STUDIES ON THE CLOSER OF THE DURAL TUNIC IN THE EYE BIRDS
(GALLINAE DOMESTICAE)

BY

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STUDIES ON THE GLOUSES OF THE
MENOPAL TONE IN THE CHICK EGG
(GALEN DOMESTIC)
Dedicated

To

My beloved parents, my wife Mary and family for their patience and forbearance while I was engaged in preparing this Thesis.
Fig. 1: Scanning electron micrograph of edges of the neural folds of chick embryo stage 9 (seven somites) at a region ventral to the somites. X 165,500.

Note: Numerous long cytoplasmic processes (CP) of neural fold (nf) and neural groove (ng).
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INTRODUCTION

In vertebrates, the nervous system develops from the ectoderm as a flat layer of cells in the centre of the area pellucida (Fig. 2). This layer of ectodermal cells, destined to form the nervous system, has been given a number of names: thus it is called the medullary plate, neural plate or the embryonic shield.

During development, the flat medullary plate folds on itself forming a neural groove and two neural folds. The lips of the neural folds meet and fuse with each other to form a neural tube. Growth changes take place in the tube to give rise to the various parts of the central nervous system.

Much interest was, and is still, directed to understand and elucidate the nature of the process of folding of the medullary plate, the underlying mechanism and the various factors influencing it.

Many theories, postulates and hypotheses have been advanced to explain the nature of folding of the medullary plate. Thus His (1874) suggested that folding was due to the more rapid growth of the nervous system as compared to adjacent structures and the flat medullary plate must fold under the mechanical necessities of the situation. Boveri (1908) and later Hamburger (1916) believed in the existence of what they called "the autonomous folding factor". This factor caused a change in the shape of the neuroepithelial cells from rectangular to trapezoidal. The latter cells had a longer base on the ventral surface of the medullary plate causing it to be longer than the dorsal surface and folding followed. Some other workers (Beccari, 1907;
as microfilaments (Harnday, 1960) and microtubules (Waddington and Perry, 1966) were also considered as possible factors contributing to the process of folding. More recently (Gouda, 1974a and b, 1976 and 1977b) somites and cytoplasmic processes originating from the neuroepithelial cells were suggested as major factors in the process of folding. The somites play their part by exerting lateral external pressure. This possible role played by the somites, is indicated by the observation that the neural folds, at the somatic region, initially contact each other at the level of somites, rather than between them.

The cytoplasmic processes which extend from the neuroepithelial cells of one side, crossing the neural groove and interdigitate with those of the other side, play their part by creating a cohesive force between the lips of the neural folds. These cytoplasmic processes appear in early stages of neurulation, disappear at later stages and are more numerous in the rostral area of the closing neural tube where somites do not exist and between somites.

In this work the closure of the neural tube in the chick embryo was investigated in an attempt to throw more light on the mechanism of folding of the medullary plate and the possible factors that could be involved in this process.

Emphasis was laid on the study of the neuroepithelial cells, extracellular spaces and substructures, somites, cervical mesenchyme, cytoplasmic processes, microfilaments and
microtubules of neuroepithelial cells as possible essential agents in the mechanism of folding.

Neural development of the neural tube was studied with stereomicroscopy, light and electron microscopy.

The role of somites, cervical mesenchyme and cytoplasmic processes was investigated experimentally. That was done either by adding or removing somites and cervical mesenchyme and by reopening of the closed neural tube. The effect of these experimental manipulations on the closure of the neural tube, was then studied.

This thesis presents the background to this problem in the form of literature review. Then studies of neural tube development are reported followed by the experimental work. The findings are finally discussed and general conclusions are made.
Fig. 2: Light microscopic photograph of chick embryo of stage 8 illustrating the developing neural tube (*). X 100.
CHAPTER I

REVIEW OF LITERATURE
REVIEW OF LITERATURE

A. NEUROULATION

Many theories have been put forward to explain the process of neurulation in vertebrates. The following postulates will be reviewed in chronological order.

1. FOLDING OF THE MEDULLARY PLATE

1) FOLDING PRODUCED BY EXTERNAL PRESSURE FROM RELATED TISSUE (Fig. 4a)

In the opinion of many research workers, folding of the medullary plate is dependent upon the surrounding environment. His (1974) related neurulation to the rapidity of growth of the nervous system but gave no explanation for neurulation at the brain region. However, this theory was supported by Roux (1995) when he noticed that laterally exerted pressure helped neurulation while its removal resulted in flattening of the medullary plate. Hiltmeterer (1963) related the changes in the form of the neural tube to the laterally placed suture and the underlying notochord.

On the other hand, Helen (1963) assumed that the upward and median movement of the epidermal layer which is attached to the lateral margins of the neural folds, together with a change of position of the mesoderm, are responsible for folding of the medullary plate. It is obvious that such a suggestion needs to be substantiated.
2) The possibility of pressure exerted from non-neural ectoderm (Fig. 5a)

It was believed that a lateral-medial pressure exerted by the non-neural ectoderm is responsible for neurulation (Saffenberg, 1924; Neirz, 1939; Welser, 1943, and Schroeder, 1976). This hypothesis was tested experimentally by Saffenberg (1924). He isolated the medullary plate by treating a frog gastrula with hypertonic sugar solution. He reported that, in some cases, the medullary plate could succeed to fold. It is difficult to explain why only some cases showed folding of the neural plate. Possibly those in which folding occurred were least affected by the hypertonic sugar solution than the rest. On the other hand, Board (1958) stated that the pressure was exerted mediolaterally because such a force is needed to separate the splanchnic material on each side from the epiblast.

3) The theory of autonomous folding (Fig. 4a and 4b)

Ruhmbler (1902) postulated a theory in which he stated that the neural plate can fold itself autonomously. He added that changes in concentration of certain substances might lead to lowering of surface tension and increased permeability of the ventral surface of the cells in the medullary plate. Consequently, the outline of each cell becomes a trapezium instead of a rectangle (Fig. 3) and the whole ventral surface of the plate becomes wider than the dorsal. This process will lead to folding. This view was supported by Ollmer (1914 and 1915) and later Nelson (1941). These authors reported that the change in shape of the cells is associated with increase in the water content of the cell. Accordingly they suggested
Fig. 3: Diagrams illustrating folding of the medullary plate due to change in shape of cells.
that folding of the plate is a result of differential inhibition of growth by the neural plate.

In 1914 and 1916, Glaser also tried to study the manner of distribution of nuclei in the inner and outer zones of the notochord plate at different stages of neurulation (Fig. 1, AB and CD respectively). He observed that the concentration of nuclei in the outer zone increased steadily as neurulation proceeded. Glaser (1914, 1916) therefore claimed that the increase in volume of the outer zone may have provided forces leading to folding. It is obvious that this assumption was based when the notochord plate was only one cell thick. No proper explanation was given on the effect of this external factor when the notochord plate was of multiple cell thick. Again, this hypothesis does not offer any explanation for only producing a laterally folded tube and not a ball of tissue.

c. The theory of differential cell growth or cell multiplication (Eifies, 48)

This theory is based on the fact that the cells of the notochord plate are continuously dividing and migrating along the dorsoventral lines. Derrick (1937) reported that cells in the middle portions of the notochord plate divided more rapidly than those in the peripheral parts of the plate. He believed that this differential growth contributed to the folding of the neural plate. Derrick further added that the same process underlies the formation of the ventrally directed curvature of the embryo. However, he did not confirm whether the number of cells in the outer
(dorsal) and inner (ventral) halves of the medullary plate differed from each other at early, mid- and late neuration.

Other workers attributed the closure of the neural tube in the chick embryo to division and subsequent migration of cells in the intermediate part of the plate (Sauer, 1935; Bellerive, 1935; Mattern, 1936; Langman and Martin, 1944 and Martin and Langman, 1955). Such mitotic activity and migration of cells were also reported in Amblystoma (Surt, 1943; Billette, 1944, and Hutchinson, 1944).

d. The theory of apical concretion (Fig. 1a)

Many workers proposed that folding of the medullary plate was due to a contractile element placed either outside the edges of the neural cells as a gel coat, or inside them as a band of microfilaments oriented parallel to the surface of the cells (Fig. 1a).

Sauer (1935) noticed that the germinal cells of the neural tube were attached to each other at their luminal surfaces by a terminal bar net. Similar bar net or layer was also observed by Holsteiner (1943, 1946). He stated that "The amphibian eggs were provided with a special curving layer. This layer has got an elastic property which allows it to expand and contract". Waddington (1958) reported similar findings but in a later paper (1965) he regarded the dense band as a "passive accumulation of superfluous cortical material".
The importance of the superficial gel layer to the process of folding was also reported in Anlystoma by Gillatt (1944) and Lewis (1947).

On the other hand, Balkisny (1950) stated that folding of the medullary plate was due to contraction of an electron dense layer which lies just underneath the folded luminal surface of the neural plate cells. The presence of this layer of microfilaments was also confirmed in Xenopus (Schreuder, 1970 and Kerfunkel, 1971); in chick (Suggeri, 1967; Nakao and Roth, 1971; Kerfunkel, 1972 and Forton and Barson, 1974) and in rat (Preece, 1972). However, the results of the present work suggest that microfilaments play an indirect role in folding of the medullary plate.

8. The theory of actively elongating microtubules (Fig. 16)

Elongation and wedging of cells have been correlated with embryonic folding. Becerra (1968) and others have considered that such changes might be sufficient to cause folding of the plate.

Cytoplasmic microtubules which appear in early neurulation and disappear in late neurulation, lead many workers to think about the possibility of their involvement in the process in Amphibia (Waddington and Perry, 1966; Becker and Schreuder, 1967 and Kerfunkel, 1971); in chick (Keseler, 1969; Nakao and Roth, 1971; Kerfunkel, 1972 and Keseler and Auclair, 1973); in mouse (Nakano and Neufeld, 1966), and in rat (Preece, 1972).
These microtubules may possibly possess other functions. According to Carnen and Kaufman (1966) they may have a secretory function, in particular secretion of cerebrospinal fluid.

These microtubules may be responsible for change in the shape of cells since they are seen in every cell that undergoes elongation, as in the lens epithelium and in cell suspensions from the optic lobe of the chick (Peters and Zunna, 1970 and Adler, 1971), in rat epidermis (Swannon, 1963), and in cultured human cells and the endosperm of the African blood lily, Haemanthus... Katharinae (Walpor, Mertosch and Freling, 1970). This view was supported experimentally by Bannai and Roth (1971), and Karlfunkel (1972).

Some workers (Bouvier and Auduair, 1973) reported that microtubules are absent during mitosis and present in other phases of the cell cycle. They stated that these microtubules may be required for the development of the elongated cell shape and for the migration of nucleoli to the basal zone after completion of cell division.

Karlfunkel (1971) suggested that the material of the microtubules might be used to synthesize microfilaments. He based his conclusion on the fact that microfilaments increase in number when microtubules decrease in number.

Bouvier and Auduair (1974) observed some threads in the chick neural tube. In 1975 they described microtubules connecting two daughter cells: "we have not yet been able to ascertain whether true cytoplasmic continuity persists between the two daughter cells via threads; if it does, then until they degenerate the microtubules may act as a transport system along which materials could pass from cell to cell".
The work of these investigators does not provide sufficient evidence to support their view that neurulation is totally dependent on the actively elongating micromeres. The results of chapter III of the present work will throw more light on this point.

II. FOLDING OF THE CEPHALIC PART OF THE NOTOBLAST FEZ (Chap. 48)

Morris and Sokoloff (1976) used light, scanning, and transmission electron microscopy to investigate the mechanism of closure of neural neural tube. They reported that "only those neural plate cells immediately overlying the notochord were found to be bottle-shaped, forming a deep midline groove. The walls of the neural plate cells remained columnar. Neural fold formation, i.e., lifting of the neural ectoderm on either side of this deepening midline groove, was associated with accumulation of the underlying cephalic mesenchyme. The volume of subplate mesenchyme enlarged both because of increasing cell numbers and accumulating extracellular matrix."

They investigated the role of the latter in neural fold development by culturing embryos at neural plate stage for 24 hours in a medium containing fungal hyaluronidase. They noticed that the matrix was drastically reduced by the enzyme. The mesenchyme cells were clumped together but continued to increase in number. The neural fold development was much poorer than in control embryos. Morris & Sokoloff (1976) therefore concluded that the extracellular matrix has a morphogenetic role in neural fold formation especially at the cephalic region."
**Fig. 4: Regeneration**

**a**
Mechanical necessities (Hia, 1974)

**b**
Differential rates of cell growth and multiplication (Derrick, 1937)

**c**
Apical microfilaments (Baumgärtner, 1960)

**d**
Elongation of microtubules (Waddington and Perry, 1966)

**e**
Autonomic folding factor (Shohl, 1962)

**f**
Cephalic mesenchyme (Morris and Solursh, 1976)
in the chick, the somites develop from a plate of secondary mesoderm (Fig. 9a). The latter appears as a lateral growth from the primitive streak during the first day of incubation (Tessela, 1937). This plate of secondary mesoderm extends anteriorly and posteriorly (Patterson, 1907; Robertson, 1929 and Waddington, 1931 - 1932).

Segmentation of the plate of secondary mesoderm then follows. Two transverse furrows appear, one on each side of the posterior end of the notochord. These furrows constitute the anterior boundaries of the first pair of the so-called paraxial somites. After about 24 hours a similar set of furrows defines the posterior boundaries of the first pair and, additional paired intersegmental slits begin to develop still more posteriorly (Patterson, 1907 and Williams, 1910).

While the third pair of transverse furrows is forming, a longitudinal furrow appears on either side of the notochord (Williams, 1910). This furrow extends posteriorly to divide the mesoderm on each side into a thin lateral plate, and a thick spinal plate known as the somite, paraxial, segmental, or vertebral plate of mesoderm. In the chick, the total number of somites are forty-two pairs (Balch, 1925; Millik, 1928). All the somites posterior to the twenty-seventh, sometimes twenty-eighth pair (Balch, 1925) or twenty-first pair (Gans, 1943), differentiate from the indefinite cell mass of the tail bud. Not all the somites formed are so called persistent ones because some of these are rudimentary and rapidly disappear. According to Patterson, (1927), Bussard, (1930) and Miesch and Huxley, (1959) the number of rudimentary somites are two or three and appear in front of the persisting first pair. The first rudimentary somite disappears between the 10- and 14- somite stages (de Beer and Waddington, 1931). The second
one begins to break down into mesenchyme at the
18 - somite stage and disappears at the latest
before the 30 - somite stage (de Beer and Berrington
1934) although Mann and Hamilton (1958) reported
this phenomenon at the 19 - somite stage.

