Analysis of the effects of *Streptomyces* hyaluronidase on formation of the neural tube

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**SUMMARY**

Chick embryos at stages 8 to 9 were treated *in ovo* with *Streptomyces* hyaluronidase (SH) to determine whether neurulation occurs normally in embryos depleted of hyaluronic acid, a major component of the extracellular matrix. Open neural tube defects occurred in 60–94% (depending on the particular enzyme batch) of the embryos treated with SH and examined after an additional 24 h of incubation. Defects were confined mainly to the spinal cord. The neural folds underwent elevation in defective regions but failed to converge and fuse across the dorsal midline. The extracellular matrix of embryos treated with SH was depleted consistently, as determined with sections stained with Alcian blue. Control experiments were done to ensure that neural tube defects were not caused by non-specific protease contamination of SH, or by digestion products of hyaluronic acid. We propose several plausible and testable mechanisms through which the extracellular matrix might influence the complex developmental process of neurulation.

**INTRODUCTION**

Neurulation is a complex developmental process which results in formation of the neural tube, the rudiment of the entire central nervous system. Experiments were first begun about 50 years ago to determine how this process occurs (reviewed by M. Jacobson, 1978). Although many different mechanisms have been hypothesized to cause neurulation, all of these mechanisms can be categorized as either intrinsic or extrinsic factors. Intrinsic factors consist of changes restricted to the neural plate. Such factors include the possible constriction of apical bands of microfilaments in neuroepithelial cells during bending of the neural plate (reviewed by Karfunkel, 1974). Extrinsic factors consist of forces originating outside of the neural plate, such as the possible migration of surface epithelial cells medially (Bragg, 1938; Gillette, 1944; Schroeder, 1970), stretching of the neuroepithelium during craniocaudal elongation of the embryo (reviewed by A. G. Jacobson, 1980) and changes in the extracellular matrix and cells underlying the elevating neural folds (Morris & Solursh, 1978a, b; Morris-Kay & Crutch, 1982). It is likely that multiple factors act in concert to cause neurulation, rather than one factor acting exclusively, since experimental evidence favouring many different factors have appeared in the literature.

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We have examined the possible roles of the extracellular matrix, principally hyaluronic acid, in neurulation in chick embryos. Hyaluronic acid is a major component of the extracellular matrix underlying the neural folds of both avian (Pratt, Larsen & Johnston, 1975; Solursh, 1976; Fisher & Solursh, 1977) and mammalian (Solursh & Morriss, 1977; Derby, 1978; Morriss & Solursh, 1978a, b) embryos, and constitutes at least 84% of the glycosaminoglycans synthesized by early chick embryos (Solursh, 1976). Hyaluronic acid is synthesized by a variety of tissues in early embryos, such as the neural tube (Hay & Meier, 1974), somites (Solursh, Fisher, Meier & Singley, 1979a), surface ectoderm (Solursh, Fisher & Singley, 1979b) and myocardium (Manasek et al., 1973). It has been suggested that an increase in the volume of the extracellular matrix beneath the neural folds might play an important role in the formation of the neural tube (Morriss & Solursh, 1978a, b). Because hyaluronic acid is capable of a high degree of hydration (Laurent, 1970), with concomitant increase in volume, it is reasonable to suggest that inflation of the hyaluronic acid component of the extracellular matrix might produce forces sufficient to assist in neurulation. We have tested this possibility by treating chick embryos in ovo with Streptomyces hyaluronidase (SH). We restricted our study to embryos at stages 8 to 9 (Hamburger & Hamilton, 1951), thereby examining formation of the spinal cord. (Formation of the brain was not studied since this process occurs at earlier stages when windowing alone produces multiple defects in a large percentage of the embryos. At those stages, special precautions must be used to ensure that the effects of SH are studied, and not some unrelated side effect; Mann & Persaud, 1979; Fisher & Schoenwolf, 1983.) We report that neural tube defects are usually present in the region of the future spinal cord of embryos treated with SH, and that the principal effect of treatment on neurulation is a failure of the elevated neural folds to converge and fuse across the dorsal midline.

**MATERIALS AND METHODS**

*Treatment of embryos in ovo*

White Leghorn chicken eggs were incubated at 38°C and 50–60% relative humidity until embryos reached stages 8 to 9. A small window was cut through the portion of the shell overlying each embryo, with standard techniques (Hamburger, 1960). The sub-blastodermic space of each embryo was injected with 20 μl of one of the following solutions: SH (200 TRU/ml in 0.9% saline; two different lots of enzyme were purchased from Calbiochem and are designated below as lots 1 and 2); SH in a solution of 3 parts 0.9% saline to 1 part albumen; trypsin (Boehringer; 0.1 μg/ml 0.9% saline); a solution of hyaluronic acid (Sigma, Grade 1; 1 mg/ml 0.9% saline) digested with 40 TRU SH and boiled for 5 min to destroy the enzyme; 0.9% saline; or a solution of 3 parts 0.9% saline to 1 part albumen. All embryos were illuminated obliquely with fibre optics and
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staged critically. Windows were then sealed with Scotch tape and eggs were returned to the incubator for an additional 24 h.

