

RESULTS

I Vital Staining

The vital-staining experiments established the following points. The prospective central nervous system of this species arises from a region immediately superior to the blastopore at stage 10½ (see also Vogt, 1929). During gastrulation the superficial layer (presumptive ectoderm) undergoes epiboly so as eventually to occupy the entire outer surface of the embryo. As a direct result of this movement, the prospective neuroepithelium is extended to occupy the entire dorsal surface by stage 12. It is not until stage 11 - 11½ that the two regions, prospective hindbrain and prospective trunk spinal-cord, become separate and distinct, and which can be vitally stained or mechanically isolated. Prior to stage 11 the neural plate shows no such spatial separations (see also Dalcq, 1964). Furthermore, the relative positions established by stage 12 are not altered by the events occurring during subsequent neurogenesis, i.e., neural fold formation and closure of the tube, other than the considerable extension of the trunk region (see also Jacobson, 1962).

From the above information and with the assistance of the published fate maps of presumptive brain regions prepared for A. mexicanum (Nieuwkoop et al., 1955a, 1955b; Jacobson, 1959, 1962; Nieuwkoop and van der Grinten, 1961), a series of diagrams was prepared indicating the position of the prospective brain regions in A. gracile at successive developmental stages (Fig. 3).

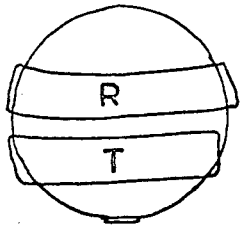
II Tissue Relationships during Gastrulation and Neurogenesis

A. Gross Dissection

A representative developmental series of A. gracile embryos, fixed in sulfuric acid, was examined by gross dissection and the results are presented in Figure 4.

The process of invagination and extension of the presumptive notochord, mesoderm and endoderm begins at stage 10 and is essentially completed by stage 12 (Fig. 4; A-F). During the earlier stages of gastrulation (10-11), regions of presumptive neuroepithelium could be identified by certain characteristic landmarks. That is, at stage 10 - 10½, two regions of ectoderm can be distinguished; one which is uninduced since it is not underlain by prechordal plate, and another region which has been induced by the migrating prechordal plate (Fig. 4; A,B). At stage 10 3/4 three regions

Figure 3. A series of fate maps indicating relative positions of presumptive rhombencephalon (R) and presumptive trunk spinal-cord (T) regions during the early stages of Ambystoma gracile development



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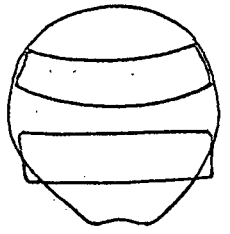

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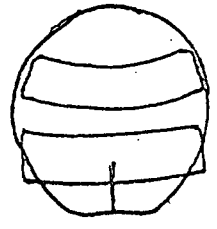

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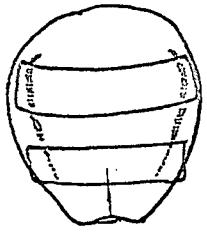

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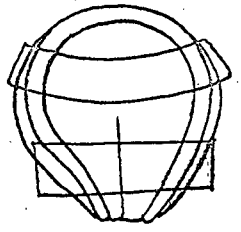

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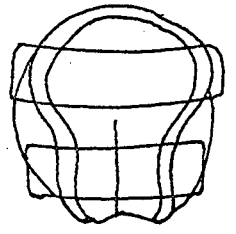

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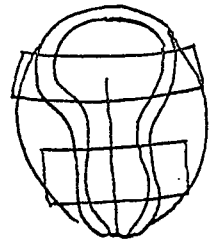

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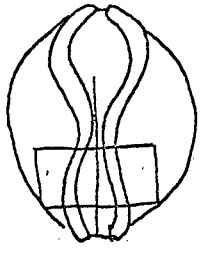

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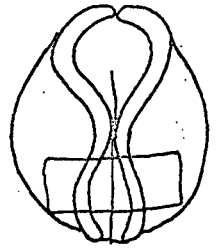

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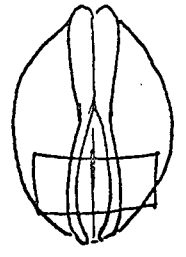

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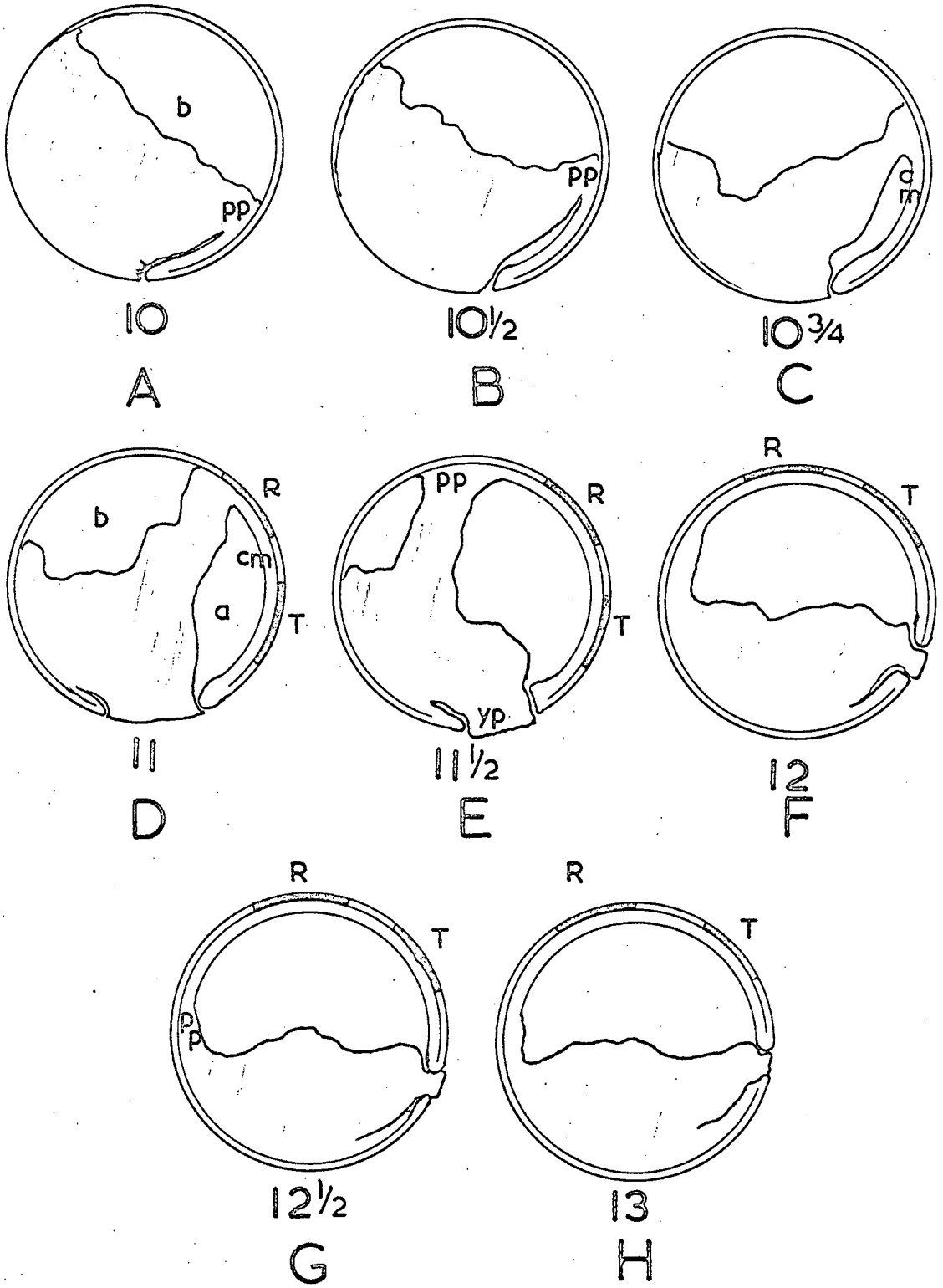
Figure 4. Diagrammatic representation of a mid-sagittal section of the gastula stages of Ambystoma gracile

a. archenteron
b. blastocoel
pp. prechordal plate endoderm
cm. chorda-mesoderm- roof of archenteron
yp. yolk plug in blastopore
R. position of presumptive rhombencephalon
T. position of presumptive trunk spinal-cord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13
H

can be distinguished; (1) the uninduced ectoderm, (2) the ectoderm underlain by prechordal plate, and, further posteriorly, (3) the ectoderm which is underlain by the anterior chorda-mesoderm, the roof of the archenteron. At stage 11½ the presumptive neural ectoderm is completely underlain by the archenteron roof and, from the author's knowledge of the previous vital staining results, the ectodermal regions which correspond to the two regions of the future C.N.S. i.e., the hindbrain and the trunk spinal cord, can now be identified (see Fig. 4, E-H).

B. Histological Analysis of Neural and Underlying Mesodermal Tissues

The tissue relationships during gastrulation and neurogenesis were studied by microdissection in the living embryo as well as by studying serial sections of operative regions, fixed immediately upon removal from the embryo. These two methods provided the following information unavailable as such in the literature except where noted. Prior to stage 11, the superficial neuroepithelium could be cleanly removed from the embryo, that is, no prechordal plate cells adhered to overlying epithelium. At stage 11, the presumptive anterior notochordal region and the associated thin endodermal layer (which together form the roof of the

archenteron) underly about two-thirds of the neural region. The invaginating mesoderm, which migrates on the thin endodermal roof of the archenteron lateral to the cephalo-caudal axis, is first distinguishable only in the posterior regions underlying the presumptive trunk at stage 11. This layer advances anteriorly by stage 12 approaching the border between the presumptive trunk and the presumptive hindbrain, while at stage 13, it underlies the latter region completely. During stages 11 - 13, the presumptive notochord begins to concentrate cephalocaudally within the archenteron roof to form a compact rod of cells beneath the overlying ectoderm. As this notochord condensation occurs, an intimate association between the condensing chorda cells and the overlying presumptive neuroepithelium takes place posteriorly so that a completely clean removal of neuroepithelium from chorda could be obtained only by carefully teasing the layers apart. This association of notochord and neural tissue became more and more intimate anteriorly throughout neurogenesis and the event was correlated with the outward appearance of a depression in the midline of the presumptive neuroepithelium. Microscopic examination of this association revealed that a spatial separation between the ectodermal and mesodermal layers

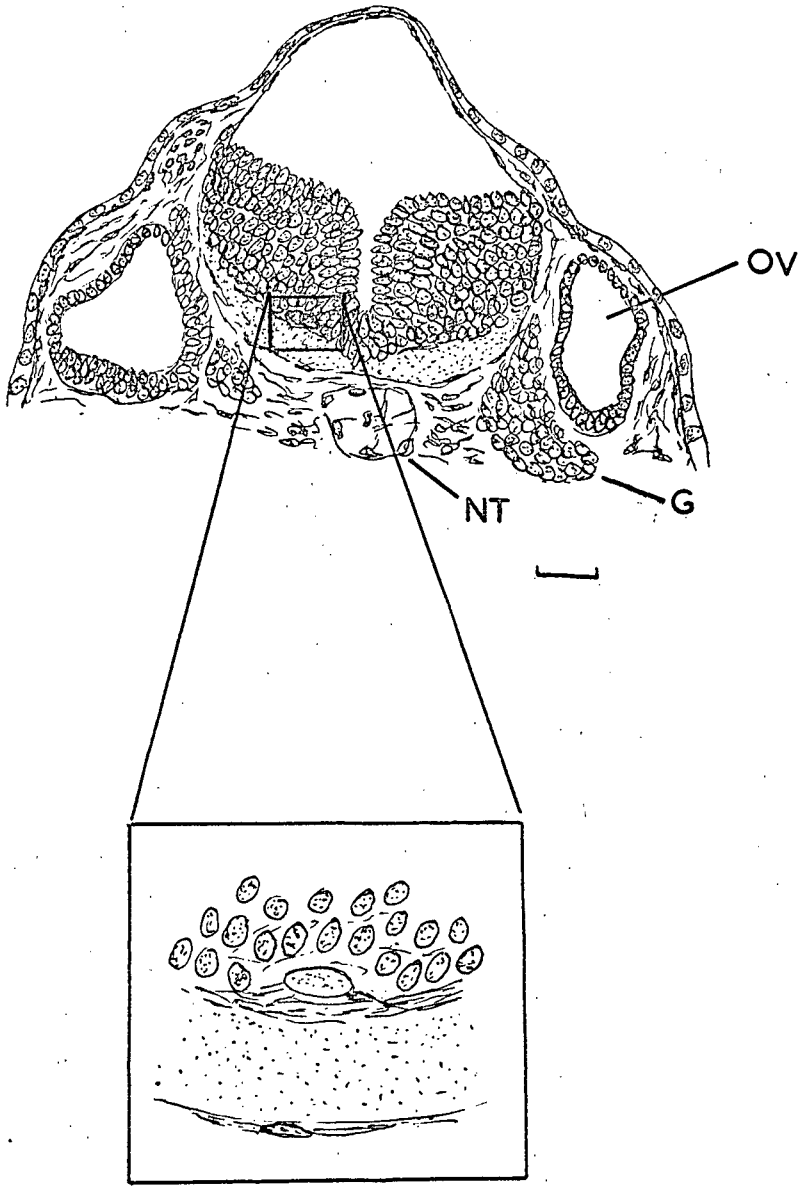
became reduced, and seemed to disappear, only in the region between the notochord and overlying ectoderm during stages 11 - 16. The observation that during the earlier stages the epithelium is more easily separated by microdissection from the underlying notochoral tissues, taken with the above result, indicates that the observed tissue separation is not a fixation artifact. During this time (stages 12-16) the advancing somite mesoderm condenses against the chorda but does not demonstrate the same intimate relationship with the overlying ectoderm at neurogenesis as does the chorda (see above). Examination of sections from stage 11 - 16 revealed that the neuroepithelium is pseudostratified columnar, except in the anteriormost regions where it is stratified with the upper layers of cuboidal (see also Gillette, 1944). The neuroepithelium is without apparent histogenesis along the cephalo-caudal axis.