FACTORS AFFECTING THE DEVELOPMENT
OF THE EMBRYON

1. THE EFFECT OF HENSEN'S NODE (PL. 5b)

Partial or complete removal of Hensen's node
resulted in failure of the embryos to develop.
(Waller, 1906; and Waller and Baxton, 1907).
This was supported experimentally by Stii (1929,
1931 and 1932 a, b) who noted that Hensen's node
is vital for the development not only of the somites
but also of the embryo as a whole. He also found
that the inducing capacity of the node appears in
early stages of development and decreases in later
stages.

The capacity of the node to induce the formation
of somites is further supported by the experiments
of Hallowes (1958), Fraser (1954), Grabowski (1952,
1955 and 1956) and Hiscott (1960).

Although the results of the experiments carried
out by the above workers appear convincing, yet
other investigators believe that Hensen's node has
no effect on the formation of somites (Balston, 1935;
Waterman, 1936 and Ballinga, 1936 a). On the other
hand Fraser (1960) claimed that the role of Hensen's
node is to lay down neural tissue which in turn is
responsible for the genesis of somites.
It has been reported that an interconnection between neural tube, notochord and somites takes place in early stages of development. This interconnection led many workers to believe that the notochord and neural tube have some influence on the development of somites (Abercrombie and Hollinshead, 1954; Bellairs, 1965 b; Branch, 1966; Prolestad, Hay and Revel, 1966; Hay, 1968; and Jacob and Carter, 1974). The importance of such interconnections was proved experimentally by many investigators. Wals (1936) and Gallina (1960) reported that removal of the stretching of notochord adjacent to the paraxial mesoderm resulted in fusion of pairs of somites across the midline. On the other hand, somites were seen to develop normally in the absence of the notochord (Abercrombie and Hollinshead, 1954; Grossman, 1956 and Bellairs, 1965 a) or after removal of the chordal centres (Spratt, 1955 and 1957 a). The role played by the neural tube was tested experimentally by Patterson and Fowler (1953) and more recently by Jink (1971). These workers noticed that caudal portions of somites would not develop if the related neural tube was removed. Similar results were also obtained by Wason, (1950 and 1960 a). The latter considered that the neural tube might act in two ways: firstly, by bringing about the accumulation of mesenchymal cells which lead to formation of somites; secondly, by affecting pleasure of the segmental plate into somites.
3. THE LOREOF SOMITE CENTRES (Figs. 54).

Using the method of cellular marking, Spratt and Gordon (1947 a) reported that the somites from which somites are formed lie in and close to the primitive streak. This zone was found to be 0.23 - 0.30 mm behind the pit of the definitive streak and head process blastoderm. Later on in 1955, Spratt carried out various isolation and solution experiments to detect what he called "somite centres". He believed that there are two centres: one on each side of the posterior half of the node. Each centre was found to extend about 0.05 - 0.06 mm under the node and 0.2 - 0.3 mm lateral to it. Spratt (1957 a, 1957 b) referred to the cells posterior to the node as "Prospective somite, somatic or lateral plate cells by virtue of their positions in the blastoderm. Otherwise, they presumably differ in no way from one another, and accordingly, somite centres and any of prospective somatic cells, prospective notochord cells or prospective lateral plate cells can give origin to somites". On the other hand, Bellairs (1963 a), reported that removal of the somite centre has no effect on the development of somities. There are three possible explanations for this finding: firstly, factors other than the somite centres are responsible for the process of somitogenesis; secondly, the somite centres might have not been removed completely and their remnants developed into normal somites; and the third possibility is that the centres were removed when the process of somitogenesis had already begun in the anterior part of the embryo. Under such circumstances the process of somitogenesis will proceed in a normal way (Catton, 1973).

4. THE EFFECT OF REGRESSION MOVEMENT (Fig. 50.).

The regression movement of the primitive...
struck was thought to play a role in the development of somites. Various authors (Peckles, 1930; Sproat and Gaddum, 1931; Fraser, 1936; Bellairs, 1933 a and 1934) agreed that the axial region alone develops as a result of direct activity of the streak. Fraser (1936) believed that the regression movement lays down neural tissue which is the important factor for somitogenesis. On the other hand, Bellairs (1933 a & 1934) noted that regression movement causes migration of the cells on either side of the primitive streak and is controlled by the area pellucida as a whole. She further added that this movement is capable of stimulating the differentiation of somites.

5. THE EFFECT OF ECTODERM, EMBRYONIC AND LATERAL PLATE MESODERM (PL. 55)

Some workers believe that the ectoderm has a role in the formation of somites (Watson, 1936; Jackson, 1936). Watson (1936) reached this conclusion when he noticed that cultured hypoblastic tissue gave rise to nervous tissue, notochord, mesoderm and somites. Similar observation was obtained by Jacobson (1936) who found that in the presence of the streak, the presumptive mesoderm is invaginated from the epiblast.

It was reported that the endoderm in the vicinity of Hensen’s node is important for growth in general. Waddington (1938) and Suevic (1932) investigated such importance by removing the endoderm layer in embryos of primitive streak to head fold stages. They noticed cessation of growth and abnormality of developed somites. Fraser (1936) reported that the endoderm, especially in the region of Hensen’s node,
influenced general growth. On the other hand, Belaidis (1963 a) carried out an experiment in which she removed all three layers (ectoderm, mesoderm and endoderm) in the nodal region. She found that somites still developed normally.

The importance of the lateral plate mesoderm to the genesis of somites is not excluded. Spratt (1957 b) pointed out that "somite centro + prospective lateral plate cells can give rise to somites". This is in agreement with Belaidis' findings (1963 a).

5. OTHER FACTORS (p. 51)

Some workers believed that segmentation of the paraxial mesoderm is a predetermined process, or in other words: it is due to the activity of inherent factors which are already present before the onset of segmentation (Harnby and Semlyen, 1939). This view was supported by Webster (1973). She stated that "segmentation of the presegmental mesoderm is not dependent on its continued association with any of the immediately surrounding tissues - the ectoderm, endoderm, lateral plate, neural tube and notochord. It is an inherent property".

Other factors, of chemical nature were also reported to affect somatogenesis. Fraser (1939) reported that certain phospholipids, such as phosphatidyl choline, inhibit somitogenesis. Other phospholipids such as choline, lecithin, lecithin, and myo-inositol inhibit somitogenesis. According to Rathke (1934) and Horne et al (1955), compounds of lecithin inhibit somite segmentation.
while addition of leucine was found to increase the rate of acetylation (Boucher, 1959). Other substances like reduced sulphur (SH group) of glutathione, methionine and especially cysteine were found necessary for normal development (Bradman and Washington, 1955; Proctor, 1960a and b; and Stone, 1966). Similar importance was given to purine (Bradman and Perry, 1953).

Some of the views reported above are inconclusive since it is difficult to be certain of the complete removal or isolation of preservative tissue at a time when the whole embryo is very small. There is evidence of active movement of population of cells (Nelson, 1953), as well as visible connections between cells of the three layers (Bourochevski and Bellesco, 1966; Bellesco, 1968 and Scheridan, 1966). Trelaut, Hay and Reval, 1966; Hay, 1969; Jacob and Christ, 1973 and Lam, 1974 a, b). It seems improbable that a single factor by itself could be held responsible for gastrulation when there is abundant evidence of interactions between cells and their environment (England, 1969).
KEY TO FIG. 8:

Diagrams illustrating the developing chick embryo and the earlier structures implicated by the various theories advanced to explain somatogenesis.

cot. = ectoderm
cod. = endoderm
H.n. = Hensen's node
lmt. = lateral
n.t. = neural tube
notch. = notochord
pres. = presumptive
S.c. = somite center
FIG. 2 SOMATOCENESIS

Presumptive areas (after Pasteels, 1937)

The effect of Hensen's node

The effect of neural tube and notochord

The effect of somite centres

Regression movements (after Pasteels, 1937)

The effect of ectoderm, endoderm and lateral plate mesoderm

The effect of amino acids
C. METHODS AND MEDIA OF CULTURING PARTS OR WHOLE OF CHICK EMBRYO IN VITRO

Owing to the inaccessibility of chick embryos in vivo, several attempts have been made to use in vitro methods (Carrel and Djerassi, 1911). The following gives some methods of culturing whole or part of chick embryos. These are described in chronological order.

1. GLASS VESSEL OR WEAVER METHOD (Pflüger, 69)

Aschauer (1906); Volmer (1906); Schmidt, (1937) and Rommoff (1943) used the glass vessel method to carry out their experiments on chick embryos in vitro. This method consists of pouring the yolk and the surrounding albumen into a glass vessel having a rather greater capacity than that of the ordinary egg shell. The yolk is arranged so that the blastoderm floats uppermost, and a wire or celluloid ring is placed over it to prevent the yolk from floating to the surface. The vessel is filled up with albumen and covered with a glass lid, and placed in the incubator at a temperature of 40° C.

2. HANDLING EMBRYO METHOD (Pflüger, 6-8)

In this method, the blastoderm is explanted into drops of nutrient medium suspended from coverslips. The medium is composed of egg extract. This technique gave better visualization of the embryo but the development was much less than that in other methods of culturing (Gower and Chippollett, 1932 and others). It seems that the main reason is the inessential surface of the drop of nutrient medium with that of the expanding blastoderm.
3. CULTIVATION (p. 5-3)

Monhertet and Whipple, 1927; Schmidt, 1930
and others used a medium of plasma clot for
culture: their chick embryos. They used the
method of Carroll and Harrows (1911) of obtaining
the plasma. This method is to draw blood from
the external jugular vein of cat, dog or rabbit
through a glass cannula, coated with olive oil, ..
into ice cold paraffin tubes to prevent clotting.
The blood is then centrifuged and the supernatant
plasma refrigerated.

The blastoderm and the vitelline membrane
around it were removed and put in Locke's solution,
kept at 37°C on a water bath. The blastoderm was
freed from the vitelline membranes and adherent yolk.
The blastoderm was then floated into a cover glass
with its dorsal surface in contact with the cover
glass. The excess of Locke's solution was removed.
A few drops of plasma were put on the blastoderm
and when this was congealed the cover glass was
inverted over a hollow glass slide containing a
drop of water and the margins were sealed with
paraffin. The specimen was then incubated at
37°C.

A similar method was employed by Wolff and
Sisson (1935) but they used clotted paraffin
extract instead of a plasma clot between the watch
glasses.
4. **A METHOD USING ALBUMEN**

S innovate (1919), Spratt (1917) and others used a medium composed of albumen to cultivate chick embryos of definite age struck and had processed stages. They were of the opinion that such a medium is essentially adequate for the development of the chick embryo provided that optimal pH condition were permanently maintained.

5. **A METHOD USING ALBUMEN, COW'S MILK, SOLUTION**

S innovate (1914) cultivated chick tissues in vitro using a medium consisting of egg albumen and trypsinized pepsin solution in equal part + 1.5% sugar in Ringer's solution.

In 1919, Spray added varying proportions of egg yolk, Ringer's solution and extract of capsules tissue to egg albumen medium. He reported that the medium was unsatisfactory because it was not possible for him to see what was taking place within the medium.

6. **WATER GLASS METHOD**

Ruddington (1931, 1932) described a simple method for cultivating chick embryos in vitro. He put a layer of cotton wool with a hole in its centre in the floor of a sterile petri dish. This layer was conditioned with boiled water to ensure adequate humidity. A watch glass was then put on the centre of the layer. A medium composed of 1/3 plate of salt + salting extract of foetal tissue (1 day chick embryo, stained in salting and centrifuged, the supernatant was then allowed to settle). The supernatant was then placed over the clotted medium. Spratt
(1947 b) also used a medium consisting of fischer's albumin and fischer's agar.

7. EGG SHELL AND ALBUMEN (Ch. 5-7)

Syderly (1926), Price and Fowler (1940) used another method for culturing chick embryos in vitro. Syderly removed about a square inch of shell from the large end of the egg, together with a little albumen. The egg was then placed vertically in an incubator just tall enough to clear the egg. Syderly (1926) reported that the embryos developed normally from the 24th to the 110 hours of incubation.

8. VITELLINE MEMBRANE METHOD (Ch. 2-3)

Ras (1955) described this technique mainly to overcome some of the limitations associated with the previous techniques. He carried out the whole technique under sterile conditions. The egg at a required stage of development was opened by tapping round the broad end with the handle of a needle. Thick albumen was removed with blunt forceps. The yolk sac and remaining albumen were poured into a deep dish containing sufficient gassed & carbonated saline to cover completely the yolk, which rotated so that the blastoderm came to the top. A watch glass was then placed in the dish ready to receive the blastoderm. The vitelline membrane was then cut with scissors along a line running slightly above the equator of the yolk. Thereafter the membrane with the adherent blastoderm was placed in the watch glass, blastoderm side upwards, and held in place by a glass ring, with a diameter of 32 mm. The vitelline membrane was stretched around the glass ring and
the egg was treated with saline. The watch glass was then transferred to a Petri dish. The bottom of the Petri dish was covered with water and cotton wool which acted, as in clot techniques, as a moist chamber. All but a few drops of saline were removed from above the blastoderm. The saline under the membrane was also removed and replaced with thin albumen. The whole set was then put in an incubator.

Now's method is an extremely effective method. It is also simple to use, because it does not involve preparation of culture media, with their attendant sterilization problems.

The survival of an embryo depends on the fact that many cell particles to be seen within the cytoplasm of the cells during the early stages of development. The source of nourishment however is largely exhausted by the time, in a normal embryo, when the circulation becomes and nutrition is drawn from the blood now circulating in the area vasculosa. The failure of this second source of nutrition in this and other in vitro methods, accounts for the fact that survival of embryos in vitro is usually short periods.

The efficiency of Now's technique was tested in this study. Eight embryos cultured in vitro using this method were compared with other eight embryos cultured in vivo. Over all specimens no abnormalities in development could be detected (see data p. 1).
Fig. 6: Diagrams illustrating the various methods of culturing chick embryos in vitro.