Assay for protease activity

An assay for protease activity was used to determine whether significant levels of protease contaminated our particular batches of SH. The assay used was modified from Caputo, Schröde, Kimura & Hascall (1980) and is based on the lysis of fibrin clots. Fibrinogen/agar-coated plates were prepared as follows: a mixture containing 1 gm agar (Difco Noble) in 75 ml 0·05 m-Tris buffer (pH 8·6) plus 0·014 m-calcium lactate was boiled for approximately 15 min, until the agar dissolved, and then cooled to 60°C. A second solution, containing 80 mg fibrinogen (Sigma, Type I) in 25 ml of 0·025 m-Tris buffer (pH 8·6) minus calcium lactate (the fibrinogen solution was made up fresh and centrifuged briefly to remove the undissolved fibrinogen), was then added slowly to the agar solution. The agar/fibrinogen mixture was subsequently incubated for 1 h, in an 80°C water bath, and poured into 60 mm Falcon dishes (5 ml/dish). Plates were allowed to cool at room temperature and then used immediately or stored refrigerated for up to 2 weeks in a humidified chamber. Small wells were made in the agar/fibrinogen gels by pushing the tip of a glass pipette (tip diameter = about 3 mm) down to the level of the plastic dish, and sucking out a small island. Wells were then filled with 10 μl of enzyme solutions (SH; pronase, Calbiochem, grade B; trypsin; pronase + 1:3, albumen:0·9% saline; or trypsin + 1:3, albumen: 0·9% saline), and plates were incubated at 37°C for 24 to 36 h.

Processing embryos for microscopy

Three different fixation protocols were used to prepare embryos for examination by light microscopy. In preliminary experiments, control embryos and embryos treated with SH were fixed for 2 h with Carnoy’s fixative (Humason, 1972) containing 0·5 % cetylpyridinium chloride, dehydrated with ethanol, cleared with Histosol, embedded in Peel-A-Way paraffin (53–55°C) and sectioned serially and transversely at 8 μm. Sections were stained with 1% aqueous Alcian blue (pH 2·5) to assess the effectiveness of SH on the depletion of glycosaminoglycans (i.e., principally hyaluronic acid; Ohya & Kaneko, 1970). This method consistently demonstrated a marked reduction in the amount of extracellular matrix present in embryos treated with SH (cf. Figs 3, 4), confirming previous investigations (Fisher & Solursh, 1977; Solursh et al., 1979a, b). In addition, in embryos treated with SH we found that: (1) the blood islands failed to differentiate into circulating blood cells and anastomosing vitelline blood vessels, (2) expansion of the blastoderm over the yolk was inhibited, and (3) the heart rate was slowed markedly. Consequently, the presence of these characteristic features was used exclusively in subsequent experiments as evidence that the matrix was depleted by SH (i.e., virtually 100% of the embryos injected
sub-blastodermically with SH exhibited these features, indicating that the matrix was depleted consistently), and embryos were processed with different fixation protocols to obtain better morphological preservation and/or improved staining of the cellular components of the embryonic tissues. The following two protocols were used for this purpose. Some embryos were fixed with Bouin's fluid overnight, decolorized with 70% ethanol saturated with lithium carbonate, dehydrated, cleared and embedded in Peel-A-Way paraffin. Sections were cut at 8 μm and stained with Gill's hematoxylin. Other embryos were fixed for 2 h with 2% glutaraldehyde, 2% paraformaldehyde (in 0.1 M phosphate buffer at pH 7.2), washed with buffer, postfixed for 1 h with osmium tetroxide, dehydrated with ethanol and divided into two groups. One group of dehydrated embryos was transferred to propylene oxide and embedded in Epon/Araldite (Kushida, 1971). Blocks from these embryos were cut at 1 μm, and sections were stained with methylene blue/azure II. The second group of dehydrated embryos was critical-point-dried from liquid CO₂ and photographed intact as stereopairs with a Nikon SMZ-10 stereomicroscope.

RESULTS

Controls for possible protease contamination

SH, at the same concentration used to deplete the extracellular matrix in ovo, was tested with the fibrin-clot protease assay and failed to show any protease activity (Table 1). By this same assay, pronase or trypsin showed lysis, 24–36 h later, with concentrations as low as 0.1 μg/ml. When pronase or trypsin was dissolved in 1:3 albumen:saline, protease activity was inhibited with concentrations as high as 10 μg/ml (Figs 1, 2). Thus, by dissolving enzymes in albumen:saline mixtures it was possible to inhibit protease activity at a concentration 100× the lowest concentration detectable with our assay.