III Morphology of Normal Stage 40 Hindbrain and Trunk Spinal-Cord

The appearance of a typical section through the hindbrain region is seen in Figure 5. The rhombencephalon is distinguishable from all other regions of the amphibian brain by virtue of its wide ventricle, thin epithelial roof with much thicker ventral walls and the associated

Figure 5. A typical section through a normal stage 40 embryo showing characteristic hindbrain morphology, bilaterally positioned otic vesicles (OV), ventral notochord (NT) and auditory ganglia (G).

inset: A Mauthner cell, a giant neural cell in the floor of the hindbrain. Note that the white fibrous coat demonstrates two layers; a thinner layer of neurofibers running at right angles to an outer longitudinal layer (Traced from a projection; scale equals 0.1 mm)

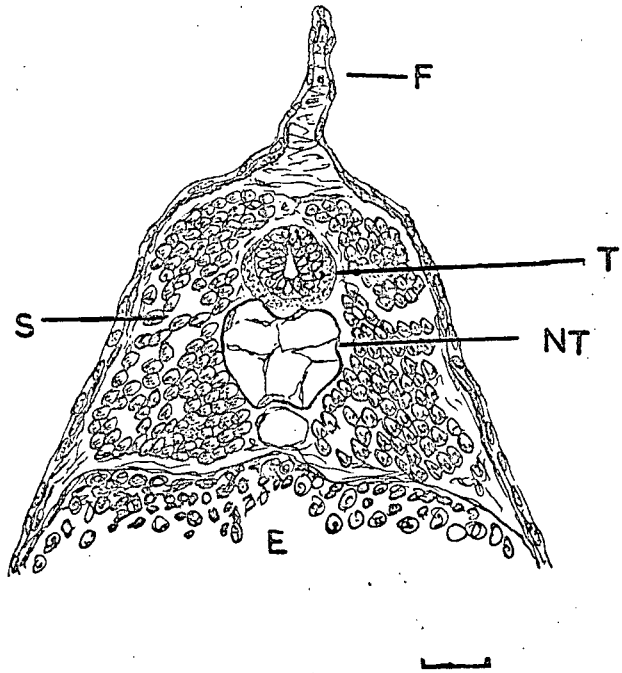

OV
NT
G

dorso-laterally positioned otic (auditory) vesicles. Furthermore, the two giant cells of Mauthner (M-cells) located at the level of the VIII root fibers and VII visceral sensory root (Herrick, 1914, 1948) characterize the medulla oblongata or rhombencephalon of Ambystoma and were found in A. gracile control embryos (see Fig. 5). The outer white fiber coat comprising the floor of the hindbrain partly contains longitudinal fiber-tracts (Eyal-Giladi, 1954).

A typical section through the trunk spinal-cord regions reveals (see also Balinsky, 1965) a tube, the lateral walls being thicker than either the roof or the floor (Fig. 6). The white matter (or fibers) is restricted to the ventro-lateral walls and fibers pass out to segmentally arranged spinal ganglia. The trunk neural tube shows giant ganglionic or Rohon-Beard cells arranged segmentally along the dorsal mid-line. These correspond to a large extent to the segmented somite tissue (Dushane, 1938) which is juxtaposed to the lateral walls of the trunk spinal-cord. The trunk region is characterized by the appearance of a dorsal fin which shows internally a ladder-like arrangement of the mesenchymal and pigment cells (see Fig. 6). In the isolates, fins were considered present only when the internal mesenchyme pattern appeared similar to the above.

Figure 6. Section through the anterior trunk region of a stage 40 embryo. Note ladder-like arrangement of mesenchyme cells and fibers forming the core of the dorsal fin (F). (Traced from a projection; scale equals 0.1 mm)

E. endoderm
F. dorsal fin
NT. notochord
S. somite muscle
T. trunk spinal-cord


F
T
S
NT
E

IV Results of the Operative Series

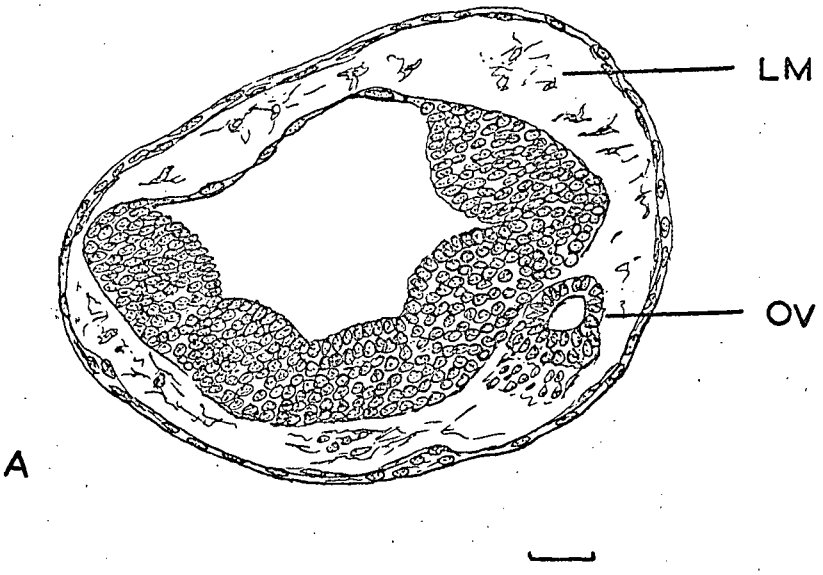
A. General Considerations

Before presenting the results obtained in each series, a general consideration of the criteria used in this investigation is necessary. Only those vesicles remaining viable, that is showing no evidence of cell sloughing or cytolysis at the end of the developmental period, were processed for microscopic examination. For the purposes of this investigation the term neural histogenesis refers to the appearance of neural cells, which are recognized by the cell arrangement (oriented radially about a lumen), nuclear chromatin pattern and staining affinities of these cells as compared to normal control embryos. Furthermore, this neural tissue can be either organized, i.e., present as a neural region resembling the hindbrain or forming a neural tube-like structure (see Fig. 7), or present as an unorganized neural mass either remaining solid or containing numerous lumina (Fig. 8). A condition, which has been observed in experimentally injured brains as well as in cultures of neural tissue, has been termed polymyely (Townes and Holtfreter, 1955). In both of these situations, i.e., organized or unorganized neural tissue, neural fiber formation (fibrogenesis) is observed to occur in varying amounts as part of neural histogenesis.

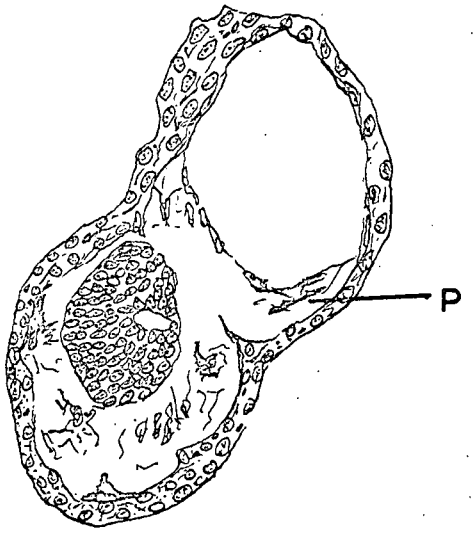
Figure 7. Examples of organized neural histogenesis

A. section through an ectodermal vesicle demonstrating a hindbrain-like morphology. Note associated otic vesicle (OV) and loose mesenchyme (LM). (Traced from a projection; scale equals 0.1 mm).

B. section through an ectodermal vesicle demonstrating neural tube-like morphology. Note scattered mesenchyme and pigment cells, (melanophores) (P). (Traced from a projection; scale equals 0.1 mm).

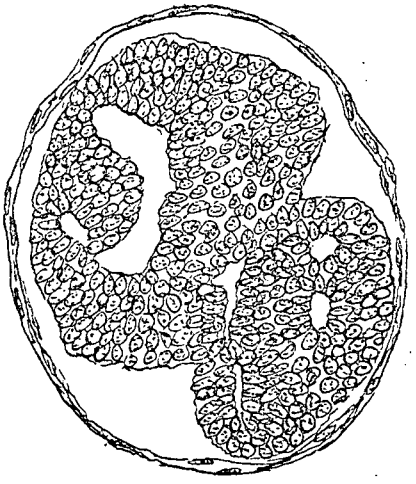


A



B

Figure 8. Section through an ectodermal vesicle demonstrating unorganized neural histogenesis. Note many lumina. (Traced from a projection: scale equals 0.1 mm).



The following specific criteria were used to classify the experimental results: 1) the neural structure(s) formed should be morphologically identifiable in comparison with corresponding structures in control embryos (see Fig. 7); and 2) the neural structures must occupy one half or more of the vesicle's volume.

The data gathered in this investigation are presented in both tables and histograms. The former are presented to indicate the types of differentiations observed in one vesicle isolated at any given developmental stage. The histograms indicate, in percentages, the cases isolated from each developmental stage which predominantly exhibit one of the following levels of differentiation: 1) only atypical epidermis; 2) unorganized neural tissue; 3) organized neural tissue; 4) a secondary induction associated with either unorganized or organized neural tissue.

B. Control Series

Uninduced prospective neuropithelium (8 cases), when isolated and cultured, always formed an irregularly shaped ectodermal vesicle which upon histological examination, revealed only undifferentiated or atypical epidermis (see also Holtfreter, 1933, 1938). Furthermore, these vesicles showed no visible sign of induction without inducer

(Barth, 1941; Holtfreter, 1945), or local activations, i.e., small neuroid placodes in superficial regions of the outer vesicle wall brought about by exposure to culturing medium (Hori and Nieuwkoop, 1955; Sala, 1956).

C. Presumptive Rhombencephalic Isolates Cultured Alone (R)

The results for this series are presented in Table I and in Figure 9.

Of the isolates examined from stage 11 (12 cases), eight cases developed only a solid mass of undifferentiated or atypical epidermis, whereas four cases showed, in addition, a centrally located unorganized neural mass. These latter cases demonstrated a tendency for the superficial epidermis to contain regions of cuboidal cells, though devoid of a microscopically visible basement membrane. In all cases examined no evidence of any secondary neural derivativies, e.g. melanophores, could be seen.

The isolates examined from stage 12 (32 cases) produced a large increase in unorganized neural histogenesis showing a larger neural mass with more lumina than in the previously described series. In addition, two isolates differentiated well organized hindbrains. Only one case appeared restricted to forming only atypical epidermis, although nine others had some of this tissue associated with

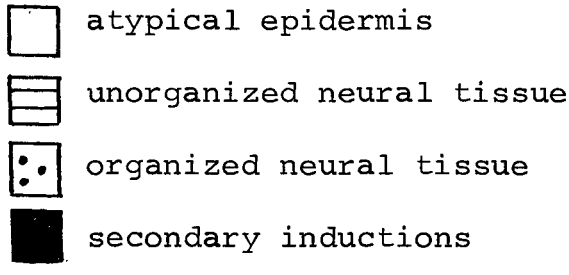
TABLE I

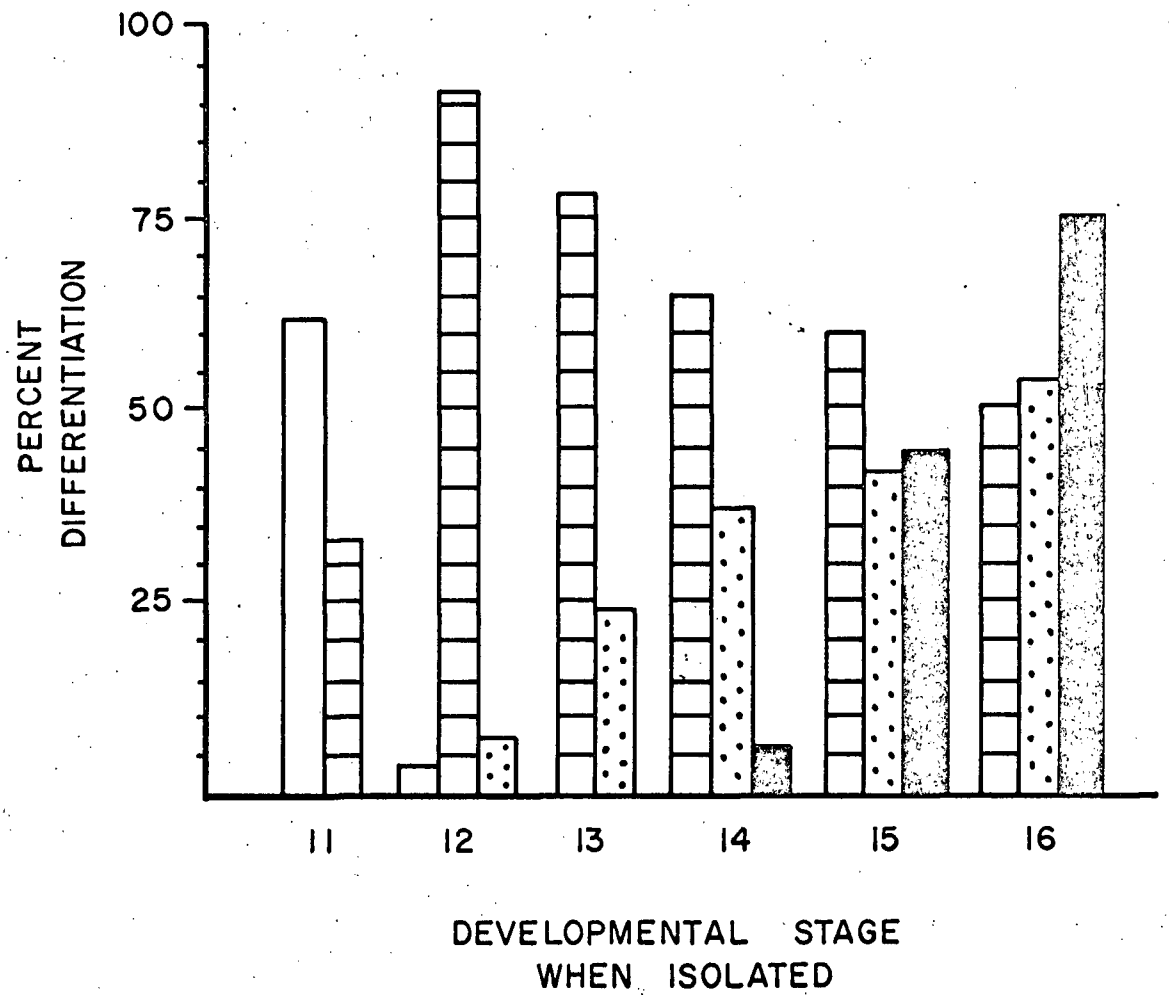
Differentiation in Isolates of
Presumptive Rhombencephalon Cultured Alone

Stage	Total number of Isolates	Atypical epidermis	Neural histogenesis unorganized	Neural histogenesis organized	Secondary inductions
11	12	10	4	0	0
12	32	10	29	2	0
13	13	2	10	3	0
14	17	2	11	6(1)[a]	1
15	25	1	15(6)	10(5)	11
16	19	0	9(7)	10(7)	14

a. the number in parenthesis (1) indicates that part of the total cases (6) showing an associated secondary induction.