1. **Glass Vessel or Beaker Method**

   ![Diagram of Glass Vessel or Beaker Method]

   - Albumen
   - Plastoderm
   - Glass ring
   - Yolk
   - Wire mesh

2. **Tapping Drop Method**

   ![Diagram of Tapping Drop Method]

   - Coverslip
   - Nutrient medium
   - Plastoderm
3. CLOTH METHOD

7. BIRD SHELL CAP METHOD

8. VITELLINE MEMBRANE METHOD

(AFTER LEM. 1935)

Fig. 6: Methods of culturing chick embryos in vitro
CHAPTER II

SCANNING ELECTRON MICROSCOPICAL
STUDIES ON THE CLOSURE OF THE NEURAL
TUBE OF CHICK EMBRYO
STEREOSCANNING ELECTRON MICROSCOPICAL STUDIES ON THE CLOSURE OF THE NEURAL TUBE OF CHICK EMBRYOS

Introduction

The role of cytoplasmic processes (Coulon, 1974a and b, 1976a and b and 1977a) in the closure of the neural tube has received little attention. These cytoplasmic processes protrude from the apices of the neuroepithelial cells of one neural fold and interdigitate with those of the other side. It was suggested that such interdigititation is responsible for the folding of the neural folds and their further approximation to each other. Such view was also supported by Baumcroft and Bobitza (1975). They applied the term "specialized threads" to describe these cytoplasmic processes.

In this work the surface topography of the neural tube and the cytoplasmic processes were studied at various stages and in various levels of the closing neural tube using stereoscanning electron microscopy. Special attention was paid to changes in their general appearance, shape, length and number to see whether any of these features could support their involvement in the closure of the neural tube.

Materials and Methods

Forty specimens (3 embryos of each stage 7 to 11 inclusive) were prepared for stereoscanning electron microscopy. All specimens were fixed in Karnovsky's solution (buffered to pH 7.2 - 7.5) for 24 hours, immersed in cacodylate buffer (pH 7.2 - 7.5) for 24 hours, then post fixed in 2% osmium tetroxide for 30 minutes, rinsed twice in water for 5 minutes and finally dehydrated in
acetone (50%, 70% and 100% ten minutes in each concentration). After dehydration all specimens were dried to a critical point using liquid CO₂ and then coated with gold-palladium alloy (60% gold and 40% palladium) in an Edwards's coating unit model No. 306. The specimens were then examined with the Cambridge Scientific instrument, "Stereoscan" scanning electron microscope (S3 -10). They were photographed with a tilt ranging between 35° and 45°. The E.M. operated within 10 - 20 KV. Micrographs were made on 35 mm. film at original magnification ranging between x11.2 - 13,500 and photographically enlarged four times in print, giving a final magnification of = 244.8 to 54,000.

METHOD AND OBSERVATIONS

a) Stages 7 and 8 (Procraditory Step)

At stage 7 the neural fold and neural groove were well formed at the cephalic or brain region (Fig. 7a). Usually, the formation of the neural tube had just begun and there was an indication of the developing neural groove. The first somites appeared as two ectodermal swellings just distal to the cephalic neural groove.

In stage 8 host first somites (Fig. 7b) the neural folds are well formed. Few and rather thin cytoplasmic processes ran across the neural groove. Cilia were found protruding from the neuroepithelial cells on the floor of the neural groove. Clusters of unknown bodies, varying in shape and size were also observed.

At the region of the first somite (Fig. 7c), the cytoplasmic processes were abundant. They were
thick and short. Microvilli were also seen as well as the unknown bodies. Surface topography of neuroepithelial cells could be discerned. They were fusiform or rectangular in shape.

Caudal to the first somite (Fig. 7d), the appearance of the neuroepithelial cells forming the walls and the floor of the neural groove were evidently fusiform or rectangular. The cytoplasmic processes were thin, short and slightly more in number. At that level, the unknown bodies were also present. Microvilli were seen emerging from the surface of the neuroepithelial cells especially at their margins.

At stage 6 the cranial part of the developing neural tube became wider and thicker and the neural groove extended more caudally (Fig. 8a). Caudal to first somite (Fig. 8b), the neural folds were well formed with numerous small processes protruding from the lips.

At the level of first somite (Fig. 8c) the neuroepithelial cells of the neural groove were found in all forms. No cytoplasmic processes or unknown bodies could be seen.

Between first and second somites (Fig. 8d) cytoplasmic processes, cilia and microvilli were present together with the unknown bodies. Their shape and number were probably similar to those seen at stage 7.

Caudal to the last somite (Fig. 8e) the neural folds were well formed, the neural groove was deep and contained numerous unknown bodies. No cytoplasmic processes were found crossing the neural groove. However, microvilli were found in the neuroepithelial cells.
At stages 9 and 10, the neural folds and neural groove extended more caudally, the cranial part became longer and the posterior neuropore developed (Figs. 3a and 10a). The neural folds developed their segmentation. At the cranial part of the neural groove some projections were found joining the neural folds (Fig. 10a).

Anterior to somites, in stage 9 (Fig. 3b) cytoplasmic processes were numerous, thick and long. Some of them crossed the neural groove and interdigitated with each other.

Unknown bodies were also found in numerous numbers. At the same level in stage 10 (Fig. 10b) the number of the cytoplasmic processes had increased. They were rather thinner but more of them were found crossing the neural groove. Several unknown bodies were present.

At the level of the first somite in stage 9 embryo (Fig. 3c) a cluster of unknown bodies filled the neural groove, and the cytoplasmic processes were less in evidence. That observation applies also to the same level of stage 10 embryo (Fig. 10c).

The appearance of the neural groove in stage 9 and 10 embryos, at the level between first and second somites (Figs. 3d and 10d) and caudal to last somite (Figs. 3e and 10e) was probably very much smaller. Unknown bodies were still present. Still, microvilli and cytoplasmic processes were observed. The cilia projected from the centre of the neuroepithelial cells (Fig. 10f). They were uniform in thickness and were devoid of terminal hair stick appearance. The cytoplasmic processes protuded at neuroepithelial cell junction. They were characterised by drum stick end and ANF bone (Figs. 3e and 10e). Many oogonia were observed on the surface of the neuroepithelial cells.
c) **Stage 11 (Stage of limb formation)**

At this stage, the neural folds had joined each other forming a complete neural tube (Pl. 11a), which had a segmented appearance.

Rostral to first somite (Pl. 11b), the cytoplasmic processes became less in number as compared to previous stages.

At the level of the first somite (Pl. 11c), the neural groove had closed. Some unknown bodies were seen on the surface and inner, rather short, cytoplasmic processes emerged from the cells.

Basically similar appearances were observed between first and second somites (Pl. 11d) and caudal to the last somite (Pl. 11e).

**SUMMARY AND CONCLUSIONS**

At the earlier stage of development examined (proparotory stage - stage 7 and 8) the neuroepithelial cells were either fusiform or rectangular in shape.

Microvilli, similar to those reported by Porth and Barsan, 1974 projected at cell junctions. Sialins were also found in the neuroepithelial cells. There was no change in general feature or distribution of microvilli or of sialins at different sites of the developing neural tube or at different stages of its development.

The cytoplasmic processes were found at all stages of the developing neural tube. They were processes which were thinner than sialins, with drumstick terminal end bulbous base. They arose from any part of the cell surface. These cytoplasmic processes were probably the neural processes of fusiform cells (Coady, 1974).
The cytoplasmic processes were few at stage 7 and became more obvious at stage 8. They were mostly present at the region of the developing brain or between somites. At the region of somite they were few. These processes projected from the cells of the side of the neural folds towards the neural groove to crowd it and interdigitate with those of the opposite side.

At stages 9 and 10, the cytoplasmic processes became longer and thicker and were still more numerous at regions devoid of somites. That is at the brain region and in between somites. At that stage, the neural folds came closer to each other.

At stage 21, the cytoplasmic processes became shorter and fewer in number. The neural folds came into contact.

The appearance and distribution of the cytoplasmic processes and the manner by which they crowd the neural groove to interdigitate with those of the other side suggest that they may, in some manner, influence the approximation of the neural folds and probably create a cohesive force between the lips of the neural folds. The cytoplasmic processes may creep along each other or shorten in such a way as to close the neural groove. The fact that the cytoplasmic processes tend to increase in number in regions devoid of somites, lend support to the hypothesis that they become more essential to closure of the neural tube at these sites.

The accompanying electron microscopic study demonstrated the general appearance of the developing neural tube, the shape of the cell, the processes of cell, microvilli and cytoplasmic processes. The appearance, distribution and relation of the latter processes were suggestive of the function they play in the closure of the
neural tube. The presence of openings or holes in some of the neuroepithelial cells suggest that they may be fragments of the neuroepithelial cells or some secretory products of these cells.

Rounded, spherical or oval bodies of different size were observed at all stages filling the neural groove, either single or in clusters. These bodies were more numerous at regions between somites rather than at the level of somites. They were also present in greater number at later stages of development. The nature of these bodies remains unknown. These unknown bodies were reported by Tarin (1972) in Xenopus laevis and by Souda (1974a) in chick. It had suggested that these unknown bodies could be cellular debris or yolk cells (Souda, 1974b).

In an attempt to investigate the nature of these unknown bodies (Souda, unpublished work) developing chick embryos were washed thoroughly before examining them by scanning electron microscopy. The bodies did not disappear from the neural groove. Though their nature remains unknown we could exclude the possibility of their being microorganisms because of the strict aseptic technique used.
Fig. 7.: Scanning electron micrographs of dorsal surface of developing neural tube of chick embryo of stage 7.

**Note:** Cilia (c), cytoplasmic processes (cp), microvilli (mv), neural fold (nf), neural groove (ng), neuroepithelial cells (nc) and unknown bodies (u).
At the sonite region X 42,000

Caudal to the sonite X 5160
**FIG. 8**: Stereoscanning electron micrographs of dorsal surface of the closing neural tube of chick embryo of stage 8+.

**Note**: Cilia (c), Cytoplasmic processes (cp), Microvilli (mv), Neural fold (nf), Neural groove (ng), Neuroepithelial cells (ne) and Unknown bodies (b).
Between first and second sonite X 25,200

Caudal to the last sonite X 2520.
Fig. 9: Stereoscopic electron micrographs of dorsal surface of the closing neural tube of chick embryo of stage 9.

Note: Cilia (c), Cytoplasmic processes (cp), Microvilli (mv), Neural fold (nf), Neural groove (ng), Neuroepithelial cells (ne) and Unknown bodies (b).
Between first and second sonite X 48,000.

Causal to the last sonite X 24,000.
Fig. 10: Stereoscanning electron micrographs of dorsal surface of the closing neural tube of chick embryo of stage 10.

Note: Cilia (c), Cytoskeletal processes (cp), Microvilli (mv), Neural fold (nf), Neural groove (ng), Neuronal epithelial cells (ne), Openings (o) and Unknown bodies (b).
Between first and second sonite x 50,000;

Caudal to the last sonite x 50,800.
Fig. 11: Stereoscopic electron micrographs of dorsal surface of the closing neural tube of chick embryo of stage 11.

Note: Cilia (c), Cytoplasmic processes (cp), Microvilli (mv), Neural fold (nf), Neural groove (ng), Neuroepithelial cells (nc), Opinions (o) and Unknown bodies (b).
CHAPTER III

LIGHT AND ELECTRON MICROSCOPICAL
STUDIES ON THE CLOSURE OF NEURAL
TUBES OF CHICK EMBRYOS
INTRODUCTION

In this study the developing neural tube was investigated by light and transmission electron microscopy at various sites and at different stages of development. This was done in an attempt to understand the histological and fine structural changes that may occur during development. Special emphasis was placed on changes in cell size, shape and arrangement as well as extracellular spaces and subcellular organelles. Microfilaments, microtubules, subapical vesicles and cellular junctions were also followed by electron microscopy.

The work was activated by the implication of the rapid growth of the nervous system as compared to surrounding structures (Stricker, 1974); changes in the shape of the neuroepithelial cells (Rumbler, 1905 and Gliessner, 1914 and 1916); Differential rate of growth and cell multiplication (Berridge, 1937); contraction of cytoplasmically located microfilaments (Balinsky, 1960); elongation of microtubules (Waddington and Perry, 1966); cytoplasmic processes (Gowin, 1974) and cervical mesenchyme (Harris and Solursh, 1976) as being factors playing a role in the closure of the neural tube.
Fifty four embryos of stages 7 – 12 inclusive were used in this study. Each stage contained nine embryos. All were fixed in Karnovsky's solution (appendix p.11) buffered to pH 7.2 – 7.5 for 24 hours. Then the Karnovsky's solution was replaced by cacodylate buffer pH 7.2 – 7.5 for another 24 hours. The blastodermes were then separated carefully from the vitelline membrane. They were postfixed for 30 minutes in 2% osmium tetroxide, dehydrated in ascending concentrations of alcohol (30 minutes in each of 30%, 50% and 70% followed by two changes of absolute alcohol each for one hour). With a razor blade the area opaque was trimmed off. The specimens were lifted carefully on a spatula and excess alcohol was removed with a blotting paper. The specimens were allowed to flatten on the emaide (see appendix p.11) in an aluminum foil container. The floating specimens was covered with a drop of emaide. The containers were left overnight in an oven at 60°C. The hardened emaide plates were removed from the dishes and the stage of the embryo was reshaped under the dissecting microscope and specimens were labelled. Suitable specimens were sectioned at different levels of the closing neural tube. (Brain region, somite regions and between somites). Semi-thin sections (1Mm) were stained with toluidine blue and studied with light microscopy. Thin sections of 300 Å were stained with lead acetate and studied with Siemens transmission electron microscope and appropriate microphotographs prepared.
RESULTS AND OBSERVATIONS

LIGHT MICROSCOPY

I. SECTIONS 7 AND 8 (BASIL NERVATURE):

a) AT THE BRAIN REGION (Fig. 12c)

The neural folds were formed. It was six or five cells thick. Cells of various shapes were observed: most of the cells were fusiform with their apical processes reaching the ventral and dorsal surfaces (central and peripheral processes respectively). Other cells were wedge shaped with their bases toward the ventral surface. Some other cells were irregular in shape. Other than the apical processes, side processes were noticed coming from the cell-bodies and extended into the extracellular spaces. Extracellular spaces which separated the cells especially at the ventral portion of the neural fold. Dorsal vesicles appeared as small spherical empty spaces. They were found on the dorsal surface of the neural groove. Irregular mesenchymal cells were seen. Some of these cells had cytoplasmic processes connecting them to the neuroepithelial cells.

b) AT THE SQUIRT REGION (Fig. 12b)

Most of the cells in that region were fusiform with central and peripheral processes reaching up to the dorsal and ventral surfaces. The number of cells per unit area were probably less than that of the cranial level. Few irregular and wedge shaped cells were also observed. The extracellular spaces were well in evidence especially at the ventral border of the neural fold. The subdorsal vesicles were fewer than those at the cranial level. Side collagen processes were also observed. The mesenchyme was much cellular with a clear zone demarcating it from the neural fold. Some cytoplasmic processes joined the mesenchymal cells to the neuroepithelial cells were also found.
c) CAUDAL TO THE LAST SCHWALBE (Fig. 13b)

The neural fold was thinner and probably less cellular than that at the previous two levels. It contained mainly fusiform cells with characteristic central and peripheral processes. Irregular or wedge shaped cells were scarce. Extracellular spaces were less extensive than that at the two previous levels with an even distribution throughout the depth of the neural fold. Side cytoplasmic processes were also present. The subdorsal vesicles were not present. The surrounding mesenchyme was cellular with processes joining its cells to the neuroepithelial cells. The clear zone separating the mesenchyme from the neuroepithelial cells became wider and clearer.