Table 1. Summary of the various concentrations of enzymes tested for proteolytic activity, as determined by the lysis of a fibrin clot.

<table>
<thead>
<tr>
<th>Agents tested</th>
<th>Concentrations</th>
<th>Detectable lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomyces</td>
<td>200 TRU/ml</td>
<td>No</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pronase or Trypsin</td>
<td>100 μg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>10 μg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>0.1 μg/ml</td>
<td>Barely (at 36 h)</td>
</tr>
<tr>
<td></td>
<td>0.01 μg/ml</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.001 μg/ml</td>
<td>No</td>
</tr>
<tr>
<td>Pronase or Trypsin + Albumen</td>
<td>100 μg/ml</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>10 μg/ml</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>1 μg/ml</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>0.1 μg/ml</td>
<td>No</td>
</tr>
</tbody>
</table>
Effects of hyaluronidase on neurulation

Embryos were injected with various solutions to test whether the effects of SH on the extracellular matrix and on neurulation were related to the depletion of hyaluronic acid, or to a protease contaminant. Sections stained with Alcian blue demonstrated a marked reduction in the extracellular matrix (including cell surface coat materials) of embryos treated with SH, with or without albumen, as compared to control embryos injected with saline or an albumen:saline mixture (cf. Figs 3–5). Furthermore, embryos treated with trypsin appeared identical in all respects to control embryos (Fig. 6). (Perhaps trypsin was inactivated by endogenous albumen in the sub-blastodermic space.) These experiments demonstrate that the extracellular matrix is depleted by SH, not by a protease contaminant. However, it is unlikely that SH depletes only the hyaluronic acid component of the extracellular matrix since SH has been shown to remove sulphated glycosaminoglycans (possibly as part of hyaluronic acid-containing complexes) as well as hyaluronic acid (Fisher & Solursh, 1977; Morriss and Solursh, 1978b). Thus, our experiments test the effects of removal of hyaluronic acid, and possibly other components of the extracellular matrix intimately associated with hyaluronic acid, on formation of the neural tube.

Neurulation in control embryos

Although the morphogenesis of the neuroepithelium has been described already (Schoenwolf, 1982), this process will be reviewed briefly here to aid the reader in the interpretation of embryos exhibiting neural tube defects.

Neurulation occurs in three stages: formation of the neural plate; bending of the neural plate, with formation of the neural groove and neural folds; and fusion of the neural folds. Likewise, bending of the neural plate occurs in two steps: elevation of the incipient neural folds, and convergence of the definitive neural folds. Elevation occurs similar to the closing of a hinge, with the supranochochal cells forming a locus of bending (Fig. 7), whereas convergence involves the formation of distinct bilateral furrows on the future luminal side of the dorsolateral walls of the neuroepithelium (Fig. 7: arrowhead). These furrows always formed at the precise level at which the surface and neural ectodermal layers of each neural fold diverged from one another (Fig. 7: arrow). Elevation serves to bring the neural folds dorsally, forming a V-shaped neural groove; convergence, to displace the folds medially, so that they come into apposition along the midline. Fusion ensues, after elevation and convergence are completed, establishing a closed neural tube covered by surface epithelium. Neural tube defects result when elevation, convergence or fusion fails to occur properly.

Effects of SH on neurulation

 Frequencies of neural tube defects

Most embryos treated with SH exhibited neural tube defects (Table 2). However, the frequency at which these defects formed was related to the particular
Table 2. Summary of the effects of various treatments on closure of the neural groove. Neural tube defects = dysraphic (open) defects.

<table>
<thead>
<tr>
<th>Solutions injected sub-blastodermically</th>
<th>No. of embryos with closed neural tubes</th>
<th>No. of embryos with neural tube defects</th>
<th>% of embryos with neural tube defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronidase (lot 1)</td>
<td>24</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>Hyaluronidase (lot 2)</td>
<td>3</td>
<td>51</td>
<td>94</td>
</tr>
<tr>
<td>Hyaluronidase (lot 2) + saline/albumen mixture</td>
<td>0</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin (0.1 \mu g/ml)</td>
<td>14</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Hyaluronic acid digested with hyaluronidase (lot 2) and boiled for 5 mins.</td>
<td>24</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Saline or saline/albumen mixture</td>
<td>64</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

batch of enzyme used. Neural tube defects formed in 60% of the embryos treated with our first enzyme lot, whereas 94% of the embryos treated with our second lot had neural tube defects. Surprisingly, both batches of enzymes appeared to be equally effective in depleting the matrix, since no differences could be detected in sections (from embryos treated with the two batches of enzyme) stained with Alcian blue. The addition of albumen to the SH solution failed to reduce the efficacy of SH in causing neural tube defects (Table 2). In addition,
neural tube defects occurred infrequently in embryos treated with trypsin (Table 2). These two experiments demonstrate clearly that the adverse effects of SH on neurulation are not due to a protease contaminant. Finally, neural tube defects were not caused by the digestion products of hyaluronic acid (i.e., unsaturated tetra- and hexasaccharides), because embryos treated with digested hyaluronic acid were usually normal (Table 2).