Figure 9. The histogenetic profile of presumptive rhombencephalon cultured alone (R).



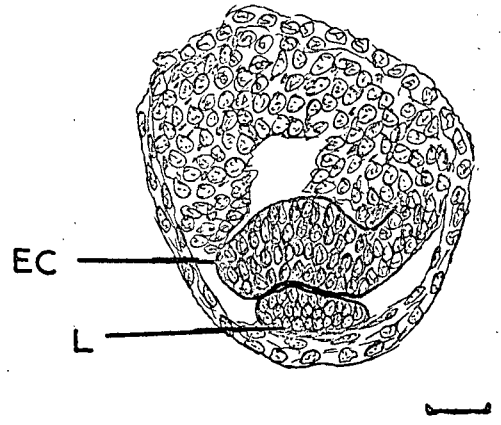

100
75
50
25
PERCENT DIFFERENTIATION
11
12
13
14
15
16
DEVELOPMENTAL STAGE WHEN ISOLATED

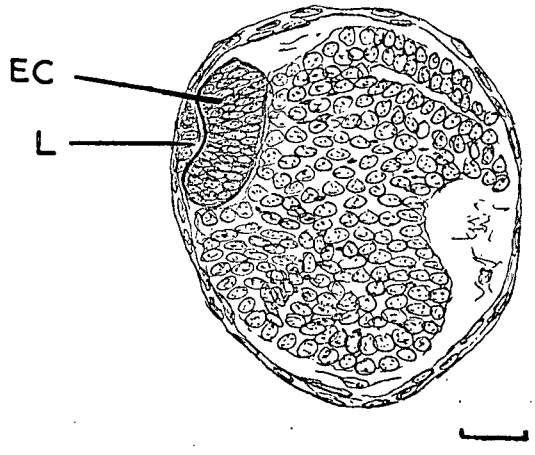
the superficial epidermis of the vesicle. The latter tissue tended to be composed of squamous and/or cuboidal cells, with mesenchymal cells, probably derived from the neural crest, adhering to the inner surface. The atypical epidermis associated with these more differentiated vesicles was limited to the most peripherial portions, i.e., furthermost from the center, of the vesicles outer wall. There were two cases not included in Table I or Fig. 9. These showed, in addition to an apparently unorganized neural mass, the development and differentiation of a morphologically recognizable eye. The neural component, the optic cup, and induced epidermal component, the lens, were both present (Fig. 10).

The isolates from stage 13 (13 cases) demonstrated neural histogenesis with a reduction in the amount of undifferentiated epidermis which is associated with the vesicle's superficial outer layer. It should be noted that, from stage 12 to stage 13, the percentage of cases which showed an organized hindbrain increased from 6% to 23% and a corresponding decrease (from 91 to 77%) occurred in the unorganized neural masses.

Stage 14 isolates (17 cases), for the most part, were similar to the previously described cases except for a slight increase in the number with organized neural tissue.

Figure 10. Two ectodermal vesicles each demonstrating eye cup (EC) and lens (L) differentiation with associated unorganized neural tissue. (Traced from a projection; scale equals 0.1 mm)


EC
L


EC
L

of the six cases developing a hindbrain, one had an associated otic vesicle.

The vesicles examined from stage 15 (25 cases) developed a larger number of secondary inductions (11 cases) which were associated equally with unorganized (6 cases) and organized (5 cases) neural masses. The organized neural system was the predominant differentiation in these isolates.

The isolates from stage 16 (19 cases) showed an equal tendency to form either an unorganized or an organized neural mass, both of which demonstrated otic vesicles. A very noticeable increase in secondary inductions over the previous stages was observed.

In summary, it should be noted that, initially (stage 12), the differentiation of neural tissue is largely unorganized (91%). The isolates from subsequent stages (13 - 16) show a reduction of the unorganized neural tissue, at first, with a subsequent increase in organized neural tissue (stages 13 - 14). With the appearance of organized neural tissue there is a corresponding increase in the number of cases showing otic vesicles (stages 14 - 16)(see Fig. 7A). However, the number of secondary inductions associated with unorganized and organized neural tissue occurred with equal frequency.

D. Presumptive Rhombencephalic Isolates Cultured with Axial Tissues

The axial tissues, notochord and somite, could be obtained from two sites along the anterior-posterior axis of the embryo: 1) directly beneath the presumptive trunk neural tissue. No discernable differences were detected in the resulting neural or mesodermal differentiations in either case. Furthermore, the stage of the embryos donating axial tissues sometimes did not correspond to the stage of the neuroepithelial donor but, again, no differences in neural or mesodermal differentiation could be detected. The number of vesicles isolated from stage 11 were insufficient and are not included in these results.

1. Presumptive Rhombencephalon plus Presumptive Notochord (Rnt)

Microscopic examination revealed that almost half of the isolates prepared for this series showed differentiated muscle tissue. This differentiation was attributed to an inability to isolate presumptive notochordal material free from presumptive somite mesodermal cells. These cases were added to (and are considered with) the Rnts series (see below). However, the remaining cases (29) provided sufficient data for consideration (see Table II and Fig. 11). Isolates from stages 12 and 13 demonstrated neural histogenesis, showing both unorganized and organized types with almost equal frequency.

TABLE II

Differentiation in Isolates of
Presumptive Rhombencephalon cultured with
Presumptive Notochord Tissue

Stage	Total number of Isolates	Atypical epidermis	Neural histogenesis unorganized	Neural histogenesis organized	secondary inductions	melano-phores
12	8	2	5	3	0	0
13	3	0	1	2	0	1
14	4	0	2	2(2)[a]	2	1
15	8	0	2	6(6)	6	5
16	4	0	0	4(4)	4	2

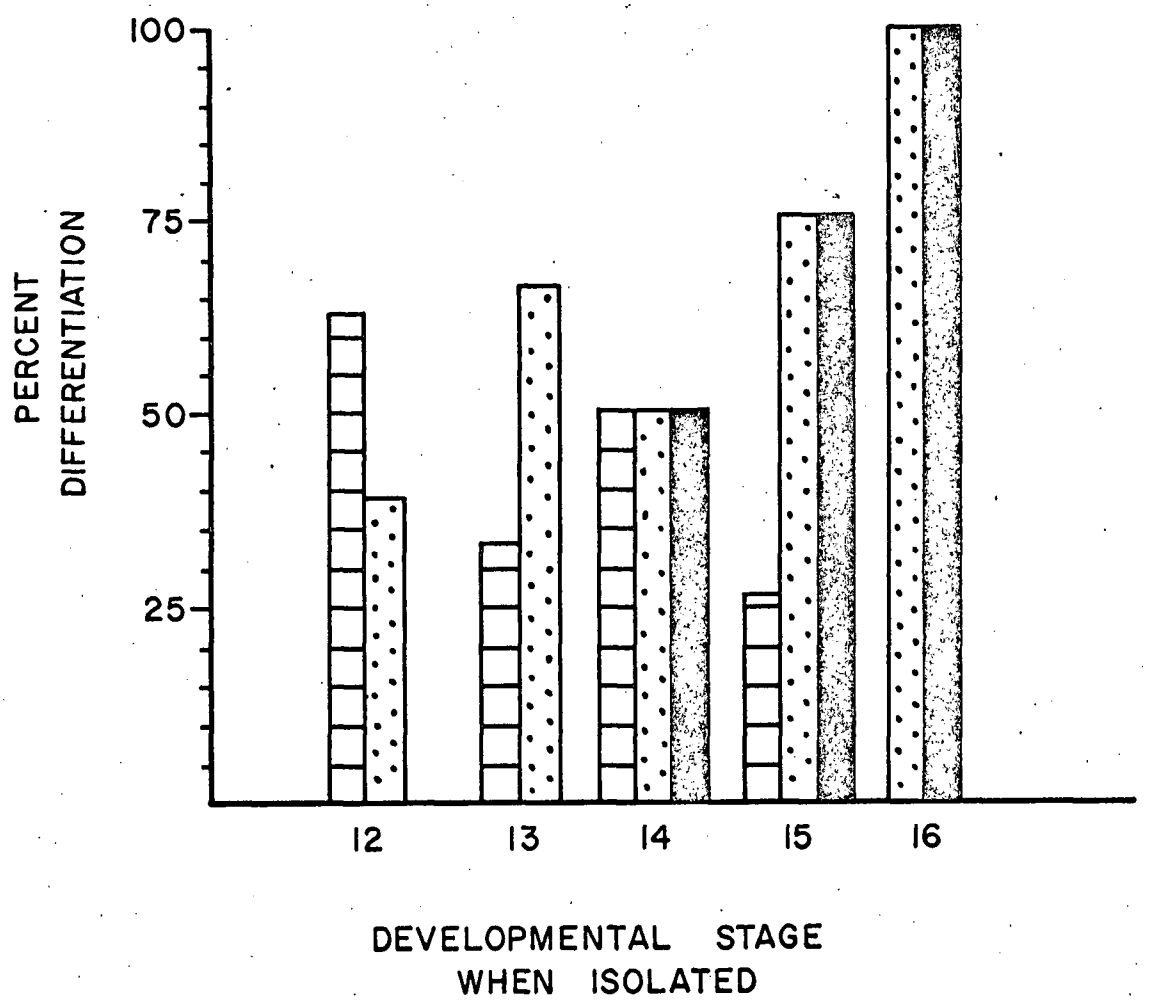
a. the number in parenthesis (2) indicates that part of the total cases (2) showing an associated secondary induction.

Figure 11. The histogenetic profile of presumptive rhombencephalon cultured with presumptive notochord (Rnt)

unorganized neural tissue

organized neural tissue

secondary inductions


100
75
50
25
PERCENT DIFFERENTIATION
12
13
14
15
16
DEVELOPMENTAL STAGE WHEN ISOLATED

Several vesicles from stage 12 showed small amounts of peripherally located atypical epidermis devoid of adhering mesenchyme, but the remainder of the outer vesicle epithelium in these and all other cases was squamous or cuboidal, with adhering mesenchyme cells. Of the four cases examined at stage 14, two had differentiated unorganized neural tissue while two demonstrated organized neural masses with associated otic vesicles. Examination of stage 15 (8 cases) revealed that a large number of isolates (6) formed an organized neural mass, all of which had associated secondary inductions. The remaining two cases formed only unorganized neural tissue.

At stage 16 all four cases demonstrated a hindbrain organization and also showed otic vesicles. In addition, melanophores were first noted in a stage 13 isolate and were found to be present in most of the subsequent isolates examined.

Beginning at stage 14, the number of cases showing hindbrain differentiations with otic vesicles increases from 50% to 75% (stage 15) and to 100% by stage 16. Furthermore, in contrast to the previous (R) series these Rnt cultures showed no secondary inductions associated with unorganized neural tissue. The apparent decrease in the percentage of cases showing unorganized neural tissue in isolates from stage 12 to stage 13, and the subsequent increase in stage 14 isolates, probably

reflects the reduced number of cases, and is not thought to have any developmental significance.

It was found that the differentiated notochord was located in a 'ventral' or 'ventro-lateral' position in relation to the differentiated hindbrain in nearly all the ectodermal vesicles examined in this series (Fig. 12). The melanophores which differentiated in this series (and in all subsequent cases) were usually distributed in the loose mesenchyme between the neural mass and the outer vesicle wall, and/or were adhering to the inner surface of the latter. Melanophores were rarely found within a neural mass or on the external surface of the vesicle.

2. Presumptive Rhombencephalon Cultured with Presumptive Somite Mesoderm (Rs)

The results of this series are presented in Table III and Figure 13.