2. STAGES 9 AND 10 (HIND EMBRYONATION).

a) AT THE BRAIN REGION: (Fig. 13c)

The neural groove was deep and rhomboid in shape. The lips of the closing neural tube approached each other. The neural fold was still about 5-6 cell thick, but was apparently thicker at the lip. The cells were rather fusiform in shape with occasional irregular or wedge shaped cells. The extracellular spaces were probably similar in size to that of stages 7 and 8, but their distribution tends to be more in the ventral rather than the dorsal aspect of the groove. Side and apical processes were present. Very few subdorsal vesicles were observed. The mesenchyme was scanty.

b) AT THE SCHWALBE REGION (Fig. 13b)

The process of folding increased as compared to stages 7 and 8 with the lips approaching each other. The neural folds were also thicker and more cellular than the same level at stages 7 and 8.
as opposed to the cranial sections of the same stage, the cells were also larger in size. They were mostly oval in shape with few cells that retained their fusiform, wedge or irregular appearance. The extracellular spaces were reduced and tended to exist on the ventral aspect of the neural groove. The subdermal vessels were very remarkable. They increased in size and number as compared to their appearance at previous stages, the mesenchyme became organised in a reticulate formation and was separated by the clear zone from the neuroepithelial cells. No cellular processes that connected the mesenchyme to the neuroepithelial cells were present.

a) CAUDAL TO THE LAST SOMITE (Fig. 13a)

The neural groove was shallow, yet rather deeper than that of the same level in stages 7 and 8. It was about 7 to 8 cells thick, thus thicker than the previous stages. The cells were large, oval or circular with a rather hydropic fuzzy appearance. The cells were closely packed with very little if any extracellular spaces. Few subdermal vessels were present. The mesenchyme was dense and separated by the clear zone from the neural fold. No cellular processes that connected the mesenchyme to the neuroepithelial cells were seen.

3. SPIGENS 11 and 12 (HINT NEUROSPINEM):

b) AT THE BRAIN REGION (Fig. 14a)

The neural tube was formed and was covered with the ectoderm. It was probably of the same thickness except at the roof which was thin. The cells were still fusiform, wedge or irregular in shape. There was a remarkable reduction in the extracellular spaces as compared to all previous stages. The little that remained was confined to the ventral aspect of the tuba. The subdermal vessels were not present. The mesenchyme was scanty.
b) AT THE MÖNTOR REGION (Fig. 14b)

The tube was well formed and was uniform in thickness. The cells were circular or oval in shape and were closely packed with no extracellular spaces in evidence. The subdorsal vessels had completely disappeared. The ektoderm covered the neural tube. The somites were well developed with their somitic cavity. The clear zone separating the somite from the neural tube was large.

c) CEIDAL TO THE LAST SOMITE (Fig. 14c)

The lips of the neural folds were just in contact with each other. Fusiform, wedge and irregular shaped cells were recognized. The extracellular spaces were less marked than in the previous stages, but nevertheless were in evidence and more as towards the ventral parts of the neural tube. No subdorsal vessels were present. The surrounding mesenchyme was cellular and dense. No clear somite formation was observed in that mesenchyme. It was also separated from the neural tube by the clear zone.

TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy demonstrated some of the general fine features of the fusiform neuroepithelial cells (Fig. 15a). The nucleus occupied the centre of the cell and the central and peripheral apical processes extended from the cell body. In some sections (Fig. 15a), side processes were seen as blunt projections of the cell membrane. The apical (central) cytoplasmic process extended across the neural groove to form tight and focal junctions with those of the opposite side (Figs 15a, b and 16b).
The microtubules started to appear at stages 7 and 8. They were present in the cytoplasm of the cell body and extended to the central and peripheral processes (Fig. 16a). They increased in number at stages 9 and 10 (Fig. 17a) and decreased at stages 11 and 12 (Fig. 15a). Some of these microtubules were seen joining the subdorsal vesicles (Fig. 17b and c).

The microfilaments appeared as electron dense bands confined to the bases of the cytoplasmic processes of all types (Figs. 15a and 17c).

The subdorsal vesicles appeared as non-membrane bound electronlucent spherical bodies confined to the ventral surfaces of the neuroepithelial cells (Figs. 17a, b and c). Some of them had ruptured into the neural groove (Fig. 17c). Some of the microtubules, as previously mentioned, joined these vesicles.

The neuroepithelial cells exhibited other fine features common to all cells such as mitochondria, endoplasmic reticulum and inclusion bodies.

SUMMARY AND CONCLUSION

The developing neural tube was investigated with light and transmission electron microscopy.

During the development of the neural tube certain histological changes and differences occurred. Special emphasis was laid on changes in the general shape of the tube, the approximation of its lips, the shape and size of the cells, the extracellular spaces and the subdorsal vesicles.
The cells were of three main types: fusiform, wedge or irregular in shape. The fusiform cells had central and peripheral processes which extended to the dorsal and ventral surfaces of the closing neural tube respectively. These cells were most numerous at the early stages of neurulation and may be at the scute level.

At later stages of neurulation, the neuroepithelial cells tended to be uniform in shape and were rather hypodense, oval and large in size.

Cytoplasmic processes extended from all types of neuroepithelial cells and some of them were seen in the extracellular spaces.

The extracellular spaces were more extensive at mid-neurulation and tended to be more on the ventral rather than the dorsal aspect of the closing neural tube. At later stages, the extracellular spaces disappeared and the cells were very closely packed. Parallel to the increase in the extracellular spaces at mid-neurulation, the size of cytoplasmic processes extending from one cell to the other were by necessity longer.

Subdorsal vesicles, which were spherical empty spaces on the dorsal surface of the closing neural tube were present since early neurulation, increased remarkably in number in mid-neurulation and almost disappeared in later stages.

A clear zone was always present between the neuroepithelial cells of the neural folds. Cellular processes connecting the neuroepithelial cells to the neuroepithelial cells, were observed at certain stages.
Transmission electron microscopy had demonstrated the relation of the central cytoplasmic processes to the body of the neuroepithelial cells. It has also demonstrated that these processes cross the neural groove and form focal and tight junctions with those of the opposite side.

Microtubules extended from the cell body and the cytoplasmic processes were seen. These microtubules were obviously more numerous in mid-neuromelization and had almost disappeared at late stages. Some of these microtubules were seen joining the subluminal vesicles. It is possible that they transport some secretory product of the neuroepithelial cells.

Subluminal vesicles were also seen by transmission electron microscopy as electron-lucent spherical bodies. Some of which demonstrated their rupture into the neural groove, thus may be releasing a secretory product.

Microfilaments, which appeared as electron dense filarontous structures were found on the branches of all cell processes. Such microfilaments were also reported by (Salmén, 1960) who stated that they were only confined to the apices of the neuroepithelial cells.

Changes in the shape and distribution of the neuroepithelial cells during the closure of the neural tube do not suggest, by themselves, any role they could play in the closure of the neural tube. The distribution of the extracellular spaces being larger and more extensive on the ventral rather than the dorsal aspect, suggest that the neuroepithelial cells are pushed apart to a greater extent in the ventral rather than the dorsal aspect of the medullary plate, thus creating a greater outer circumference among the
modulary plate to fold on itself. The extracellular substance (s) is most probably a secretory product of the neuroepithelial cells.

The tight and focal junctions between the central epithelial processes from each side of the neural tube may create a dynamic process which causes the sliding or weaving of these processes along each other. They also create a cohesive force that keeps the neural folds together.

The microfilaments may be responsible for the protrusion of the epithelial processes.

The significance of the subepithelial vacuoles and their appearance and disappearance in later neurulation remain uncertain. However, it was suggested by Horwitz and Anfinsen (1966) and Condon (1974a) that they could contain cerebrospinal fluid.
Fig. 12. 1mm toluidine blue transverse sections of chick embryo of stage 7 at the brain region (a); at the somite region (b) and caudal to the last somite (c), X 800.

Note: Clear white zone (s); connection (x); dorsal and ventral surfaces of the folding medullary plate (d and v respectively); endoderm (ed); extracellular space (e); fusiform cell (f); irregular cell (i); mesenchyme (m); neural folds (nf); neural groove (ng); side process (sp); vesticle (l) and wedge shaped cell (v).
Fig. 13: 1 mm toluidine blue transverse sections of chick embryo of stage 9 at the brain region (a); at the somite region (b) and caudal to the last somite (c). X 800.

Note: central canal (c); curved process (p); extracellular space (s); somite (m); verticle (l) and white zone (w).
Fig. 14: 4mm toluidine blue transverse sections of chick embryo of stage 11 at the brain region (a); at the somite region (b) and caudal to the last somite (c), × 800.

Note: bodies (b); central canal (c); ectoderm (et); extracellular space (s); fusiform ectodermal cell (f); irregular ectodermal cell (i); left neural fold (lf); vesicle (l) and white zone (w).
Fig. 25: TEM micrographs of transverse sections of folding medullary plate of chick embryo stage 6 (a, b and c). X 180,000.

Note: Central canal (c); focal junction (d); fusiform cell (f); microfilaments (mf); microtubules (mt); vesicles (v) and tight junction (t). X 180,000.
**Fig. 15**: TEM micrographs of transverse sections of closing neural tube of chick embryo stage 9+ (a, b, c and d). × 120,000.

**Note**: Central canal (c); central process (p); extracellular space (e); focal junction (j); microfilaments (mf); microtubules (mt); peripheral process (pp); tight junction (t) and vesicle (v).
Fig. 12. TEM micrographs of transverse sections of closed neural tube of chick embryo stage 11 (a, b, c and d). X 120,000.

Note: Central process (cp); focal junction (f); fusiform epidermal cell (f); microtubules (mt); peripheral process (pp); side process (sp) and tight junction (t).
CHAPTER IV

STUDIES ON THE EFFECT OF ADDITION AND REMOVAL OF SOMAPE ON THE CLOSURE OF THE NEURAL TUBE IN THE CHICK EMBRYO
STUDIES ON THE EFFECT OF ADDITION AND REMOVAL OF SOMITES ON THE CLOSURE OF THE NEURAL TUBE IN THE CHICK EMBRYO

INTRODUCTION

S somites are considered to play an essential and major role in the process of closure of the neural tube (Louda, 1974a and 1974b). They are believed to do so either by the sheer exertion of lateral mechanical pressure or by some means of chemical induction.

It has been suggested (Louda, 1974a and b) that the neural tube is very much narrower at the region of somites than between somites. The alternating differences in width produce the appearance of segmentation of the neural tube.

The present investigations were based on measurements made in the normally developing neural tube at various points.

In this study the role of somites in the closure of the neural tube was investigated experimentally. From some developing embryos somites were removed while to others extra somites were added. The effect of such manipulation on the closure of the neural tube was observed on organs specimens and by measuring certain parameters of the tube in transverse histological sections. The aim of these measurements was to detect, mainly, any difference in the distances between the lips and medial margins of the neural tube that could have occurred due to the addition or removal of somites, changes in the thickness of the neural folds and the floor plate were also considered.

The results of these investigations are reported and discussed in this chapter.
MATERIALS AND METHODS

Chick embryos were obtained from 174 white Leghorn eggs and divided between two main categories: an experimental and a control.

The experimental embryos were subdivided into four groups:
1. 24 embryos at stage 6 to which somites were added.
2. 31 embryos at stage 7+ to 8 to which somites were added ventral to first somite.
3. 17 embryos at stage 8+ to 9+ to which somites were added ventral to first and second somites.
4. 20 embryos of stage 8+ to 9+ from which somites were removed.

The control embryos were also subdivided into four groups:
1. 13 embryos were allowed to develop in ovo.
2. 12 embryos were cultured in vitro using New's technique (1958).
3. 40 embryos had a single endodermal implant but no further surgical manipulation.
4. 19 embryos had somites removed and immediately replaced.

Donor and host embryos were cultured in vitro using New's technique (1958).

Preparation of somites for transplantation:

Donor embryos of stage 6+ to 7+ were taken from the incubator and put on a dissection microscope. The tips of two dissecting knives were applied to the
Midline of the neural tube and lateral pressure applied. That could separate the two columns of somites; right and left with their related neural fold, ectoderm and endoderm from each other. A longitudinal cut was then made on the lateral side of each column to separate it from the rest of the embryo. Transverse cuts were also made rostral to the first somite and caudal to the last somite in the column. The column was sucked into a pipette and transferred to normal glucose saline (PH 6.2) in a Petri dish. The column was then cut into blocks each containing two somites. The somites in each block were then separated from the neural fold, ectoderm and endoderm and were then ready for transplantation.