A majority of these isolates demonstrated the ability to develop organized neural masses and secondary inductions occurred in almost all of these cases. Compared to the previous (Rnt) series, the secondary inductions are evident in 40% of the stage 12 isolates, 74% of the stage 14, and increase to 78% and 87% in the stage 15 and 16 isolates, respectively. A limited number (10 out of 41) showed only an unorganized neural mass while four cases (10%) developed only

Figure 12. Section through an ectodermal vesicle demonstrating hindbrain organization and a 'ventral' notochord (NT). (Traced from a projection; scale equals 0.1 mm)

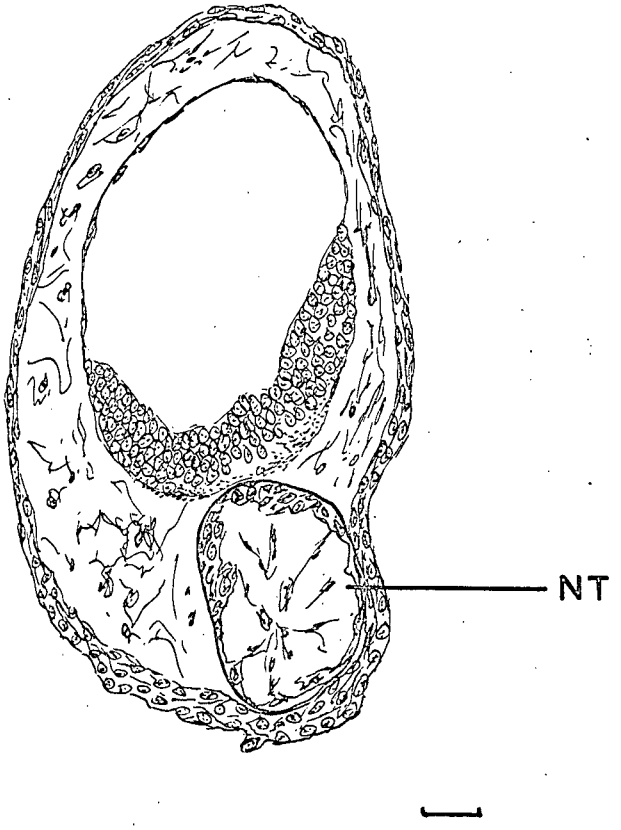

NT

TABLE III

Differentiation in Isolates of
Presumptive Rhombencephalon Cultured
With Presumptive Somite Tissue

Stage	Total number of isolates	Atypical epidermis	Neural histogenesis unorganized	Neural histogenesis organized	Secondary inductions	Melano-phores	muscle	Nephric tubules
12	10	0	5(1)[a]	5(3)	4	4	0	3
13	6	0	3	3(3)	3	1	1	2
14	8	0	1	7(6)	6	3	0	3
15	9	0	3(2)	6(5)	7	5	3	3
16	8	0	1	7(7)	1	5	4	3

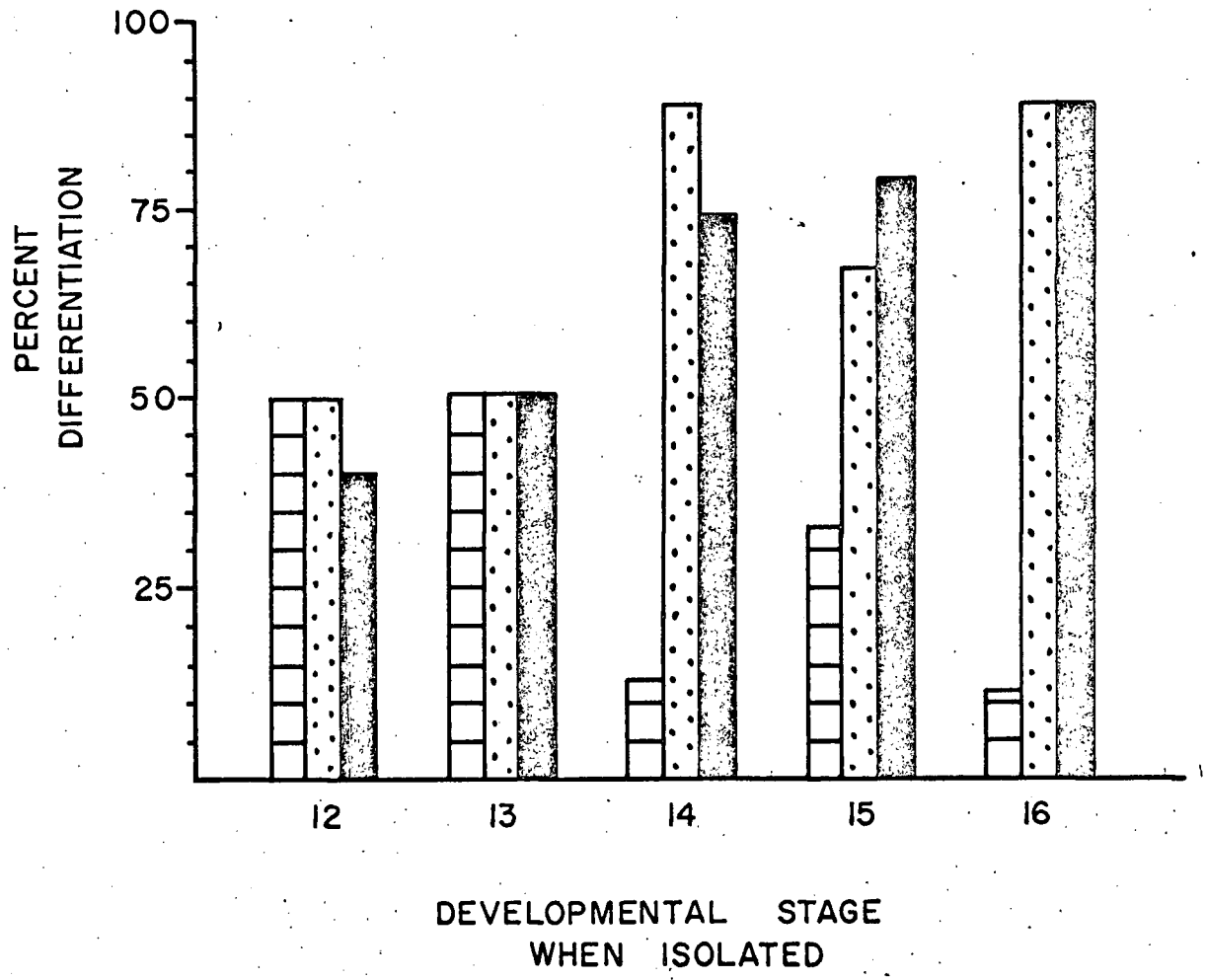
a. the number in parenthesis (1) indicates that part of the total cases (5) showing an associated secondary induction.

Figure 13. The histogenetic profile of presumptive rhombencephalon cultured with presumptive somite mesoderm (Rs).

unorganized neural tissue

organized neural tissue

secondary inductions

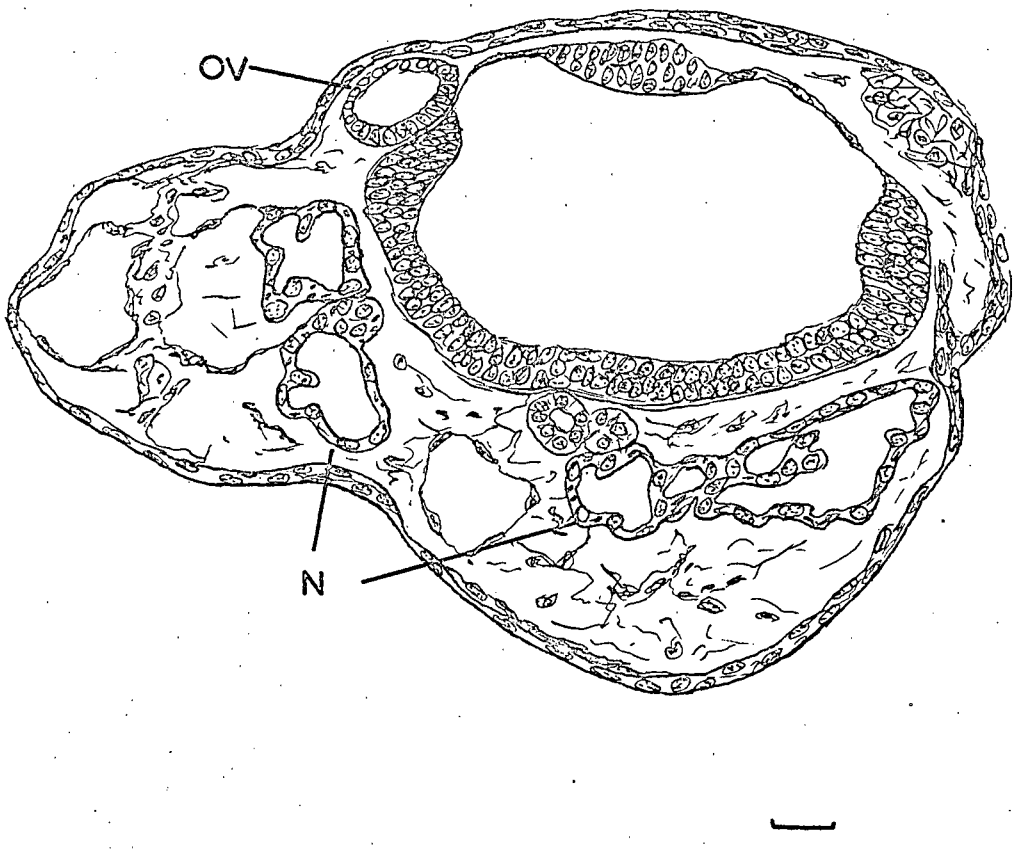

100
75
50
25
PERCENT DIFFERENTIATION
12
13
14
15
16
DEVELOPMENTAL STAGE WHEN ISOLATED

hindbrain organization with no secondary inductions. Of the number of cases showing secondary inductions (24) only three are associated with unorganized neural tissue.

Only eight of the 41 cases demonstrated any definite muscle tissue while the remaining cases revealed large masses of yolky cells representing the undifferentiated somite tissue. This observation is in agreement with those of Muchmore (1958; 1964) indicating the inhibitory role of the brain on muscle histogenesis. In addition to, and in some cases instead of, muscle histogenesis, the differentiation of nephric tubules was observed (Fig. 14). These tubules, originating from the somite mesoderm (Yamada, 1937; Muchmore, 1957; Finnegan, 1961), are characterized by the acidophilic staining of the cytoplasm and the series of thin convoluted tubules.

In all the cases examined in this series, the somite tissue (whether undifferentiated, or differentiated as muscle or nephric tubules) was found 'ventral' and 'lateral' or both, to the hindbrain. In the case of unorganized neural tissue, no axis could be established but the added tissues were limited in their distribution and usually apposed to one side of the neural mass.

Figure 14. An ectodermal vesicle demonstrating nephric tubule differentiation. Note also differentiated hindbrain with an associated otic vesicle (OV). (Traced from a projection; scale equals 0.1 mm)


OV
N

3. Presumptive Rhombencephalon Cultured with Presumptive Notochord and Presumptive Somite Tissues (Rnts).

The results of this series are presented in Table IV and Figure 15. Of 57 vesicles prepared for this series, 47 produced an organized neural mass and 90% of these cases showed associated otic vesicles. Of the 10 cases showing only unorganized neural masses, half also had otic vesicles. In contrast to the somite added (Rs) series, all of the Rnts vesicles demonstrated muscle histogenesis. Muchmore (1958, 1964) demonstrated the inhibitory role of brain tissue as compared with trunk neural tissue on somite histogenesis, while noting the enhancement of muscle histogenesis when notochord and trunk neural tissue are combined. The present observations also show that presumptive hindbrain tissue inhibits muscle histogenesis and that the addition of notochord seems to neutralize this effect of brain tissue, thus allowing muscle histogenesis to occur in these isolates. The differentiation of nephric tubules was limited to a few cases. In many of the cases examined in this series, a few large fibrous cells within the cellular mass of the hindbrain were evident but these cells were not associated with any recognizable ganglia and could not be identified positively as Mauthner cells. In two cases (stage 11½ and 12½

TABLE IV

Differentiation in Isolates of
Presumptive Rhombencephalon Cultured With
Presumptive Notochord and Presumptive Somite Tissues.

Stage	Total number of isolates	Atypical epidermis	Neural histogenesis unorganized	Neural histogenesis organized	Secondary inductions	Melano-phores	Muscle	Nephric tubules
12	17	4	4(2)[a]	13(12)	14	13	17	1
13	9	1	2	7(7)	7	6	9	2
14	5	0	0	5(3)	3	5	5	0
15	11	0	1(1)	10(9)	10	11	11	2
16	14	0	3(2)	11(10)	12	13	14	5

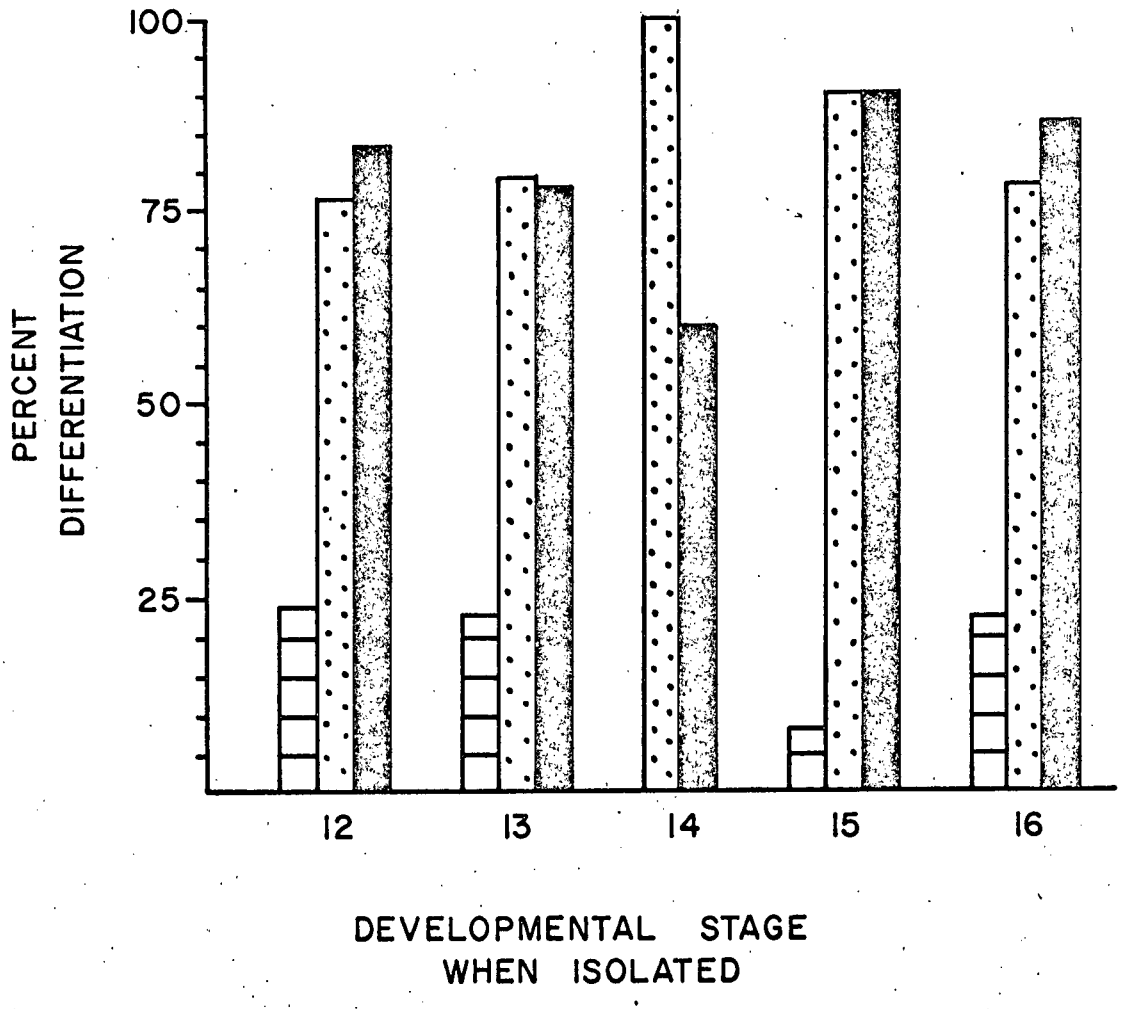
a. the number in parenthesis (2) indicates that part of the total cases (4) showing an associated secondary induction

Figure 15. The histogenetic profile of presumptive rhombencephalon cultured with presumptive notochord and presumptive somite tissues. (Rnts)

unorganized neural tissue

organized neural tissue

secondary inductions


100
75
50
25
PERCENT
DIFFERENTIATION
12
13
14
15
16
DEVELOPMENTAL STAGE
WHEN ISOLATED

isolates), the level of neural histogenesis and organogenesis with associated otic vesicles approached that seen in the normal embryo (Fig. 16).

In all of the cases examined the notochord assumed a 'ventral' position with respect to the differentiated hindbrain and the muscle tissue was spread out laterally from the notochord (e.g. Fig. 16). In those cases having unorganized neural masses, the notochord and somite tissues showed a normal relationship with each other, both tending to orient to one side of the neural mass.

E. Presumptive Trunk Spinal-Cord Isolates Cultured Alone (T)

The results of this series are presented in Table V and Fig. 17. Of the 15 isolates prepared from stage 11, eight developed as an entirely solid mass of atypical epidermis while the remaining seven demonstrated an unorganized neural histogenesis. In these latter cases a superficial cuboidal layer also differentiated although some portions of the epidermis still remained atypical. Two additional cases are not included in Table V since they each differentiated an unorganized neural mass with an associated eye. The eye possessed an optic cup and lens.

The isolates examined from stage 12 (17 cases) showed an increase over the previous stage in the number

Figure 16. Section through an ectodermal vesicle demonstrating normal hindbrain morphology. Note that otic vesicles and notochord are oriented 'normally' with respect to the hindbrain. (Traced from a projection, scale equals 0.1 mm).

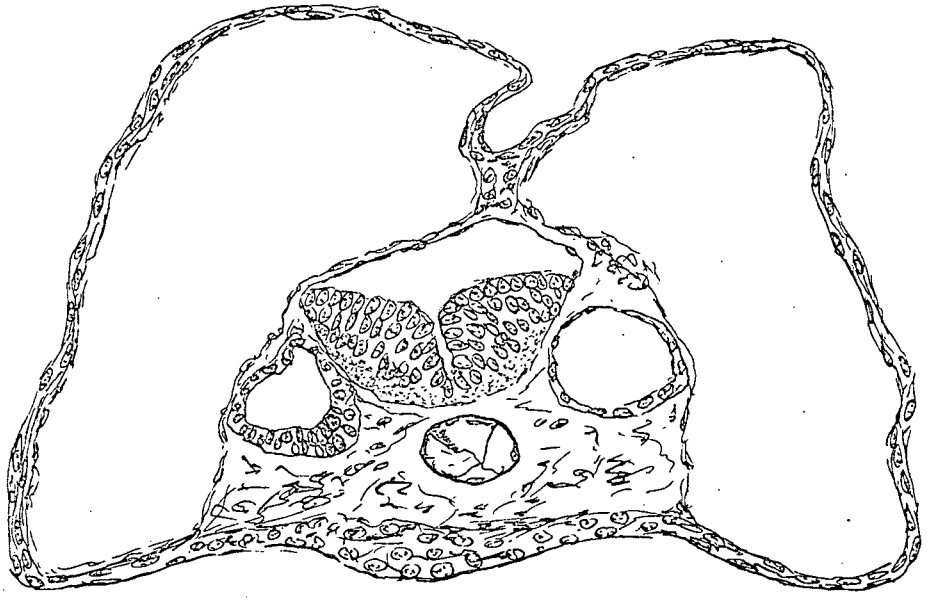


TABLE V

Differentiation in Isolates of
Presumptive Trunk Spinal-Cord Cultured Alone

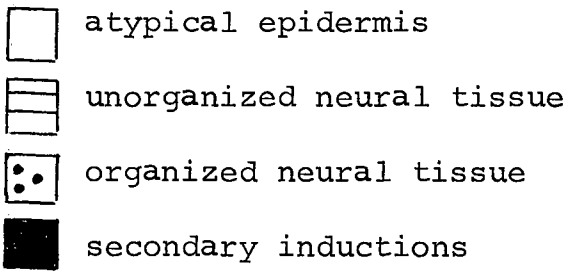
Stage	Total number of isolates	Atypical epidermis	Neural histogenesis unorganized	hindbrain	neural tube	Secondary inductions	Melano-phores
11	12	7	5	0	0	0	0
12	17	7	14(1E)[a]	3	0	1	4
13	25	1	21(3E)	3(2E) b	3	5	12
14	20	1	13	3	4	0	18
15	21	2	14(2E)	3 c	7(2F)	4	20
16	19	0	9	7 b	4(1E)	1	17
17	8	0	1	2(1E)	5	1	8
18	8	0	2	1 b	6(1F)	1	8
19	13	0	4	2	7(2F)	2	13

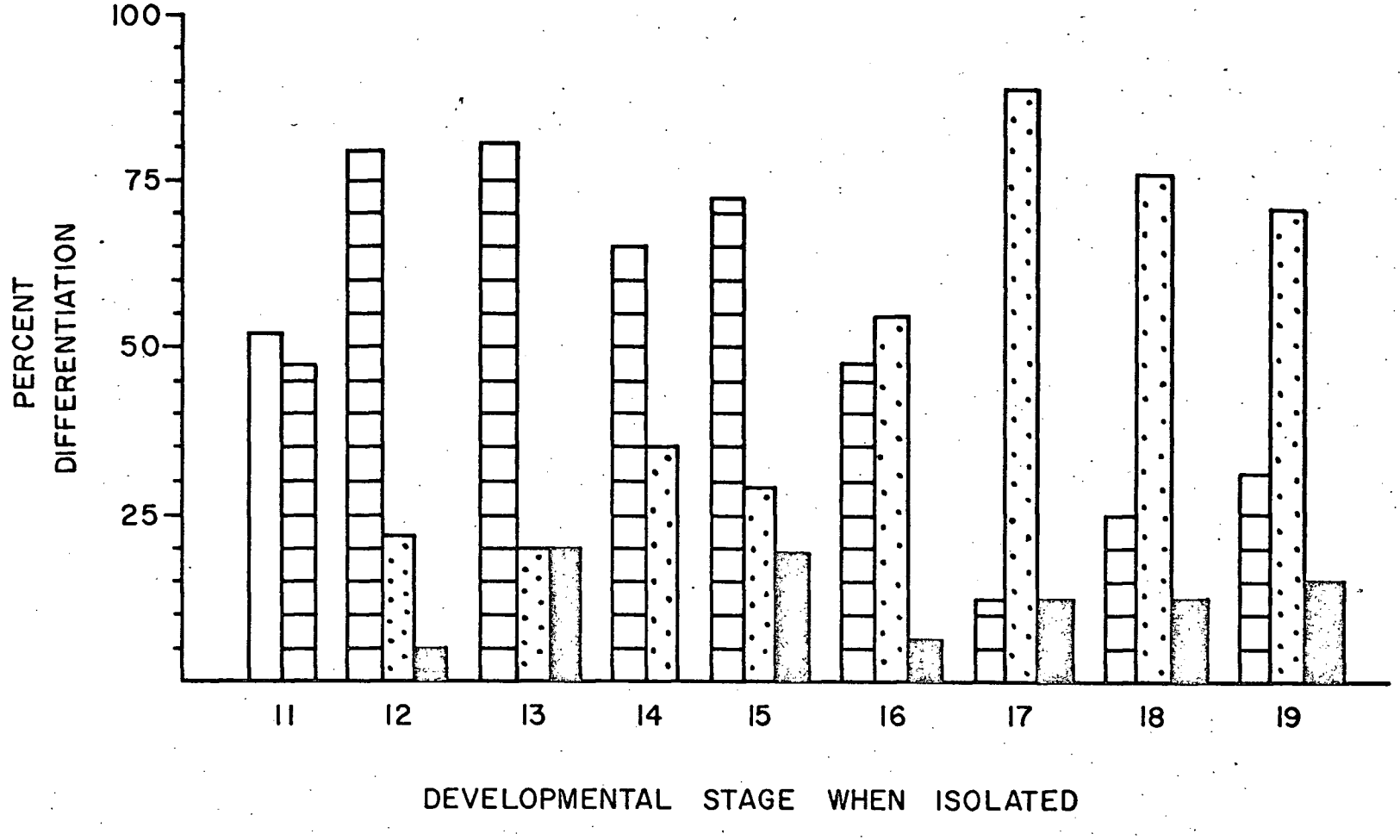
a. The number in parenthesis (1) indicates that part of the total cases (14) showing an associated secondary induction and the letter indicates the class of induction; E, otic vesicle; F, Fin.

b. includes one case which demonstrated both types of organized neural tissue.

c. includes three cases which demonstrated both types of organized neural tissue.

Figure 17. The histogenetic profile of presumptive trunk spinal-cord cultured alone (T).




100
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PERCENT
DIFFERENTIATION
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18
19
DEVELOPMENTAL STAGE WHEN ISOLATED

forming an unorganized neural mass. Half of the vesicles evidenced limited amounts of peripherally located atypical epidermis, and a small number (three cases) showed an organized neural mass of hindbrain-like morphology. One isolate, having an unorganized neural mass differentiated an otic vesicle. One vesicle differentiated a morphologically recognizable eye structure and was discarded. About 25% of the observed cases revealed the differentiation of melanophores.

The stage 13 isolates (25 cases) revealed 21 vesicles showing an unorganized neural mass of which three had an associated otic vesicle. There were five vesicles which demonstrated organized neural masses. Two formed a hindbrain and also possessed otic vesicles, two showed a tube-like neural structure, i.e. units with one lumen and with walls of more or less even thickness (see Fig. 7B), and one case had developed both. Approximately 50% of these stage 13 cases differentiated neural crest as evidenced by the appearance of melanophores.

The majority of vesicles isolated from stage 14 (13 out of 20) demonstrated an unorganized neural mass, and, of the seven which formed organized neural tissue, three were hindbrain and four were tube-like. There were no secondary inductions, but an increased number (90% of these cases) demonstrated melanophores.

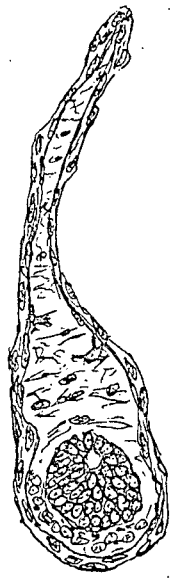
Examination of the 21 cases isolated from stage 15 indicated that 14 demonstrated unorganized neural tissue, with 2 isolates differentiating otic vesicles. Of the remaining seven cases, four were neural tube-like, with fins, and three demonstrated hindbrain-like and neural tube-like neural masses. The number of cases showing melanophores was 20 (95%).

The stage 16 isolates demonstrated an equal tendency to form both unorganized and organized neural tissue. Among the cases of organized neural tissue, six showed hindbrain three formed a neural tube and one had both types of neural structures. Only one case of fin induction (see Fig. 18) was observed in a vesicle having a neural tube-like structure, but 90% of all the cases had melanophores.

The majority of the vesicles isolated from stage 17 (7 out of 8 cases) demonstrated an organized neural mass; two possessed a hindbrain and five showed neural tube morphology. One case had developed a hindbrain-like mass with associated otic vesicles. In this group, all cases demonstrated melanophores.

Only two of the stage 18 isolates (8 cases) failed to differentiate an organized neural mass. The predominant neural structure to appear was a neural tube and two of these

Figure 18. An example of fin formation in an ectodermal vesicle with a neural tube-like differentiation. Note ladder-like arrangement of mesenchyme cells and fibers in core of fin. (Traced from a projection; scale equals 0.1 mm).



cases evidenced a fin-like differentiation. Only one case showed both a hindbrain-like and neural tube-like organogenesis. As in the stage 17 isolates, all vesicles developed melanophores.

The stage 19 isolates were similar to the stage 18 series described above, although the percentages of vesicles falling into each category varied somewhat (see Fig. 17). The stage 19 isolates demonstrated an increase in the number of cases of unorganized neural tissue and a corresponding decrease in organized neural tissues, contrary to expected results. These patterns are established in the latter stages (17, 18, 19).