Operative procedure:

1. Addition of somites:

An appropriate left side, endodermal incision was made in host embryo cultured in vitro using Nell's technique. With a pipette and through that incision a block of two somites was introduced and pushed by the tip of a cautery knife to lie between the endoderm and ectoderm. The host embryos were reincubated to stage 9-10 (six to seven somites stages).

This procedure was carried out in group 1, 2 and 3 of the experimental embryos.

2. Removal of somites:

A left side endodermal incision was made in embryos of stages 9-10 (3 to 5 somites) cultured in vitro using Nell's technique (1955). Through that incision three or four somites were cut away and removed using a curved cautery knife. The embryos were reincubated to stage 9-10.

The experimental embryos of group 4 were subjected to this treatment.
Fig. 18: For explanation of numbers see text.
Further processing:

The specimens were fixed in Bouin's fluid for 24 hours, dehydrated in ascending grades of alcohol and cleared in oil of cedar wood for 24 hours. Camera lucida drawings were then made of all the specimens (raw data pp 1-4; group 1: pp 27-30; group 2: pp 33-41; group 3: pp 50-54; group 4: pp 65-74, pp 81-85; control groups).

From each group six specimens (of stage 2-3 and 3 of stage 9) were selected for histological processing. 10μm transverse paraffin sections were prepared and stained with H & E.

Measurements:

The following measurements of the developing neural tube (Fig. 10) were made in H & E transverse paraffin sections at x 400 magnification (x 10 eye piece and x 40 objective).

1. Thickness of the floor of the neural tube. This was measured where the floor was crossed by a line joining the mid-point between the lips of the neural fold to the centre of the notochord (Fig. 10.2).

2. The distance between the medial margins of the neural folds at a point 20 μm from the floor plate (Fig. 10.3).

3. The thickness of the neural folds at a point 20 μm from the floor plate (Fig. 10-4 and 5). 

4. The distance between the lips of the neural folds (Fig. 10-1) at a point 20 μm ventral to a line touching their crest.
Fig. 12: For explanation of letters see text.
Measurements were made in sections obtained from the following levels of the developing neural tube (Fig. 19).

a. 30μm rostral to the first somite
b. At the level of somites
  c. Between somites
  d. 30μm caudal to the last somite

RESULTS AND OBSERVATIONS

GROSS OBSERVATIONS

Control Groups of Embryos

13 embryos cultured in vivo (raw data pp 1-2) and developed normally (Fig. 20).

12 embryos cultured in vitro using Novak's technique (raw data pp 1-2) developed normally from bend process stage to sixteen somites stage (Fig. 21).

13 embryos with single endodermal incision (raw data pp 34-39) had developed normally (Fig. 22). Their general appearance was normal. The wound healed and seven to thirteen somites appeared normally and at the appropriate stage of development. The heart started to beat at later stages and the neural tube was well formed and closed.

16 embryos from which somites were removed and immediately replaced (raw data pp 65-74) developed normally (Fig. 23a).
In 15 of them, however, the neural tube remained open. Somites in the operated side were normal in 7 embryos. Fusion of somites, lateral curvature, distortion in shape, connection with surrounding mesenchyme, partial disappearance of somites, or abnormal appearance occurred in few specimens (Figs. 22a and 23b).

Experimental groups of embryos:

Group 1: (Addition of somites to stage 6 embryos – Raw data pp 1-12) of the 24 embryos used, eleven developed normally (Figs. 24 and 25a) while thirteen specimens were either spilled during manipulation or developed abnormally (Fig. 25b) and were rejected from this study.

In the normally developed embryos, the wound healed in six specimens and remained open in five. Opposite the graft, the neural tube had a wide lateral concavity and the graft grew larger in most specimens.

The neural tube was closed in nine specimens and remained open in two specimens.

Group 2. (Addition of somites rostral to host’s first somite – Raw data pp. 17-20),

15 embryos developed normally (Figs. 26 and 27a). The somites were normal in appearance, shape and segmentation. The neural tube either closed completely or had one or more points of contact. In few specimens, the neural tube remained open. The wound healed in most specimens and the graft increased in size.

15 embryos in this group developed abnormally (Fig. 27b).
Group 1. (Addition of somites between host's first and second somites - Raw data pp 22-41).

16 embryos developed normally (Figs. 28 & 29), the wound healed in most of them and in thirteen normal somites appeared.

In all the specimens the neural folds were in contact with each other except at the region of the posterior neuropore and rostral to host's first somite.

The graft increased in size in all specimens in nine of which the neural tube showed a marked lateral concavity opposite the graft.

Group 2. (Removal of somites - Raw data pp 50-54).

9 embryos in this group developed normally as far as the general appearance was concerned. In all these nine embryos the neural tube remained open (Fig. 30). In five of the above embryos, no new somites were reformed to replace those which had been removed. In the other four embryos, two developed a complete new set of somites, while the other two re-formed one somite to replace the removed ones.

The operation had no effect on the somites on the opposite side or caudal to the area of the operation.

Measurements:

Measurements are tabulated in Raw data volume as follows: Group 1, pp 13-21; group 2, pp 22-42; group 3, pp 42-54; group 4, pp 57-60; control in vivo, pp 61-64; control in which somites were removed and replaced, pp 75-78. Average of means and standard deviations were calculated and tabulated in Raw data pp 79. The Student t test for all measurements were also calculated and tabulated in
The results of these measurements are graphically illustrated in figures 31, 32, 33, 34 and 35.

1. **The thickness of the floor** (raw data pp. 79, text fig. 31 and table 7 column 2)

The greatest thickness of the floor was found in embryos cultured in ovo (c). It was less in embryos in which amnionites were removed (group 4) and in those in which amnionites were removed and immediately replaced (control group 3). The thickness of the floor was very much reduced in embryos to which amnionites were added (group 1, 2 and 3).

In all groups of embryos, the thickness of the floor was the same when measured at the level of amnionites or between them. Any differences were not statistically significant.

2. **The distance between the medial margins of the neural folds** (raw data pp. 79, text fig. 32 and table 7 column 3).

The distance between the medial margins in embryos to which amnionites were added (groups 1, 2 and 3), was more or less similar to controls (groups 0 and 4). It was very much reduced in embryos from which amnionites were removed (group 4).

The distance between the medial margins was significantly less at the level of amnionites when compared to the distance between amnionites. This was true in both control groups (0 and 4) and the experimental groups (1 and 2). Differences were not significant in groups (3 and 4).
The thickness of the neural folds (Raw data pp 72, Figs. 33 and 34 and table I, text).

The thickness of the neural folds on both sides of the neural tube; right and left (Figs. 33 and 34 respectively) were similar in all control and experimental groups of embryos.

In all experimental groups of embryos, the thickness of the neural folds was significantly less than that in the controls.

There was no significant difference in the thickness of the neural folds when measured at the level of somites or between them (Figs. 35 and 36).

4. The distance between the lips of the neural folds (Raw data pp 73, Fig. 35 and table I, text).

The distance between the lips of the neural folds was significantly reduced in embryos in which somites were added at stage 6 or removed at host first somite (groups 1 and 2 respectively) and in those in which somites were removed and immediately replaced (group 3). That is when the distance was compared to that in embryos cultured in vivo (control group 0). In embryos from which somites were removed (group 4) that distance was also reduced when measured between somites. It was increased in that group when measured at the level of somites. It also increased in embryos in which somites were added between first and second somite (group 3).

In all groups of embryos, with the exception of group 4, there was no significant difference in the distance between the lips of the neural folds when measured at or between somites.
The distance between the lips of the neural folds was least in embryos to which somites were added at stage 6. (group 1) or control to host first somite (group 2). It was also true between somites in embryos from which somites were removed (group 4).

**SUMMARY AND CONCLUSION**

The role of somites on the closure of the neural tube was investigated experimentally. Somites were either added to or removed from developing embryos and the results of such manipulations were studied in gross specimens and by taking measurements at various parameters in the developing neural tube.

The experimental controls were designed in such a way to exclude the effect of *in vitro* culturing using New's technique, simple endodermal incision and surgical manipulation of somites by their removal and replacement, on the closure of the neural tube.

It was concluded from these controls that the closure of the neural tube could occur normally in embryos cultured *in vitro*, as well as in those with simple endodermal incision or from which somites were removed and immediately replaced. It was felt therefore that further experimentation *in vitro* could be valid.

The experimental embryos were subdivided into four groups:

1. Embryos to which somites were added at stage 6,
2. Embryos to which somites were added to control to host first somite,
3. Embryos to which somites were added between first and second somites, and
4. Embryos from which somites were removed.
The addition of somites to developing embryos speeded up the process of closure of the neural tube.

The implanted somites or graft had persistently increased in size. A large lateral concavity appeared in the neural tube opposite the growing graft. That could have been due to an exaggerated lateral pressure exerted by the graft.

The neural tube failed to close in embryos from which somites were removed. In some of these embryos, a new set of somites re-formed. The new somites were mostly smaller than normal and seemed to have no effect on the neural tube.

The thickness of the floor of the neural tube was the same at the level of or between somites. It was decreased by the addition or removal of somites. That change was, however, less marked when somites were removed and immediately replaced. It seemed therefore that the thickness of the floor was not related directly to the presence of somites.

The distance between the medial margins of the neural tube was significantly less at the level of somites than in between somites in normally developing embryos as well as in those in which somites were added, removed or removed and replaced. The addition of somites to stage 6 embryos or ventral to host first somite had no effect on the distance occurred. The removal of somites had markedly reduced the distance between the medial margins.

The thickness of the neural tube was the same on both sides, right and left as well as at the level of somites and between somites. That thickness was reduced by all experimental manipulations regardless of whether somites were added or removed.
The distance between the lips of the neural folds was reduced by the addition of somites to stage 6 embryos or rostral to host first somite. It, however, increased in these embryos to which somites were added between first and second somite.

The distance between the lips of the neural folds was significantly less at the level of somites than in between somites. That suggested that the neural groove was narrower at the region of somites. It was reduced further by the addition of somites to stage 6 embryos or rostral to host first somite. It, however, increased slightly in these embryos to which somites were added between first and second somite. The removal of somites also caused a marked reduction in that distance when measured between somites.

In normally developing embryos, the neural tube is narrower at the region of somites rather than between somites.

The addition and removal of somites at later stages has a similar effect. They both cause an increase in distance between the lips of the neural folds.

The thicknesses of the neural fold and the floor of the closing neural tube were reduced by all experimental manipulations. It was possible that such treatment reduced the rate of cellular proliferation.

It is felt, in the main, that somites play a major role in the closure of the neural tube, especially at early stages of development.
Fig. 20: Light microscopic photograph of chick embryo of stage 11 cultured in ovo.

Note: Closing neural tube (nt) and somites (s). X 100.
Fig. 54: Light microscopic photograph of thick embryo of stage 11 cultured in vitro.

Note: Closing neural tube (nt) and somites (s). X 100.
Fig. 22: Whole mount control chick embryo in which the endoderm was incised to see the effect of such incision on closure of the neural tube. X 50.

Note: The normal appearance of the neural tube (nt) and somites (s).
Fig. 23: Whole mount control chick embryos in which three to four somites were excised and replaced to see its effect on closure of the neural tube. X 30.

Note: Neural folds (NF); excised and replaced somites (↑) and the gap (g).
Fig. 24: 10 mm Transverse section of host chick embryo of group 1 after the reincubation period. N. & E. x 200.

Note: graft (g); neural fold (nf) and mesenchymal cells (??).
Fig. 25: Whole mount chick embryos of group 1 specimens in which somites were added to stage 5 hosts followed by 9 hours reincubation. × 30.

Note: Edges of the wound (e), graft (g) and the abnormal appearance of somites in the operated side of some specimens (X).
**Fig. 26:** 10 mm transverse section of host chick embryo of group 2 after the reincubatory period. H. & E. X 200.

**Note:** Condensed mesenchymal cells (-----) in the host's first somite (s) and the blocks within the graft (g).
Fig. 27: Wholemount chick embryos of group 2 in which somites were added rostral to host's first somite followed by seven hours reincubation. X 30.

Note: Graft (g) and condensed neuroepithelial cells between the graft and the cranial somites (↑).
Fig. 28: 10 mm Transverse Section of host chick embryo of group 3 after the reimplantation period. H. & E. X 200.

Note: The graft (g).
Fig. 20: Whole mount chick embryo of group 3 in which somites were added between host's first and second somites followed by seven hours reincubation. X 30.

Note: The graft (g) and normal segmentation of the somite neuroderm (M).
Fig. 10: Wholemount chick embryo of group 4 in which the cranial three to four somites were removed followed by reincubation for a period ranging between 4.5 to 9 hours. X 30.

Note: The neural fold (NF); regeneration of the removed somites (R) and the wound (W).
Key to graphs No. 31 to 35:

group 1 = Addition of somites to stage 6 embryos.

group 2 = Addition of somites rostral to host's first somite.

group 3 = Addition of somites between host's first and second somites.

group 4 = Removal of somites.

r = Removal and replacement of somites.

c = In ovo control specimens.

b = 30 mm rostral first somite + caudal to somites.

a = At the region of somite.
Fig 33: MEAN THICKNESS OF FLOOR OF CORDING NEURAL TUBE (WITH STANDARD DEVIATIONS) IN TWO CONTROL SPECIMENS AND EXPERIMENTAL CUES, T.1. GARK EMBRYOS STAGES 8 TO 9 (SIX TO SEVEN SOMITES). THREE EMBRYOS IN EACH STAGE.
Fig 12: MEAN DISTANCE BETWEEN MEDIAL MARGINS OF NEURAL FOLDS (WITH STANDARD DEVIATIONS) IN QTO CONTROL SPECIMENS AND EXPERIMENTAL GROUPS. T.S. HIND LIMB STAGES 9-10 (SIX TO SEVEN SHERRIS). THREE EMBRYOS IN EACH STAGE.
Fig. 2: Mean thickness of right neural pult (with standard deviations) in in vivo control and experimental cords. H.S. chick embryos stages 9 to 9 (six to seven somites). Three mice in each group.
Fig. 25: LEAN THICKNESS OF LEFT NEURAL FOLDS (WITH STANDARD DEVIATIONS) IN OVO CONTROL SPECIMENS AND EXPERIMENTAL ONES, T.G. CHICK EMBRYOS STAGES 9- TO 9 (SIX TO SEVEN SISTERS), THREE EMBRYOS IN EACH STAGE.
FIG. 35: MEAN DISTANCE BETWEEN NEURAL PULS 20 H.H. VENTRAL TO DORS. ( WITH STANDARD DEVIATIONS ) IN OXY CONTROL SPECIMENS AND EXPERIMENTAL CYES. 2.S. CHICK EMBRYOS STAGES 9- TO 10 ( SIA TO SEVEN SOMITES ). THREE EMBRYOS IN EACH GROUP.
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**Removal of metal:**
- Second method: damage, stop, exit, and activation.
- Second method: damage, stop, exit, and reactivation.