A comparison between this series and the R alone series (see Figs. 17 and 9, respectively) reveals that both show a large percentage of cases containing unorganized neural tissue initially (stages 11 - 13), while the later isolates (stages 14 - 16) show an increase in organized neural tissue with a corresponding decrease in unorganized structures.

In summary; the differentiation tendency of presumptive trunk spinal cord is largely for unorganized neural tissue (stages 11 - 15), while the later stages show an increase in the number of cases with a neural tube-like configuration. None of the 36 isolates in this series which

differentiated a neural-tube-like mass demonstrated the characteristic morphology of the spinal cord seen in the normal embryo. Throughout the entire series, however, numerous cases also demonstrated hindbrain morphology, and otic vesicles were found associated with either unorganized or hindbrain-organized neural tissue (except in one case). All cases of fin induction were found in conjunction with tube-like neural masses. Evidence has indicated a direct relationship between neural crest differentiation and fin induction (Twitty and Bodenstein, 1941; Bodenstein, 1952). In this investigation a very large percentage of cases demonstrated melanophores; however, few fins developed.

F. Presumptive Trunk Spinal-Cord Cultured with Axial Tissues

The axial tissues added to the isolated presumptive spinal cord were taken from the region underlying the isolated neural tissue. This procedure could be accomplished either by isolating the neural tissue alone and adding the underlying tissue in whatever combination desired, or by excising the neural tissue together with underlying mesoderm, and then removing unwanted cells or cell layers. In either case, the experimental system revealed no detectable differences in the results.

1. Presumptive Trunk Spinal Cord Cultured with Presumptive Notochord (Tnt)

The results of this series are presented in Table VI and Fig. 19. A large percentage (almost 75%) of these cases revealed the differentiation of muscle tissue. It is felt that this tissue could arise either from cells mechanically introduced along with the notochord tissue, or as a differentiation from the more posterior regions of the trunk neural plate (see Ford, 1949), a small portion of which may have been included in each of these isolates.

Examination of the Tnt series revealed that all but 3 differentiated an organized neural mass. This neural histogenesis was of both types (hindbrain in 18 cases and neural tube in 28) with associated otic vesicles and fins. The latter induction was only differentiated in cases showing a neural tube-like neural mass, whereas the otic vesicles were associated (except in stage 12) with hindbrain structures. The later stages (17 and 18) demonstrated no secondary inductions. No cases in this series demonstrated nephric tubule differentiation, thus confirming the source of the mesoderm as other than anterior somite. The notochord always differentiated and was observed to be adjacent to the neural mass. The notochord was usually associated with the thinner wall of the neural tube (Fig. 20).

TABLE VI

Differentiation in Isolates of
Presumptive Trunk Spinal-Cord Cultured
With Presumptive Notochord Tissue

Stage	Total number of isolates	Atypical epidermis	Neural histogenesis unorganized	hindbrain	neural-tube	Secondary induction	muscle	Melano-phores
12	7	0	0	4(1E)[a]	b 4($\frac{3E}{1F}$)	5	5	7
13	8	0	2	3(3E)	b 4(2F)	5	6	6
14	3	0	1	0	2(2F)	2	3	3
15	8	0	0	5(1E)	b 4(3F)	4	6	8
16	8	0	0	3	5(1F)	1	6	8
17	7	0	0	3	4	0	4	7
18	5	0	0	0	5	0	4	5

a. the number in parenthesis (1) indicates that part of the total cases (4) showing an associated secondary induction and the letter indicates the class of induction; E, otic vesicle; F, fin.
b. includes one case showing both types of organized neural tissue.

Figure 19. The histogenetic profile of presumptive trunk spinal-cord cultured with presumptive notochord (Tnt).

unorganized neural tissue

organized neural tissue

secondary inductions

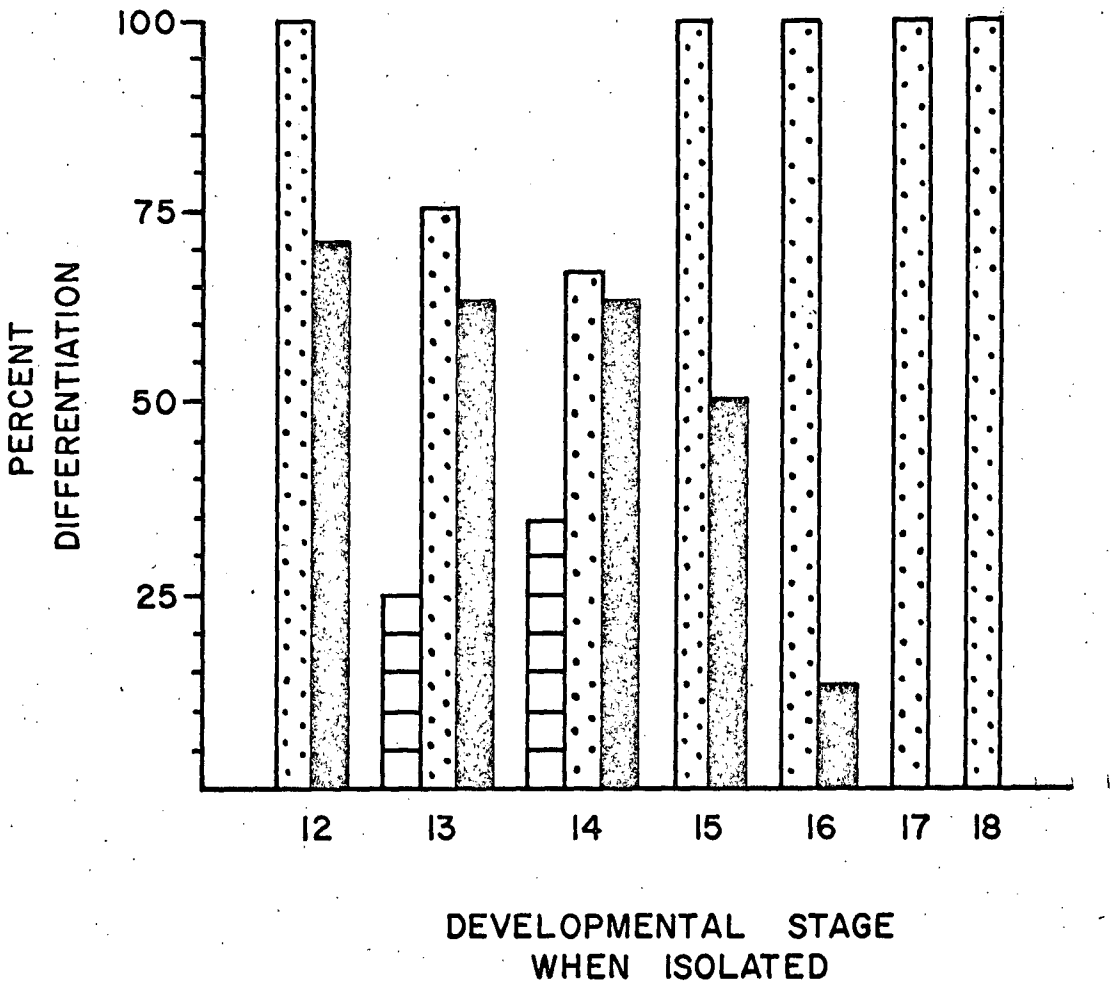
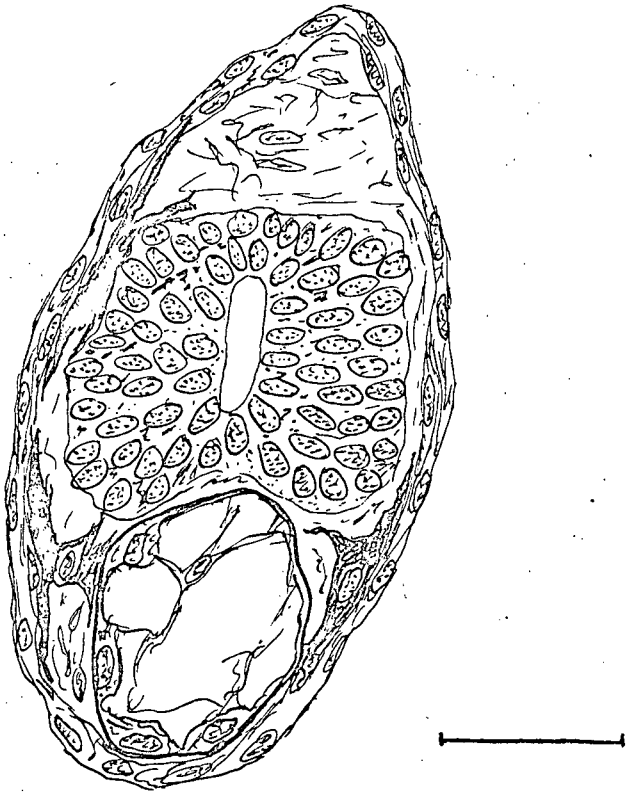

100
75
50
25
PERCENT DIFFERENTIATION
12
13
14
15
16
17
18
DEVELOPMENTAL STAGE WHEN ISOLATED

Figure 20. Section through an ectodermal vesicle demonstrating the relationship of neural tube to notochord. (Traced from a projection; scale 0.1 mm).



2. Presumptive Trunk Cord Cultured with Presumptive Somite Mesoderm (Ts).

The results of this series are presented in Table VII and as Fig. 21. The isolates prepared from stage 12 embryos (22 cases) showed, on microscopic examination, that 14 vesicles had produced an unorganized neural mass. One of these cases also showed an otic vesicle and another a fin as a secondary induction. Of the eight cases in which organized neural tissues had developed, five were hindbrain-like (of which two formed otic vesicles), and three formed a neural tube. Only 23% of these cases showed any muscle histogenesis, whereas about 90% demonstrated melanophore differentiation. With the addition of notochord (Tnts) at this stage, the percentage of cases demonstrating myogenesis increased (to 92%) and all demonstrated melanophores.

The stage 13 isolates (6 cases) failed to demonstrate any secondary inductions, but 83% of the cases differentiated organized neural tissue, as compared with 45% of stage 12 isolates. Of all the cases, half had developed muscle tissue, all showed melanophores, and nephric tubules were observed in five cases.

The results from the stage 14 and 15 isolates were identical and are considered together. In each stage, five explants were prepared. Two of these developed an

TABLE VII

Differentiation in Isolates of
Presumptive Trunk Spinal-Cord Cultured with
Presumptive Somite Mesoderm

Stage	Total number of isolates	Atypical epidermis	Neural histogenesis unorganized	hindbrain	neural tube	Secondary inductions	muscle	Melano-phores	nephric tubules
12	22	0	14(2E)[a]	5($\frac{1E}{1F}$)	3	4	5	20	0
13	6	0	1	3	2	0	3	6	5
14	5	0	2(1F)	0	3	1	2	5	4
15	5	0	2(1F)	0	3	1	5	5	1
16	8	0	0	1(1E)	7	1	6	8	0
17	6	0	0	1	5	0	5	6	4

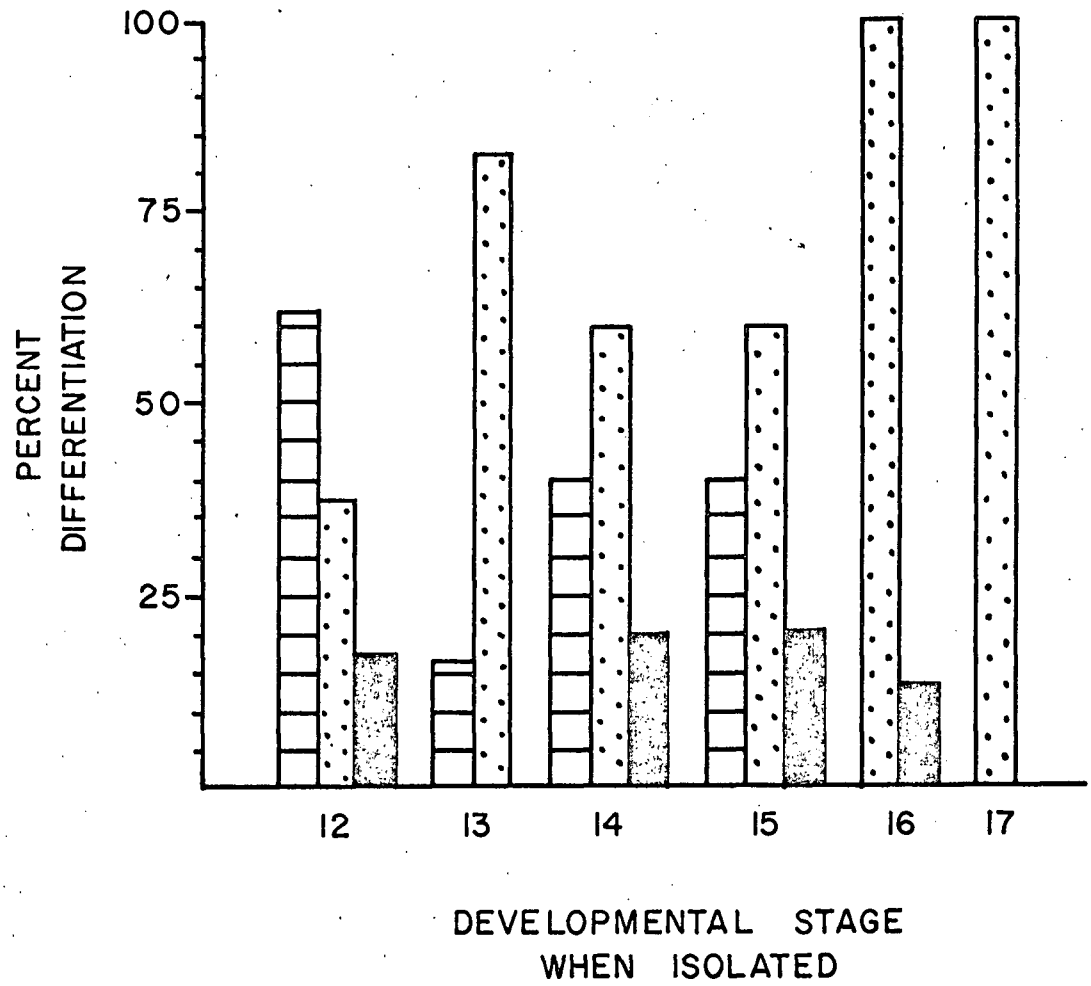
a. the number in parenthesis (2) indicates that part of the total cases (14) showing an associated secondary induction and the letter indicates the class of induction; E, otic vesicle; F, fin.

Figure 21. The histogenetic profile of presumptive trunk spinal-cord cultured with presumptive somite mesoderm (Ts).

unorganized neural tissue

organized neural tissue

secondary inductions


100
75
50
25
PERCENT
DIFFERENTIATION
12
13
14
15
16
17
DEVELOPMENTAL STAGE
WHEN ISOLATED

unorganized neural mass; one showed, in addition, a fin. The remaining vesicles (3 cases) were organized into tube-like neural masses. The observed differences between the two stages are with respect to muscle differentiation. Two cases from stage 14 and all cases from stage 15 (5 cases) showed muscle fibers. Nephric tubules were evident in four of the stage 14 isolates and in only one case from stage 15. All cases from both stages showed the differentiation of melanophores. It should be noted that one case from stage 15 isolates showed a neural tube morphology approaching the spinal-cord seen in the normal embryo of stage 40.

Of the eight cases examined from stage 16, all developed organized neural tissue, seven formed neural tubes and one had hindbrain organization, plus an otic vesicle. All cases showed melanophores. No nephric tubules were evident, but 75% of the cases showed muscle histogenesis. Those vesicles examined from stage 16 isolates (6 cases) showed no secondary inductions, one lacked muscle histogenesis, four showed nephric tubules and all differentiated melanophores. The predominant organized neural structure was of the neural tube type. It was of interest to note that, in one case, muscle histogenesis had been supplemented by recognizable segmentation.

In the cultures examined from this entire series, 50% demonstrated muscle histogenesis, always found lateral to the neural mass (Fig. 22), whereas only 17% of the Rs isolates demonstrated muscle tissue. The percentage of cases demonstrating somite histogenesis increased between stages 12 - 14 and remained at a constant level in the later stages (15 - 17). No cases of nephric tubule differentiations were observed in the Rs series, compared with 27% of the cases in the Ts series. This observation is consistent with the idea that brain tissue is inhibitory to somite differentiation, i.e., myogenesis. The neural tube morphology demonstrated in these isolates did not resemble the trunk cord in normal embryos and, in most cases, resembled the neural mass in Fig. 22.

3. Presumptive Trunk Spinal Cord Cultured with Presumptive Notochord and Presumptive Somite Tissues.

The results of this series are presented in Table VIII and as Fig. 23. The examination of the vesicles isolated from stage 12 embryos (17 cases) revealed 5 containing unorganized neural tissue, and 12 with organized neural tissues. Of the latter cases, eight had hindbrains, three possessed only a neural tube, and one case showed a neural mass composed of both regions. Of the 6 cases of secondary

Figure 22. Section through an ectodermal vesicle demonstrating neural tube morphology. Note bilaterally located masses of somite mesoderm.
(Traced from a projection; scale equals 0.1 mm)

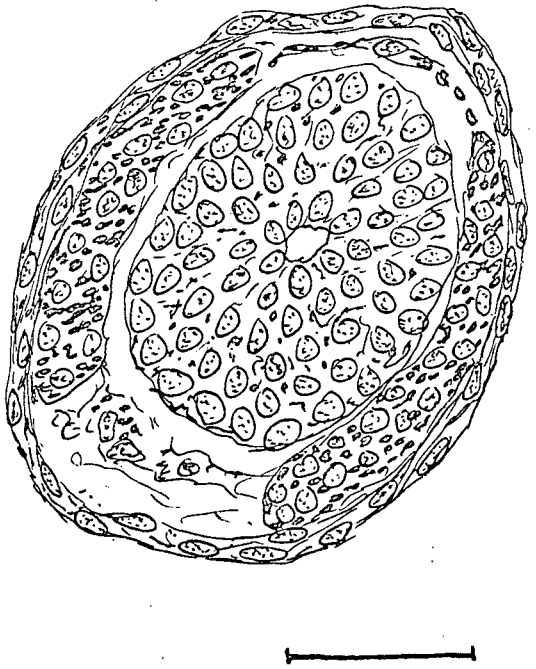


TABLE VIII

Differentiation in Isolates of Presumptive Trunk Spinal-Cord Cultured with Presumptive Notochord and Presumptive Somite Tissues

Stage	Total number of isolates	Atypical epidermis	Neural histogenesis unorganized	hindbrain	neural tube	Secondary inductions	muscle	Melano-phores	Nephric tubules
12	17	0	5(1E)[a]	9(2E)	4(4E)[b]	6	15	17	4
13	4	0	2	1(1E)	1	1	4	4	0
14	4	0	0	4	0	0	4	4	0
15	10	0	1	2	8(2F)[b]	2	10	9	1
16	8	0	0	1	8[b]	0	8	8	1
17	5	0	0	0	5	0	5	5	2
18	3	0	0	1	3[b]	0	3	3	0

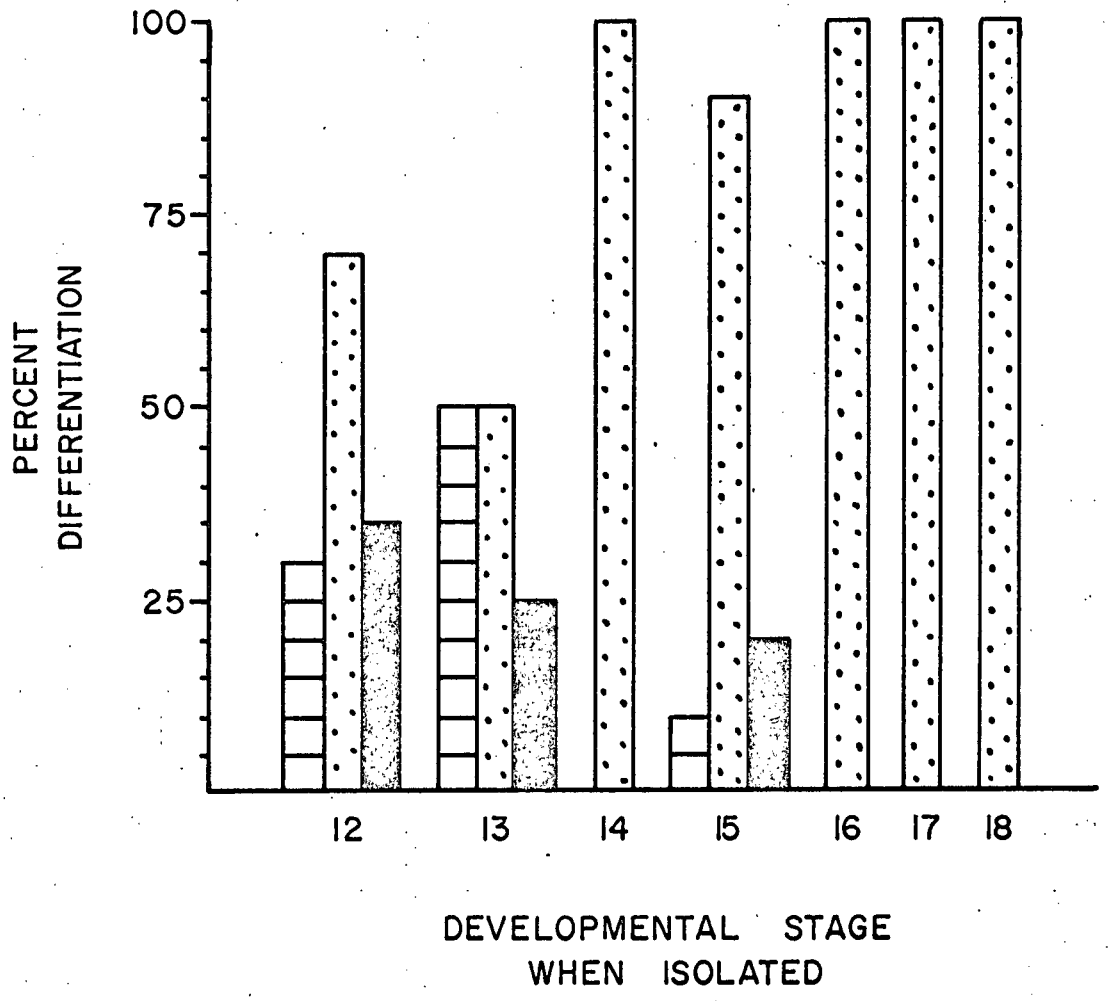
a. the number in parenthesis (1) indicates that part of the total cases (5) showing an associated secondary induction and the letter indicates the class of induction; E, otic vesicle; F, fin.
b. includes one case showing both types of organized neural tissue.

Figure 23. The histogenetic profile of presumptive trunk spinal-cord cultured with presumptive notochord and presumptive somite tissues (Tnts).

unorganized neural tissue

organized neural tissue

secondary inductions


100
75
50
25
PERCENT
DIFFERENTIATION
12
13
14
15
16
17
18
DEVELOPMENTAL STAGE
WHEN ISOLATED

inductions, the otic vesicles (3 cases) were associated only with hindbrain-like or unorganized neural tissue, whereas the fins (3 cases) were only in association with neural tubes. Melanophores were present in all cases and muscle was observed in all but one case. In three cases, the muscle tissue was segmented and four cases differentiated nephric tubules. All cases demonstrated differentiated notochord.

Of the stage 13 isolates (4 cases), two contained unorganized neural tissue and two were of the organized type. Of the latter, one formed a neural tube and the other a hindbrain with an associated otic vesicle. All four cases showed muscle histogenesis and melanophore development, as well as notochordal tissue.

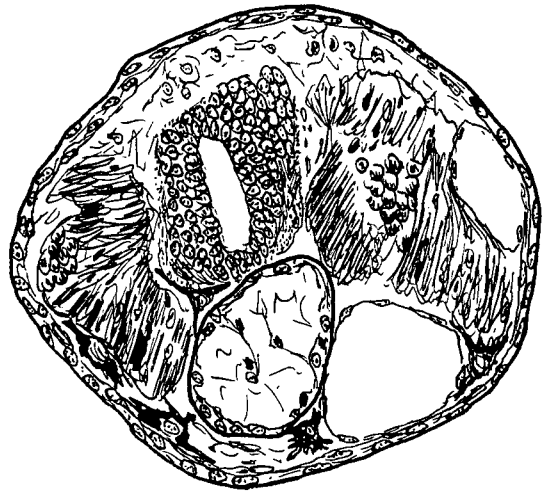
The stage 14 vesicles (4 cases) all demonstrated hindbrain organized neural tissue but with no evidence of otic vesicles or fins. All had muscle and notochoral tissue as well as melanophores.

Of the stage 15 vesicles examined (10 cases), 90% demonstrated a neural tube-like morphology, but only two of these cases had an associated fin-like structure. All the cases demonstrated muscle histogenesis, nine had melanophores and only one case showed nephric tubule differentiation.

Examination of the vesicles from stage 16, 17 and 18 revealed no secondary inductions and almost all vesicles formed an organized neural tube-like mass. The stage 16 and 18 isolates each developed one case with both neural tissue types in the same vesicle. All of the cases from these stages demonstrated muscle, notochord tissue and melanophores, while only one case from stage 16 and two from stage 17 showed nephric tubules.

In all of the vesicles examined in this series (Tnts), the notochord always differentiated adjacent to the neural mass, while the muscle tissue, formed in all but 2 cases, was usually lateral to the notochord tissue and juxtaposed to the neural mass (Fig. 24). In the isolates containing trunk neural tissue and somite mesoderm, muscle histogenesis occurred in 50% of the cases, whereas, with the addition of notochord tissue (Tnts), muscle differentiation occurred in all but 2 out of the 51 cases. This observation agrees with the previous report on the combined role of the notochord and trunk neural tissue in enhancing myogenesis in Salamanders (Muchmore, 1958). The neural tube morphology exhibited in this series was more or less similar in all cases (see Fig. 24), not quite resembling the normal configuration.

Figure 24 Section through an ectodermal vesicle demonstrating a neural tube, bilaterally situated somite masses and a 'ventral' notochord. (Traced from a projection; scale equals 0.1 mm)



DISCUSSION

The results of culturing presumptive rhombencephalon (R) alone (Table I and Fig. 10) or presumptive trunk spinal-cord (T) alone (Table V and Fig. 18) demonstrate that the intrinsic differentiation tendency of recently induced neuroepithelium from A. gracile (stage 11) (either presumptive rhombencephalon or presumptive trunk cord) is toward unorganized neural histogenesis. The ability to undergo organogenesis and induction of secondary structures (viz., otic vesicles, trunk fin) is acquired progressively by these regions at a later time (stage 13 - 14). Furthermore, while the development of normal hindbrain morphology is observed, the development of trunk cord in cultures of trunk neural tissue alone is never seen (see also Takaya, 1959). The differentiation of the neural crest, a secondary neural derivative, which is evidenced by the appearance of melanophores, occurs in the trunk-alone, but not in isolates of rhombencephalon. This seems to confirm the observation that axial (cephalo-caudal) differences exist in differentiation potentials of the neural crest (Niu, 1947). These results are contrary, in part, to results of previously reported experiments with urodele embryos (Gallera, 1958; 1959; 1960; Nieuwkoop and van der Grinten, 1961).