**Stop:**
- To prevent damage.
- To avoid further contact.

**In any condition:**
- Never touch the side of the reactor while the reactor is running.
- Close to the floor, above the floor, or in the middle of the floor.
- For distances between the floor and the reactor.

**Copy:**
- The floor.
CHAPTER 4

THE EFFECT OF THE CERVICAL MUSCLES ON THE CLOSURE OF THE NEURAL TUBE IN THE CHICK EMBRYO
Fig. 36: Diagram illustrating the site of incision and removed mesenchyme.
OUR EFFECT OF THE CERVICAL AMNESTY ON THE Closure OF THE NEURAL TUBE IN THE CHICK EMBRYO

INTRODUCTION

Somatics have been implicated in the closure of the neural tube (Gonda, 1974). However, that suggestion created the issue of the factors involved in this mechanism at the cephalic region which has no somites. Cytoplasmic processes are believed to be one of these factors (Gonda, 1974).

However, Morrisey and Soloway (1976) also believed that the cervical amnesy plays a role similar to that of somites at the cephalic region.

In this study, the cervical amnesy of developing chick embryo was excised and the effect of such an interference in the closure of the neural tube was elucidated and reported in this chapter.

MATERIALS AND METHODS

Twenty five embryos were used in this experiment: eight were cultured in vivo and used as controls. The other seventeen were cultured in vitro using Rode's technique and incubated to stages 7 (five embryos) and 7* (12 embryos). Under a pool of normal gluteal saline, the endoderm on both sides of the developing head was incised and the amnesy was carefully scraped away from the side of the mandibular plate with a curved osteact knife (see Diagram). At the end of the operation, the excess saline was removed and the specimens reincubated for 12 hours at 37.5°C.
Controls to the effect of in vitro culturing and endodermal incision from previous studies were used (Figs. 20, 21 and 22 in chapter IV).

RESULTS

Of the five embryos operated on at stage 7 and then reincubated, one specimen failed to grow, two specimens developed normally to stage 9+, and the other two developed normally as well to stage 9+.

Of the twelve embryos operated on at stage 7+, all had developed normally as far as general appearance and segmentation are concerned; one to stage 10+, two to stage 10, two to stage 10+, one to stage 11+ and six to stage 11.

The neural tube failed to close in all operated on embryos (Fig. 17). Failure of closure was most marked at the cephalic region. Segmental segmentation remained normal in all embryos on both sides of the neural tube.

Control embryos at similar stages of development, cultured in vitro showed points of contact between the neural folds at stage 9, while at stage 11 the neural tube was completely formed with an open posterior neuropore (Fig. 20).

Control embryos cultured in vitro using Hoe's technique developed normally with a closed neural tube (Fig. 21).

Control embryos with an endodermal incision showed no effect of the surgical interference on the closure of the neural tube (Fig. 22).
SUMMARY AND CONCLUSION

In this study, the effect of the cervical mesenchyme on the closure of the neural tube was studied.

An endodermal incision was made in a group of embryos cultured in vitro using Levene's technique and the cervical mesenchyme on both sides was removed and the embryos were reincubated.

The neural tube failed to close all through after the removal of the cervical mesenchyme. Failure of closure was more marked at the cephalic region.

Previous studies (chapter IX) had suggested that in vitro culturing using Levene's technique and surgical endodermal incision have no effect on the normal development of the embryos or on the closure of the neural tube.

It was concluded, therefore, that the removal of the cervical mesenchyme was responsible for the failure of closure of the neural tube.

This work lent support to Davies and Solursh (1976) hypothesis that the cervical mesenchyme plays a role in the closure of the neural tube at the cephalic region.
Fig. 37: Wholemount chick embryos used to study the effect of the cervical mesenchyme on closure of the neural tube. X 320.

Note: Failure of approximation of the neural folds (nf) in the experimental specimen (a) and normal closure in the control (b).
CHAPTER VI

THE EFFECT OF MECHANICAL SEPARATION
OF THE HEMAL FOLDS ON THE CLOSURE
OF THE HEMAL TUBE
THE EFFECT OF MECHANICAL SEPARATION OF THE NEURAL FOLDS ON THE CLOSURE OF THE NEURAL TUBE

INTRODUCTION

Some workers have studied the external topography of the neural tube ectoderm during the development of chick embryos (Gouda, 1974; Jacob and Christ, 1974 and Fortes and Sereen, 1974). They used stereoscanning electron microscopy. The presence of cilia, microvilli, cyttoplasmic processes and spherical or ovoid bodies of unknown nature have all been reported as emerging from the ectodermal cells or within the neural groove.

It has been reported (Gouda, 1974a and 1974b; 1974c and 1975c and 1977a), that these cyttoplasmic processes, especially at points of contact between neural folds, protruded from the apices of the ectodermal neuroepithelial cells (Fig. 28). They interdigitated with each other across the neural groove. Similar findings were observed by Bancroft and Bellairs (in chick embryos, 1975) and by Waterman (1976) in hamster and mouse embryos. It is suggested that these cyttoplasmic processes, together with the contact, bring about or play a role in the closure of the neural tube. This might, at least, be true at a certain stage of development.

In this study, chick embryos at stages 9- and 10, at which the neural folds were in contact, were treated mechanically in such a way so as to reopen the neural tube. The fate of further development was followed and reported in this chapter.
MATERIALS AND METHODS

Twenty six embryos of stages 6 to 10 were used in this study. Using a modification of New's technique (1955), they were cultured in vitro but with the amnion facing upwards instead of downwards. Thin albumen was painted over the blastoderm. A few drops of normal glucose saline were introduced beneath the stretched vitelline membrane.

Under a pool of normal glucose saline, the mid brain was gently pressed in the midline using a tip of a micropipette. This pressure was capable of separating the neural folds that were already in contact. The tip of the micropipette was carried along the margins of the neural folds two or three times to ensure that the neural folds were reopened. At the end of the operation, most of the saline was removed and all specimens were reincubated for 5 hours.

Twelve embryos were used as a control; four were cultured by the same method (the modified method); another four were cultured in vitro using New's technique and the rest were cultured in vivo. The time of incubation was similar to that taken by the experimental specimens.

All embryos, experimental and controls, were fixed in Bouin's fluid for 24 hours after which they were dehydrated in graded alcohols and most of the area opaca was trimmed off. While in absolute alcohol, all specimens were photographed under low power in a Nikon Ms 450 Macro Microscope, camera and studied as whole mounts (Figs. 38 and 39). The absolute alcohol was replaced by oil of cedar wood, and the specimens were left for 24 hours. The embryos were embedded in two changes of paraffin wax.
for 10 and 30 minutes respectively and rapidly hardened by floating the blocks in hot water. 10μm paraffin sections were prepared and stained with Haematoxylin and eosin.

**GRAPHIC RECONSTRUCTION**

Transversely sectioned embryos Sp. 300 - 312 (control) and sp. 255-263 (Experimental) were reconstructed at a magnification of 250. The number of sections of each embryo was counted to select the appropriate size of graph paper for the reconstruction. In every 6th sections the width of both neural folds, and the distance between their lips was measured and dotted on the graph paper at a magnification of 250. Finally the dots were joined together. The reconstructions were then photographed with pentax camera (Fig. 40).

**RESULTS AND OBSERVATIONS**

Operated on embryos, failed to develop (after reincubation) beyond stage 12. That was due to the fact that the blastoderm was limited by the stretch of the vitelline membrane.

In four embryos, a gap developed between the neural folds (Fig. 39 and Raw data p.87).

Seven of the operated on embryos failed to develop. In fifteen embryos the neural folds failed to re-close (Fig. 39) as compared with controls at same stage of development (Figs. 39 and 40).

Transverse A & X sections of experimental embryos demonstrated failure of closure both at and between somites (Fig. 41) as was expected at that stage of development in normal controls (Fig. 42). The mesenchymal cells were atypically arranged,
the nuclear stratification and staining was abnormal. The ectoderm became thinner, more wavy in outline, and stopped short at the lips of the neural folds. The somites and the lateral plate mesoderm reacted to the injury. That reaction was demonstrated by a swollen and rather circular shape of the somites, and their fusion with the neural folds.

**SUMMARY AND CONCLUSION**

Coues (1974) reported that the neural tube closes in three stages: a preparatory stage (stages 7 and 8); a stage of contact (stages 9 and 10) and stage of fusion (stages 11 and 12).

The embryos in this study were operated on at the stage of contact (stages 9 and 10). The operation re-opened the neural tubes which failed to re-close during the period of the experiment. Further development however, could not be studied because the growth of the embryo was limited by the vitelline membrane beyond stage 12.

Failure of the neural tube to re-close could have been due to a number of factors. The mechanical trauma to which the embryo was subjected might have caused that failure. The response to injury was not only limited to the neural tube but extended to the surrounding mesenchyme, ectoderm and endoderm. The somites, for instance, became swollen and rounded in shape and fused with the neural folds. Changes were also detected in the appearance of the ectoderm and endoderm following the injury.

The mechanical separation of the neural folds, on the other hand, could have ruptured or disconnected
the cytoplasmic processes which normally extend from one neural fold to interdigitate with those from the other side. Failure of re-closure of the neural tube could have followed these consequences. Whether those processes might re-form or re-connect could not be decided within the time limits of this experiment.
Fig. 38: Whole mount experimental specimens of stages 9, 9+, 10-, 10, 11- and 11.

Note: Failure of approximation of the neural folds (↑). X 350.
Fig. 29: Whole mount control specimens of stages 8, 9, 10, 11- and 11.

Note: Sensile (s), Neural fold (nf), Point of contact (p) and developing heart (h).

X 350.
Fig. 43: Graphic reconstruction of experimental specimen (a) and control one (b) of stage 11. 
Note: Failure of approximation of the neural folds in the experimental one. X 56.
Fig. 41: T. Sections of experimental chick embryo of stage 11- at the region of second somite (a) and between second and third somites (b). X 200. Note: Ectoderm (e), Endoderm (d), Somite (s) and the gap between the lips of the neural folds (†).
Fig. 42: S. Sections of control chick embryo of stage 11- at the region of second somite (a) and between second and third somites (b). X 200. Note: Closed neural tube (↑) and the covering ectoderm (e).
CHAPTER VII

STUDIES ON THE FATE OF KININ-LIKE
PLATE OF CHEST EMBRYO WHEN CULTURED
IN MICRO
STUDIES ON THE FATE OF MEDULLARY PLATE
OF CHICK EMBRYO WHEN CULTURED IN VITRO

INTRODUCTION

Rhumbler (1902) and later Glaser (1914 and 1916) stated that folding of the medullary plate was due to changes in the shape of the neuro-... epithelial cells from rectangular to trapezoid. They attributed this to a lowering of tension on the ventral surface of the medullary plate which might be due to alterations in pH (Glaser, 1914) or to changes in concentrations of certain substances (Rhumbler, 1902). They have therefore assumed that folding of the medullary plate and the formation of the neural tube were due to autonomous factors within the cells of the medullary plate, and was not related to surrounding tissues.

In this study, parts or whole of medullary plates were removed from normal chick embryos and transplanted into host chick embryos. This was done in an attempt to study the fate of the medullary plate when cultured in an abnormal external environment.

MATERIALS AND METHODS

Thirty-six white Leghorn chick embryos of stages 5 to 9+ were used. Embryos were divided into two groups: an experimental group of 30 embryos and a normal group of 6 embryos. From the experimental group, 18 embryos of stage 5 were used as donors from which the medullary plates
were recovered, and twelve embryos of stages 7+ to 9+ were used as hosts in which medullary plates were implanted.

The control embryos developed normally in vivo to compare the stage of development with that of host embryos.

Preparation of transplanted sections of medullary plate:

After 10 hours of incubation, donor embryos of stage 5 were removed from the incubator and transferred to a dissecting microscope. Using two cutanet knives, the medullary plate was removed from the embryo and sucked up into a pipette and transferred to another clean petri dish containing normal saline. The medullary plate was then sectioned transversely into 3 or 4 sections ready for transplantation.

Preparation of host:

The eggs were incubated for 19 hours after which the host embryos were cultured in vitro using Par's technique (1955), until stages 7+ to 9+ were reached. The endoderm of the left side of the hosts was incised lateral to the ventral two somites. With a pipette, one of the sections of the medullary plate prepared for transplantation was sucked up and transferred to the host near the incision and pushed towards the neural tube using the tip of a cutanet knife. The embryos were then reincubated until stages 11- to 13 were reached (Raw data p. 89).
Twelve medullary plates removed from donor embryos of stage 5, were cultured on thin albumin for 12 hours after which they were viewed by light microscopy and the general appearance was drawn (see text p. 96).

Further processing:

Host embryos, in which sections of medullary plates were implanted, were then fixed in Bouin’s fluid for 24 hours after which they were dehydrated in ascending grades of alcohol, cleared in oil of cedar wood and finally embedded in paraffin wax (melting point 56°C). 10μm transverse paraffin sections were prepared and stained with hematoxylin and eosin.

Normal embryos developed in ovo, were fixed in Karnovsky’s fluid pH 7.2-7.5 for 24 hours followed by cacodylate buffer pH 7.2-7.5 for another 24 hours after which they were postfixed in 25% osmium tetroxide for 30 minutes. Specimens were then dehydrated in 50%, 70% and 100% acetone for 10 minutes each. Finally, embryos were embedded in araldite, photographed under low power in a Nikon Multihotom, Macro/microphotography camera and studied as wholemounts (Fig. 43).

RESULTS AND OBSERVATIONS

After implantation of segments of medullary plate and reincubation, five host embryos developed normally. Their stage of development was compared to those embryos that developed in ovo. In normal hosts, the grafts appeared as a ball of tissue consisting of a mass of irregularly arranged cells (Fig. 44).
The rest of the host embryos (seven in number) developed abnormally or failed to grow.

The medullary plates cultured on thin albumen (Raw data p. 96) have also developed into irregular balls of tissues.

SUMMARY AND CONCLUSION

The medullary plate is formed of ectodermal cells which are normally arranged in a single layer of cuboidal or fusiform cells. During development the medullary plate folds to form the neural folds and further on the neural tube.

The folding of the medullary plate could take place as a result of extrinsic factors e.g., somites or surrounding mesenchyme or as has been suggested by Nambour (1942) by some intrinsic factor.

In this study, medullary plates were removed from some donor chick embryos and either implanted between the ectoderm and endoderm of developing host embryos or in thin albumen medium.

In all situations, the medullary plates developed into an irregular ball of tissue and failed to produce any feature that could suggest an attempt to form a neural fold or tube. This contradicts Glasser (1954) findings.

These findings would suggest, in the least, that a normal environment in a normally developing embryo, is essential for the folding of the medullary plate.
Fig. 43: Whole mount control embryo of stage 13 cultured in vitro.

Note: The normal appearance of neural tube (nt) and somites (s). X 350.
Fig. 44: 10 mm Transverse section of host chick embryo of stage 13 in which transverse section of medullary plate was grafted between endoderm and ectoderm. (H.E., x 200).

Note: Tendency of the graft to form a ball of tissue instead of forming a tube (†).
GENERAL DISCUSSION

The present work was undertaken in an attempt to study and throw more light on the normal development of the neural tube and the factors involved in its closure.

For the sake of clarity this discussion is tackled under the following subtopics:

A. Techniques used in this study

B. Normal development and closure of the neural tube.
   2. Light and electron microscopic studies on the closure of the neural tube.

C. Experimental studies on the closure of the neural tube.

"The effect of the somites, cervical mesenchyme, local environment, cytoplasmic processes and extracellular spaces on the process of closure of the neural tube."

A. TECHNIQUES USED

In vitro culture, using Newton's (1955) technique is used in this study because it is simple, the embryo is clearly seen, stable and easy to handle during operative procedures. Working in vivo, on the other hand, can be extremely difficult and tedious. Other in vitro techniques such as those of Wolf and Rosen (1955) in which clotted embryonic extract is used is handicapped.
by contamination and overgrowth of micro-organisms irrespective of any aseptic precautions.

In this study an attempt was made to obtain accurate measurements at various parameters of the closing neural tube made at different levels and stages of development.

It was appreciated that considerable shrinkage occurred during histological processing of the chick embryos or tissues in general. Measurements obtained in this study were of relative value. No attempt was made to study or calculate the shrinkage factor in order to obtain absolute values. However, as already stated, it was felt that such measurements had a comparative validity.

It is not always easy in transverse sections of the closing neural tube, to determine whether the part of the later under examination, is at the level of somites or between somites. This is because mesenchyme and somitic material is invariably present through out the length of the closing neural tube. Where appears as a clear zone between somites by naked eye examination, is actually occupied by a little somitic material or mesenchymal plate tissue (Couda, 1974 a and b). It is however, assumed by definition, that the part of the neural tube at somites, is that part where the somites appear with their maximum characteristic histological appearance, with a somitic cavity. Where the mesenchymal or somitic tissues is least, this part is considered to be between somites.

Abercrombie and Hollaire (1954), Hollaire (1958), Sheridan (1965), Frederick et al (1966), May (1965), Jacob and Christ (1974) and Couda (1974 a and b), claimed that the removal or
preparation of pure sciotic material for transplantation could not be possible. That was believed to be due to the interconnections between the neural tube, notochord and somites. Bancroft and Bellairs (1975) indicated that an interconnection took place between the somites, neural tube and notochord through these interconnections. Though in part of this study sciotic material was obtained from transplantation, no claim can be made that the effect of some other tissue could be excluded.

2. NORMAL DEVELOPMENT AND CLOSURE OF THE NEURAL TUBE

2.1. STEREOMINING ELECTRON MICROSCOPIC STUDIES OF THE CLOSURE OF THE NEURAL TUBE

The surface topography of the closing neural tube has been studied by many workers under stereomining electron microscopy, (Yarin 1971; Gonda 1974 a and b, 1976 a and b and 1977 a and b; Jacob and Christ 1974; Lofberg 1974; Porter and Barson 1974; Bancroft and Bellairs 1975 and Waterman 1976).

The neuroepithelial cells are fusiform or rectangular in shape. Microvilli similar to those reported by Porter and Barson (1974) projected at cell junction. Cilia were also seen projecting from the centres of the neuroepithelial cells. The general appearance of cilia and microvilli is the same at all levels of the developing neural tube and at all stages of development.
Similar processes that resemble cilia and microvilli have been observed in embryos of * Xenopus laevis* (Burris, 1971); in chick (Porter and Barson, 1974; Sumida, 1974 a and b; 1976 a and b; and 1977 a; Bencorff and Belleirs, 1975); in mouse (Naruse and Kaufman, 1966) and rat (Powers, 1972). The microvilli are small, villiform-shaped cytoplasmic processes present at cell junctions, while the cilia are medium in size, rod-shaped and protrude from the centre of the neuroepithelial cells.

Cytoplasmic processes, protruding from the neuroepithelial cells and crossing the neural groove to interdigitate with those of the other side, are a characteristic feature. These cytoplasmic processes are probably the central processes of the fusiform neuroepithelial cells (Sumida, 1974 a). Various changes occur in the appearance and distribution of the cytoplasmic processes at different sites of the developing neural tube. They are formed at stage 7, become more obvious at stage 8, increase in length and thickness at stages 9 and 10, but decrease prematurely at later stages. The cytoplasmic processes are always more numerous at the developing brain region and between somites.

The presence of these cytoplasmic processes was first reported by Sumida (1974 a and b). Belleirs and Bencorff (1975) and later on observed similar processes which they referred to as unbranched threads.
It is suggested by the present findings and some previous studies (Gooda, 1974a and b) that the cytoplasmic processes may play a significant role in the closure of the neural tube. The manner by which they cross the neural groove to interdigitate with those of the other side suggest that they may create a cohesive force and creep along each other thus approximating the lips of the neural folds. They are probably more essential for such a role in the brain region which is devoid of somites and between somites, as their distribution suggests. Bancroft and Bellairs (1973) entertained similar views. In this thesis, the separation of the already approximated neural folds by operative interference had probably disconnected the cytoplasmic processes and caused the failure of closure of the neural tube.

It is practically difficult to follow the fate of the cytoplasmic processes by stereoscopy electron microscopy, once the neural tube is closed. But these processes remain observable on the surface of the neuroepithelial cells as this work and that of Bancroft and Bellairs (1975) suggest. However, light microscopy, as will be discussed later, demonstrated the presence of these processes within the closed neural tube.

The spherical or oval bodies seen within the neural groove were also observed by other workers (Zarin, 1971; Freeman, 1972; Gooda, 1974b and Bancroft and Bellairs, 1974 and 1975). The nature of these bodies remains unknown. This raised the question of numerous possibilities: artifacts, yolk cells, degenerated cells, lipid
droplets or mucopolysaccharides. Proctor (1972), for instance, believed that these bodies were pinched off the spines of the neuroepithelial cells. Bancroft and Balfour (1979), on the other hand, considered them as fragments of degenerating or dead cells. Whether these unknown bodies, irrespective of their nature, have any part to play in the mechanism of closure of the neural tube, is difficult to determine.

Couda (1974b), however, raised such a possibility because of the fact that most of these bodies joined the cytoplasmic processes and might in this way act as a mechanical support for these processes. This relationship between the cytoplasmic processes and these unknown bodies may be supported by the fact that both these structures tend to be numerous at the same region of the neural groove; mainly at the brain region and between somites.

2. LIGHT AND ELECTRON MICROSCOPIC STUDIES

Somites start to develop by the appearance of a cavity in the paraxial mesoderm (Langman and Nelson 1966) around which cells accumulate to form the somite. Wohrer (1973) and Couda (1974a and b), however, believed that the somitic cavity appeared after the cells accumulated. The somitic cavity as ill defined structure but as cells accumulate, it becomes more definite. The lateral border of the somite is the last part of it to become so (Couda, 1974b). The anterior end of the presomatic strip is cup shaped (Wohrer, 1973) and is later filled up by adjacent cells (Couda, 1974b).
The developing neural tube appears segmented, being thicker between somites and thinner at the level of somites (Kallen, 1951; Borgquist and Kallen, 1954 and Rohenroth and Bolliaire, 1975). The increased thickness between somites is believed to be due to a higher rate of cellular proliferation in the neural tube between somites as compared to that at somites (Kallen, 1955 and Borgquist and Kallen, 1954). Watsonen et al., 1955 and Linboeth, 1965, on the other hand, believed that the neural tube became thicker where it was touched by the somites or notochord. However, Gould (1974a and b), and by taking accurate measurements of the neural tube at various levels, proved that the thickness of the neural tube was rather uniform and its segmentation was rather apparent and was due to concentric produced by the lateral pressure exerted by the somites.

The neuroepithelial cells of the developing neural tube are of three main types: fusiform, wedge and irregular in shape. The fusiform cells have central and peripheral processes which extend to the dorsal and ventral surfaces of the neural tube. The fusiform cells tend to be more numerous at early stages of neurulation especially at the somite level. Bellairs (1979) and Gould (1974b) reported similar types of cells. Some workers (Amsel, 1902; Claeys, 1914 and 1925; Haltmayer, 1942 and 1946; Dulles, 1944; Lewis, 1947; Salinsky, 1960; Waddington, 1962 and 1976; Baker and Shroeder, 1967; Shroeder, 1970; Kestnbaum, 1971, 1972 and 1974; Freeman, 1972 and Fortch and Berson, 1974) believed that the neuroepithelial cells were bottle shaped rather than fusiform. In this work very few bottle shaped neuroepithelial cells were observed.
The shape of the neuroepithelial cells gained much significance when Rhumbler (1902), Glaser (1904 and 1913) and Hobson (1941) considered it as an essential autonomous factor responsible for the folding and closure of the neural tube. They suggested that the bottle-shaped or trapezoid neuroepithelial cells caused the ventral surface of the medullary plate to become wider than the dorsal surface, thus bringing about the folding of the plate. The predominance of fusiform neuroepithelial cells, and the few number of bottle-shaped cells, as suggested by this work, tends to contradict the above-mentioned assumptions and any role the shape of cells may play in the folding of the medullary plate.

The extracellular spaces in between the neuroepithelial cells increase at mid-neuralisation and tend to be more extensive on the ventral rather than the dorsal aspects of the closing neural tube. At later stages, the extracellular spaces decreased and the cells became closely packed.

The variation in amount and distribution of the extracellular spaces during development of the neural tube suggest that it may influence the folding of the medullary plate. It is possible to assume that the extracellular spaces separate the basal parts of the neuroepithelial cells, causing the ventral border of the closing neural tube to be longer than the dorsal border, thus causing the folding process.
Subdorsal vesicles appear at early neurulation, increase in size neurulation to disappear at later stages. These vesicles were reported by many workers (Norman and Kauffman, 1965; Ruggeri, 1957; Terin, 1972; Gonda, 1974; Itoh, 1974; Waterman, 1976). The nature and function of these vesicles remain unknown. Ruggeri (1967), however, believed that these vesicles contained water that had separated from albumin, while Norman and Kauffman (1960) held the view that these vesicles transit some cellular secretory products. They based that assumption, which this work tend to support, to the presence of cytoplasmic microtubules joining these vesicles. Moreover, the number of microtubules and vesicles tends to vary proportionally. Most vesicles and microtubules are found in greater number during neurulation to disappear in later stages. It is possible, as well, that these vesicles contain extracellular fluid (Gonda and Boule, 1973).

There is a clear zone between the neuroepithelial cells of the neural folds and the surrounding mesenchyme and amniotic cells. Processes connecting the cells of these tissues. This space increases as neurulation proceeds. Many postulates had been advanced as regard the nature, content, of the space and its functional significance. Wecker (1973), Gonda (1974a) and Akerfelt and Holm (1975) observed the cellular connections traversing this space to connect the neuroepithelial cells with the mesenchymal cells. Gonda and Holm (1976) believed that this space contained an extracellular matrix which had a "neuroepithelial role" in neural fold formation especially at the cephalic region.
Electron microscopy demonstrated that the central cytoplasmic processes crossed the neural groove and formed focal and tight junction with those of the opposite side. These processes are the central processes of the inner neuro-epithelial cells. The focal and tight junctions between the cytoplasmic processes, suggest, not only the presence of a cohesive force between these processes but may also indicate that these processes slide along each other. They may also allow the cytoplasm of these cells to communicate.

Cytoplasmic microtubules joining the subdorsal vessels were also seen. Similar observations were made by Herrman and Kaufman (1966). These microtubules were probably involved in some cellular secretory function. However, Braunen (1960) and Nessler (1960) attributed the elongation of cells to these microtubules. Waddington and Ferry (1965) postulated that cellular elongation, due to the microtubules, was responsible for folding of the medullary plate. It is of interest to note, yet again, that microtubules increase up to mid-orientation to decrease in later stages. This variation goes hand in hand with that of the subdorsal vessels.

The subdorsal vessels appear in T.E.M. as non-membrane bound electron dense bodies.

Microfilaments are present in the base of central and peripheral processes of the neuro-epithelial cells. Bulinsky (1960) believed that these microfilaments were responsible for apical
contraction of the neuroepithelial cells. He also held the view that microfilaments of the neuroepithelial cells contract simultaneously causing folding of the flat medullary plate and fusion of the lips of the neural folds along the entire length of the closing neural tube. However, it is well known that the neural folds meet each other at the brain region (Streeter, 1933) and opposite anites (Gaudin, 1974b and c) and that in before complete fusion occurs. The most possible function of these microfilaments is the formation and protrusion of the cytoplasmic processes.

C. EXPERIMENTAL STUDIES ON THE CLOSING OF THE NEURAL TUBE

"The effect of the salivary, cervical, and laryngeal, local environment, cytoplasmic processes and extracellular aspect on the process of closure of the neural tube."