Gallera (1959) was able to demonstrate that the differentiation tendency of stage 11 presumptive rhombencephalon (Triturus alpestris) was toward neural crest derivatives viz., ectomesenchyme and melanophores, while isolates or grafts from later stages (12 - 13) developed irregularly defined neural masses. Gallera reported also that crest derivatives were always associated with rhombencephalic differentiations, a result contrary to those presented in this investigation. On the other hand, Nieuwkoop and van der Grinten (1961), repeating the work of Gallera (1958) in A. mexicanum, demonstrated that the presumptive rhombencephalic region becomes irreversibly determined at stage 13½, but is capable of differentiating a characteristic hindbrain morphology at stage 11. The latter observation is not in agreement with the present results. An interesting result presented, but not commented on, by these authors was the appearance of otic vesicles in stage 12 isolates and the subsequent decrease in the frequency of their occurrence during the experimental series (i.e., from stage 11 - 15). From the present investigation and others (see Jacobson, 1966), it has been shown that the competence to induce and differentiate a secondary structure (e.g. eye, ear or nose)

is progressively acquired by the tissue systems involved, although minor species differences, e.g., time of their appearance, are expected.

While according to the results of this investigation, the differentiation tendencies of the presumptive hindbrain and presumptive trunk cord cultured alone (Figs. 9 and 17, respectively) seem similar (stages 11 - 14), each region responds differently to the addition of axial mesoderm tissues. The added tissues (notochord and/or somite) demonstrate individual as well as combined effects on neural differentiation. The appearance of organized neural histogenesis and secondary inductions in the isolates of rhombencephalon (R)(stage 15, Fig. 9) is demonstrated in stage 12 - 13 isolates when somite tissue is added (Rs) (Fig. 13). The number of cases showing this enhanced differentiation is increased further at these stages by the addition of notochord (Rnts)(Fig. 15). Since the influence of notochord alone (Rnt) on neural histogenesis is not observed until stage 14 (Fig. 11), it is suspected that one of the probable roles of the notochord is to enhance somite histogenesis (see also Muchmore, 1959); the combined effect then assists neural tissue differentiation.

On the other hand, the response of the trunk neural tissue to environmental influences differs from that of the hindbrain response (stages 11 - 14). In the series with trunk cord, the addition of notochord (Tnt)(in stages 12 - 15) enhances the level of histogenesis of the neural tissue (Fig.19), while the addition of somite mesoderm (Tnts) depresses the level of histogenesis (Fig. 23). Furthermore, in these isolates (Tnt and Tnts), hindbrain organization was observed in many cases (stages 12 - 14)(Table VI and VIII, respectively). This is probably due to the fact that the anterior trunk notochord is a deuterencephalic, or hindbrain, inductor during the earlier stages (12 - 14) of neurulation (Sala, 1955). In all trunk cord isolates with added tissues, the organogenesis of the neural tissue in later stages (after stage 13) is toward neural tube. In isolates from stages 16 - 19 the neural tissue seems to develop independently of the experimental tissue environment. In the present investigation, the neural organogenesis of the trunk cord alone (T) or with added trunk axial tissues (Tnt, Ts, Tnts) never demonstrates the normal spinal-cord morphology (Fig. 6) but instead tends to form a neural tube (see Figs. 7B; 20; 22; 24). Takaya (1955) claimed that, in Triturus pyrrhogaster, isolated stage 16 brachial (anterior trunk) neural tissue will differentiate into forebrain structures in the absence of

underlying mesoderm, while the addition of varying mesodermal amounts will bring about hindbrain differentiation and/or normal spinal-cord morphology. These observations would appear to be contrary to the present results. The formative role played by the somites, i.e., thickening neural tube walls and thinning out the wall adjacent to the notochord, reported to be responsible for altering neural morphology (Takaya, 1956 a, b) does not cause the differentiation of the normal spinal-cord organization in this investigation. However, the caudalizing influence of trunk mesoderm reported by Takaya (1956a, b) did not shift the differentiating tendency of the rhombencephalon, cultured with trunk mesoderm (Rnt, Rs, Rnts), toward trunk organization. Rather, the contrary result was observed, i.e., enhanced hindbrain organogenesis.

The differentiation of a fin was a developmental tendency of the trunk cord isolates in this investigation, especially those demonstrating a neural tube. The induction and development of a fin depends on the inductive capacity of the neural crest and the competence of trunk epidermis (Twitty and Bodenstein, 1941; Bodenstein, 1952). The low frequency of fin induction in the present investigation, in spite of the many cases demonstrating neural crest differentiation, might be the fault of the present

experimental system. Earlier investigators employed embryos rather than ectodermal vesicles and it is possible that the morphogenetic movements, i.e., stretching of ectoderm and migration of mesenchyme, occurring during fin formation might have physical requirements not achieved in the present system.

This investigation has established that during early embryogenesis (late gastrulation and neurulation) both intrinsic and extrinsic factors play a role in the development of the amphibian C.N.S. The intrinsic factors are evidenced by the differentiating tendencies of the isolated neuroepithelial regions, while the enhancement or inhibition of these responses by the mesodermal tissue environment represents the extrinsic factors. The intrinsic differentiation tendency of the isolated neuroepithelium is toward neural histogenesis after being underlain by the advancing archenteron roof. The latter's influence on competent gastrula ectoderm has been examined by Saxen (1961) and a diffusible substance(s) was found that can pass a millipore filter and neuralize competent ectoderm. Furthermore, neuralization of competent amphibian gastrula ectoderm can be brought about by non-specific stimuli, e.g., pH changes (Holtfreter, 1945) or LiCl (Barth and Barth, 1963, 1964).

Recent evidence indicates that neuralizing substances can be obtained from adult fractions of adult guinea-pig tissues such as bone marrow, kidney and liver (Saxen and Toivonen, 1962); the relationship to normal inductive events has not been demonstrated.

Neural induction in the amphibian is evidenced, in this and other investigations, by the differentiation of the isolated epithelium toward neural structures; whereas prior to the contact between the epithelium and invaginating chorda-mesoderm, only atypical epidermis differentiates. Since the events of cytodifferentiation reflect, in part, specialized protein synthesis (Flickinger, 1963), a system comparable to that demonstrated in fertilization might operate in neurogenesis. Prior to fertilization the sea urchin egg is inactive biochemically and shows no incorporation of amino acids (Wilt, 1964). However, immediately following fertilization, incorporation is detected (Tyler, 1963; Gross, 1964; Wilt, 1964) and is believed to be the result of the activation of a preformed messenger-ribonucleic acid (Wilt, 1964). It is possible that in the neural epithelium, an inactive biochemical system, is "turned'on" by the influence(s) of the chorda-mesoderm, resulting in neural histogenesis.

The extrinsic influences, the product of the tissue environment, were observed in this investigation (stages 12 - 13) to either enhance or inhibit the differentiating neural tissues. Rhombencephalic development is enhanced in isolates in the presence of somite (Rs)(Fig. 13) and added notochord (Rnts)(Fig. 15). However, while trunk neural tissue differentiation is enhanced by added notochord (Tnt)(Fig. 19), the addition of somite (Tnts)(Fig. 23) inhibits neural differentiation. Therefore, the response(s) of rhombencephalon to the axial mesodermal environment appears to be different from the response of trunk spinal-cord. These observations indicate that intrinsic differences may exist in the responding tissues (hindbrain and trunk cord) with respect to the influence(s) of the axial mesoderm. The role of the mesoderm during neurogenesis has been suggested (Takaya, 1955; 1956a, b) to determine the regional character of the neural cell proliferation and cell wall thickness (see also Kallen, 1965).

An examination of other mesodermal-dependent differentiating systems may indicate the specific role of the mesoderm in neural differentiation. A series of experiments by Wilde (1955a, b, 1956, 1959, 1961) indicated that, during normal amphibian (urodele) development

(stages 11 - 14), the mesodermal tissues (specifically the roof of the archenteron) released a metabolic precursor, phenylalanine, required by the differentiating neural crest cells for melanin synthesis. This is evidence of an environmental control of cellular (neural crest) differentiation by mesoderm. Furthermore, there are indications that, under in vitro conditions, the influence of one cell type on the differentiation of another (e.g., neural crest and somite) can be mimiced by exposing the former cell type to combinations of known macromolecules (viz., nucleic acids, mucopolysaccharides, proteins) (Finnegan, unpublished; see also Moser and Flickinger, 1963; Bell, 1964; Wessells, 1964). Ambellan and Webster (1962) and Ambellan (1964) have indicated a role of added nucleotides in accelerating neural tube closure, while Finnegan and Biggin (1965) suggested that non-specific RNA causes hyperplasia of certain neural tissues. There is evidence that, in the developing chick embryo, the differentiation of the brain and notochord may be enhanced by the addition of RNA obtained from the respective tissues (Hillman and Niu, 1963). The role of the tissue environment during neurogenesis might be to elaborate metabolic precursors and/or macromolecules into the microenvironment which may be incorporated by the surrounding cell systems.

There is increasing evidence that gastrulation represents the beginning of increased metabolic activity (Brown, 1964; Brown and Littina, 1964 in amphibians; Gross, 1964 in sea urchins). In Xenopus laevis there is a substantial increase in RNA synthesis (stage 10) in the invaginating chorda-mesoderm (the inductor) followed by another increase in RNA metabolism in the overlying presumptive neuroepithelium (stage 11 - 12)(Bachvarova et al., 1966). Such increased metabolic activity by one tissue (mesoderm) could result in an increase of small molecules (amino acids, nucleotides) and of macromolecules (mucopolysaccharides, proteins) in the environment which may be preferentially utilized by differentiating regions of another tissue (the amphibian C.N.S.).

Regional differences in the developmental patterns within the nervous system might be a property of the reacting system (the epithelium) and not of the mesoderm. Inherent differences in differentiation tendencies between the presumptive rhombencephalon and trunk spinal-cord are observed (stage 12 - 14), in the presence of notochord and somite tissues. These differences in neural response might be preformed and predetermined. Curtis (1960; 1962a, b; 1963) demonstrated that, in the amphibian, Xenopus laevis, the

cortex of the fertilized uncleaved egg contained morphogenetic information. Damage to, or transplantation of, regions of the cortex could result in developmental abnormalities in the developing embryo. Cortical organization is important in the regionalization and regeneration in Stentor (Tartar, 1961). Cortical organization may, in the amphibian, as in the invertebrates (see Raven, 1958, 1961), lead to biochemically differentiated cytoplasms. As Waddington (1962) has suggested, biochemical differences, inter- and intra- cellular, can lead to differential gene activity and subsequent differentiation. It is then possible that a mosaic pattern of organization could be present in the presumptive neuroepithelium of A. gracile along the future cephalo-caudal axis prior to stage 11; alteration by metabolically active invaginating and underlying cells may thus bring about the observed progressive neural differentiations.

In summary, during late gastrulation (stage 11 - 12) and neurulation (stage 13 - 19) in A. gracile, the presumptive hindbrain and presumptive trunk spinal-cord demonstrate differentiation tendencies, initially for unorganized neural histogenesis, and subsequently for neural organogenesis. There is a progressive, gradual and cumulative

effect on the differentiation of the prospective regions of the amphibian central nervous system brought about by exposure to the tissue environment viz., notochord and somite mesoderm. While the differentiation tendencies appear similar initially (stage 11 - 14), intrinsic differences between rhombencephalon and trunk cord neural tissues are evident when these tissues are cultured in the presence of notocord and somite tissues. These added tissues show either enhancement or inhibition, both morphologically and temporally, of neural histogenesis and organogenesis.

SUMMARY

1. The differentiation tendencies of parts of the amphibian C.N.S. were investigated in an attempt to establish the regional differentiation capacity of Ambystoma gracile neural ectoderm.

2. Presumptive neural tissues (hindbrain and trunk spinal-cord) were isolated and cultured alone or with combinations of axial mesoderm (notochord and somite).

3. The tissue isolates were cultured in vitro as ectodermal vesicles for three weeks or for a period corresponding to the developmental time of a stage 40 embryo.

4. Prior to induction (stage 11) isolated rhombencephalon and trunk cord formed only atypical epidermis.

5. Subsequent to induction (stage 11) both regions demonstrated unorganized neural histogenesis, while the formation of hindbrain became evident at later stages (12 - 14).

6. By stage 15 - 16 the histogenesis and organogenesis of the isolated hindbrain region resembled that of the control whereas the isolated trunk cord only formed a neural tube.

7. The presence of somite mesoderm enhanced hindbrain differentiation considerably, notochord was effective to a limited extent, while the combined effect was greater than somite alone.

8. The addition of notochord to trunk cord enhanced histogenesis to a greater extent than either somite alone or the combination of notochord and somite.

9. The trunk neural tissue, whether alone or in combination with mesoderm, never demonstrated normal spinal-cord morphology and seemed to develop independently of the environment during the later stages of neurulation. (16 - 19).

10. The rhombencephalic differentiation was enhanced by the addition of somite tissue alone and by both somite and notochord. The trunk response was enhanced by the addition of notochord. The suggestion that inherent differentiation tendencies exist within the two brain regions is presented.

11. The possible role of the mesodermal tissues in conditioning the neural tissue microenvironment with metabolic precursors is discussed.

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APPENDIX

Using the method of Skory (1952 Automatic machine method of calculating contingency X^2. Biometrics 8: 380-382.) an r x c contingency test was performed on the results obtained in this investigation (Tables I - VIII). The appearance of unorganized, organized and secondary otic differentiations in each experimental series and at each developmental stage were compiled and subjected to statistical analysis. The probability values obtained from this analysis are presented below.

Experimental series	Developmental stages 12*	13	14	15	16	12-16
R Rnt Rst Rnts	>.01	<.025	>.005	>.1	>.005	R >.005 Rnt >.050 Rs >.9 Rnts >.005
Rnt Rs Rnts	>.005	<.500	>.900	<.5	<.5	

	12	13	14	15	16	17	12-17
T Tnt Ts Tnts	>.005	>.025	<.010	<.9	<.9	<.95	T >.005 Tnt >.005 Ts >.005 Tnts >.9
Tnt Ts Tnts	<.025	<.5	<.5	<.1	-	-	

* each developmental stage includes the categories of unorganized, organized, and secondary otic formation.