Theories have been advanced to relate folding of the medullary plate to external pressure from related tissues. His (1974) believed that such forces were created by rapid growth of the nervous system. Roux (1905) found that excessive lateral pressure, ...some manner, accelerated neurulation while the removal of such force, caused flattening of the medullary plate. A similar view was held by Holtfreter (1933) and Streeter (1933). The latter authors attributed the external pressure to the laterally placed somites and the underlying notochord.

The developing neural tube appeared segmented, being thicker between somites and
thinner at the level of somites (Kallen, 1953; Borgquist and Kallen, 1954; Gould, 1974a and b and Hancroft and Balleine, 1975). The increased thickness between somites is believed to be due to a higher rate of cellular proliferation in the neural tube between somites as compared to that at the level of somites (Kallen, 1950 and Borgquist and Kallen 1954). Waterston et al., 1955 and Llewellen, 1956, on the other hand, believed that the neural tube become thicker where it came in contact with somites or notochord. However (Gould, 1974a and b) and by taking accurate measurements of the neural tube at various levels, proved that the thickness of the neural tube was rather uniform and its segmentation was apparent and was due to the concavities produced by laterally exerted pressure by the somites.

In this study various operative procedures were carried out in developing chick embryos. The sections were either added or removed through an endodermal incision.

The operative manipulation and the surgical endodermal incision were believed to have no disturbing effect on the development of the embryo as a whole or in the closure of the neural tube. Meckel was the in vitro culturing using New's technique.

The addition of somites to developing embryos speeds the closure of the neural tube and the graft grows and causes a lateral curvature of the neural tube. This lateral curvature is believed to be due to laterally exerted pressure created by the graft.
Removal of somites or cervical mesenchyme causes failure of the neural tube to close. Although new somites may develop to replace the surgically removed somites, these new somites are usually smaller in size and do not affect the closure of the neural tube.

It seems, almost definitely, that the presence of somites is essential for the closure of the neural tube. Somites probably do this by exerting mechanical pressure.

The issue was raised that somites might affect the rate of cellular proliferation of the neuroepithelial cells of the medullary plate and neural folds (Derrick, 1937). According to her, the rate of cell multiplication and growth was more in the neural folds rather than the floor and could therefore produce folding. Haffen (1953) and Vogtquist and Zeller (1954) assumed that the highest rate of cellular proliferation occurred in the neural fold opposite the somite.

In this study various parameters of the neural tube were measured in normally developing embryos as well as in those in which somites were either added or removed.

In normally developing embryos, the lateral walls of the neural tube are thicker, but the whole tube remained thinner in thickness throughout its length. In other words, the thickness of the tube at the level of somites and between them is rather the same. The distance between the neural margins and the lips of the neural folds is less at the level of somites. These findings demonstrate
that caudal to the brain region, the rate of cellular proliferation is uniform throughout the neural tube, and is not affected by the presence of somites at certain sites as suggested by (Kellen, 1953 and Bierquist and Kellen, 1954). Further studies could render this issue more certain.

The neural tube margins and the clefts of the neural folds at the level of somites support the assumption of a laterally exerted mechanical pressure at this site.

The addition and removal of somites produce a change in the thickness of the neural fold, the distance between the lips of the folds and their neural margins at various regions. Both the addition and removal of somites decrease the thickness of the neural folds whether the thickness is measured at the floor or the lateral walls. The relationship between somites and thickness of the neural fold can not be determined by this study.

The addition of somites to developing embryos had significantly reduced the distance between the lips of the neural folds. This same effect was however produced by the removal of somites. In the first place, the addition of somites could have caused an increase in the laterally exerted mechanical force. The removal of somites was expected to cause an increase rather than a decrease in the distance between the lips. That could have been due to the fact that somites were removed at a rather later stage of development when other factors such as extracellular spaces and cytoplasmic processes might have played a more major role.
The effect of local environment was investigated by implanting medullary plates to host embryos and cultivating others on thin albumin. In all circumstances, the medullary plate developed into a ball of tissue and failed to produce any resemblance to a normally developing neural tube. It must be mentioned, however, that Glaser (1931) found that medullary plate cultured in vitro developed into a neural tube.

The mechanical separation or reopening of a closed neural tube causes failure of re-closure. This failure can be due to the mechanical trauma to which the embryo subjected or the separation may rupture the cytoplasmic processes.

It is concluded that the folding of the medullary plate and the closure of the neural tube are due to a number of factors that may operate together or at different stages of development. The main factor seems to be the presence of somites and cervical mesenchyme. These probably exert a lateral mechanical force. Other intrinsic factors are probably involved as well. The cytoplasmic processes extending from one neural fold to the other help in their approximation. The extracellular spaces, which are most abundant in the ventral aspect of the tube may also play an important role. It is felt that the somites and the cervical mesenchyme may be operating at early stages while the cytoplasmic processes and extracellular space appear at later stages of development. These latter two factors may also be more essential as regions devoid of somites. It is also felt that the shape of the cells and rate of cellular proliferation do not have a place in this process.
CHAPTER IX

SUMMARY
SUMMARY

The factors affecting the closure of the neural tube have been studied in the chick embryo (Gallus domesticus) using in vitro techniques.

Normal development was studied using stereomicroscopy, light and transmission electron microscopy.

The effect of addition or removal of somites, the cervical saccocysts and reopening of the closed neural tube was also studied.

Measurements of various parameters of the neural tube i.e. thickness of the floor, thickness of the neural folds, and distance between the lips and medial margins of the neural folds, both in normal and experimental embryos were made and statistically analysed.

The findings of these studies were presented and discussed.
CHAPTER X

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APPENDIX

FLEDDUS AND EMBEDEGING MULLTURE

A. ABBRASION MIXTURE (Dowen and Waiblingen 1967)

0.5 g sodium
0.1 g potassium dichromate
100 ml distilled water

METHOD OF POLYCHROMA SITZING NO SLIDES

1. Immerse each slide in 1% hydrochloric acid in 70 alcohol and polish subsequently with a clean fluff-free cloth.

2. Divide the ribbons with a scalpel into convenient lengths.

3. Float the sections on a little amount of the adhesive mixture, and flatten them by gentle heat from a hot plate.

4. Drain away most of the adhesive mixture by slightly tilting the slide.

5. Dry the mounted sections overnight in an incubator at 37°C.

B. ARALDITE ADHESIVE MIXTURE (Glowart and Glowart, 1950)

26.0 ml Araldite I (Epoxy resin)
26.0 ml Araldite II (hardener)
30.0 drops Araldite III (accelerator)
25.0 drops Araldite IV (01-2-Butylphthalate)
C. **ROUIN'S FLUID** (Rouin, 1937)

75.0 ml Saturated aqueous picric acid
25.0 ml Formalin 40%
5.0 ml Acetic acid

This mixture can be stored.

"Rouin causes partial or complete lysis of red blood cells and collagen fibres may be swollen, it does not cause excessive hardening and gives brilliant staining with cytoplasmic stains. Glycogen is well preserved, particularly with an alcoholic variant of the above mixture but the kidney is badly preserved and some cytoplasmic granules may be dissolved. Rouin's fluid is a micro-anatomical fixative or a cytological (nuclear) fixative when used for the demonstration of chromosomes.

Blocks are fixed for 6-24 hours and transferred to 70% alcohol. The yellow staining of tissues is sometimes an advantage with very small specimens but should be removed from sections by treatment with alcohol followed by 2.5% sodium thiosulphate before using basic staining dyes, otherwise a precipitate will be formed" (Brury and Wellington, 1967).
3. **KARNOVSKY'S FIXATIVE** (Karnovsky, 1965)

1. Dissolve 42.3 g of sodium cacodylate in 100 ml distilled water to make 0.2 M solution.

2. Add 7.25 ml of conc. HCl to one litre distilled water to make 0.2 M HCl.

3. Make final cacodylate buffer by adding 2.7 ml of 0.2 M HCl to 50.0 ml of 0.2 M sodium cacodylate, and make up to a final solution of 200.0 ml with distilled water.

4. Dissolve 10.0 g of paraformaldehyde powder in 175.0 ml water by heating to 60 - 70°C and stirring.

5. Add a few drops of 1% NaOH (40.0 g of NaOH in 1000 ml distilled water) with stirring until the solution alcine.

6. Cool and then add 150.0 ml of 25% glutaraldehyde, and make the volume up to 750.0 ml with 0.2M cacodylate buffer, pH 7.4 - 7.6. The final pH has to be about 7.2.

7. Add 0.37 g of CaCl₂ anhydrous and then filter the solution.

"The osmolarity of this fixative is about 2010 milliosmol per kg. Despite this high osmolarity, shrinkage is unusual except when the fixative is perfused or applied to free-floating cells and monolayers. Tissue fluid in slabs of tissue probably dilutes the fixative as it
penetrates, reducing the cemellarity somewhat. Myelin figures are less commonly seen than with formaldehyde or glutaraldehyde fixation alone, and all cell components are well preserved except lipid droplets, which are extracted. Microtubules are particularly well preserved. It is surmised that the formaldehyde penetrates faster than the glutaraldehyde and temporarily stabilizes structures which are subsequently more permanently stabilized by glutaraldehyde" (Drury and Wellington, 1967).

2. **Normal Glucose Saline**

   2.0 g Sodium chloride
   1.0 g D-Glucose anhydrous
   1000 ml Distilled water

3. **Osmium Tetroxide** (OsO₄) (Drury and Wellington, 1967)

   "Osmium tetroxide is supplied as pale yellow crystals in sealed tubes of 0.5 or 1 gram and is used as a 0.5 to 1 per cent solution in water. It is a poisons chemical and the vapour from both... crystals and solution is irritant and dangerous. Exposure of the eyes to the vapour should be avoided by the use of close-fitting goggles, and containers of the solution should be kept tightly stoppered. Preparation of 25 solution is carried out as follows:

1. Remove the label and all traces of gum from four 0.5 g tubes with water, but not hot water.

2. Dry the tubes with a clean cloth, store with a glass slide and keep in the middle."
3. Drop the eight halves with contents into a dark-glass bottle containing 100.0 ml of pure distilled water. The crystals may take some time to dissolve but heat should not be used to hasten solution. The bottle should have a glass stopper and be stored in a cool place.

**NOTE**

Cadmium tetraoxide is easily reduced to the gray or black lower oxide by light, warmth or organic matter and once used solutions should not be returned to the stock bottle. An effective way of preventing reduction is by the addition of one drop of saturated aqueous mercuric chloride to each 10.0 ml of solution.

Like formaldehyde, cadmium tetraoxide forms additive compounds with protein. Most lipids including collagen are blackened by the reduction of the cadmium tetraoxide to the lower oxide. Its penetration is poor and fixation is liable to be uneven. Minute objects, aneus of cells and thin sections may be fixed by the vapour, without immersion in the fluid.
A. SOLUTIONS

(1) SHERRING’S HEMATOXYLIN (Drury and Wellington, 1967)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin</td>
<td>2.0 cc</td>
</tr>
<tr>
<td>Absolute Alcohol</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>Glycerin</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>(16.0 - 14.0 cc)</td>
</tr>
<tr>
<td>Potash alum in excess</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve the hematoxylin in the alcohol before adding the other ingredients. The stain was ripened naturally by allowing to stand in a large flask, loosely stoppered with cotton wool, in a warm place and exposed to sunlight. The flask is shaken frequently and ripening takes several weeks.

(2) EOSIN (aqueous)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water soluble eosin</td>
<td>0.5 cc</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

3. METHOD OF STAINING

1. De-wax sections
2. Stain in hematoxylin for 2-30 minutes.
3. Wash well in running tap water for 2-3 minutes. The sections may be examined microscopically at this stage to confirm a sufficient degree of staining. If insufficient, return to the stain.
4. Remove excess stain by decolorization (differentiation) in 0.5-1% hydrochloric acid in 70% alcohol for a few seconds.

5. Regain the blue colour and stop decolorization by washing in alkaline (running tap water for at least 5 minutes). The stain should again be checked microscopically until proficiency in naked-eye control of decolorization has been gained by experience with stain and tissues.

6. Stain in 1.0% aqueous eosin for 1-3 minutes.

7. Wash off surplus stain in water.

8. Examine microscopically. Cytoplasm and muscle fibres should be deep pink, collagen a lighter pink. Red blood cells and eosinophil granules should be a bright orange-red.

9. Dehydrate in alcohol and clear in xylene as outlined in the general scheme, bearing in mind that aqueous eosin is removed from tissues by water and low grade alcohol less readily by absolute alcohol. The degree of staining of eosin is thus easily controllable and a slight over-staining when sections are examined prior to dehydration will be removed during the passage through alcohol.
10. Mount in D.P.X.

C. RESULTS

Nuclei - blue to blue-black.

Karyomeres - dark blue.

Cartilage - pink or light blue to dark blue depending on type and the stain used, being darkest with Brielich's haematoxylin.

Cement lines of bone - blue with Brielich's haematoxylin. Calcium and calcified bone-purplish blue.

Basophil cytoplasm (Plasma cells and osteoclasts) purplish.

Red blood cells, phagosomes, eosinophil granules, granuloc cell granules, syrinx granules, keratin - bright orange-red.

Cytoplasm - wanders of pink.

Muscle fibres, thyroid colloid, thin elastic... fibres, decalcified bone matrix - deep pink.

Collagen and osteoid tissue - light pink (Drury and Wellington, 1967).
1.0 g
1.0 g
1.0 g
2.0 g
0.20 ml

Lead nitrate
Lead acetate
Lead citrate
Sodium citrate
Distilled water

4. Method of preparing the solution
1. Heat the above mixture to 40°C and stir for one minute.
2. Add 3 g sodium citrate and stir again.
3. Add 24 ml of sodium hydroxide followed by 80 ml of distilled water.
   The solution must be kept in the fridge.

5. Method of staining
1. Put a drop of the stain on a flat plate of wax.
2. Hold the grid with a fine forceps and identify the side on which the section is stucked.
3. Put the grid with the section facing downwards on the top of the stain and leave for 6 - 10 minutes.
4. Pick the grid and rinse it vertically in a jet of distilled water 6 - 10 times.
5. Put the grid with the section facing upwards on a piece of tissue and fluff to remove any excess water.
3. The section can be viewed immediately with the T.M.M. or stored in a grid's box.

The raw data of the observations is in (Vol. 2).