Neural tube defects were present in only 14% of the control embryos (injected with saline or albumen:saline) (Table 2). This percentage is in accord with our previous studies on the effects of windowing on neurulation (Fisher and Schoenwolf, 1983).

Gross features of embryos with neural tube defects

Treatment of embryos with SH resulted in a high percentage of embryos exhibiting non-closure-type neural tube defects, as stated above. Examination of stereopairs of defective regions revealed that the neural folds usually elevated normally, but convergence often failed to occur (Fig. 8). In many of the embryos with neural tube defects, the elevated neural folds actually diverged, flaring laterally (Fig. 9). Thus, the formation of neural tube defects in embryos treated with SH was due principally to a failure of the elevated neural folds to converge toward the dorsal midline.

Many of the embryos treated with SH also exhibited areas where the elevated and converged neural folds failed to fuse across the midline, suggesting that SH might have inhibited fusion directly, or that apposition was delayed beyond the critical point at which fusion takes place. Fusion occasionally failed to occur at various levels along the length of the spinal cord, but much more frequently fusion was inhibited only in the area of the anterior neuropore (Fig. 10).

Histological features of embryos with neural tube defects

Embryos treated with SH exhibited several characteristic histological features. The most obvious effect was a reduction in the sizes of the extracellular spaces, as reported by others (Fisher & Solursh, 1977; Solursh et al., 1979a,b). This reduction occurred between adjacent organ rudiments (e.g., the spaces between the surface ectoderm and somites), and within organ rudiments (e.g., the spaces between cells of the somites, especially in the region of the forming sclerotomes;

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Fig. 8. Light micrographic stereopair of a stage-13 embryo treated with SH at stage 8. Arrowhead, elevated neural fold of the defective region; asterisk, tail bud. ×40.

Fig. 9. Light micrographic stereopair of the caudal part of a stage-15 embryo treated with SH at stage 9. The neural folds of the defective region (arrowhead) are everted. Asterisk, tail bud. ×40.

Fig. 10. Light micrographic stereopair of the head of a stage-12 embryo treated with SH at stage 8. The elevated and converged neural folds have failed to fuse in the region of the anterior neuropore. ×60.
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9

10
spaces between surface ectodermal cells) (cf. Figs 11, 12). A second unique histological feature present in embryos treated with SH was a marked dilation of the embryonic blood vessels. In the region of the spinal cord both the dorsal aortae and postcardinal veins were enlarged (Fig. 12). In some embryos, the aorta and postcardinal vein on the same side of the embryo communicated with one another broadly, distorting the intervening somites. A final histological feature present in embryos treated with SH was the presence of intracellular inclusions (Fig. 12: arrowheads), which, presumably, indicated that cell death was under way. It is reasonable to suggest that faulty development of the circulatory system was probably the cause of this apparent death, since (as stated above) both the structural and functional development of the extraembryonic (vitelline) circulation was inhibited severely in embryos treated with SH.

In embryos with neural tube defects, a short transitional zone was present, between the closed and open portions of the neural tube, in which the dorsal half of the neurocoele was expanded laterally. The portion of the neurocoele near the cranial end of the transitional zone was covered by two types of cells: a thin roof plate, consisting of neuroepithelium, and cells of the surface ectoderm. Near the caudal end of the transitional zone, the neurocoele was covered only by surface ectodermal cells.

Sections through areas of the open neural tube confirmed observations on whole mounts, that the neural folds were elevated throughout the craniocaudal extent of the defective area. Furthermore, the dorsal halves of the walls of the open neural tube were everted in many embryos (Fig. 12).

One final unusual, histological feature was sometimes present in areas containing neural tube defects. The surface ectodermal cells, immediately adjacent to the neuroepithelium, extended ventromedially as an epithelial sheet (especially near the caudal end of the defective area), often reaching as far as the notochord (Fig. 13: arrowheads). Shallow grooves, which flanked the neural folds in defective regions, were usually associated with these surface ectodermal extensions (Fig. 13: asterisk).

DISCUSSION

Closure of the neural groove occurs in three steps in chick embryos: elevation

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Fig. 11. Plastic section through the spinal cord of a stage-15 control embryo. ×400.

Fig. 12. Plastic section through the defective neural tube (future spinal cord) of a stage-15 embryo treated with SH at stage 9. The neural folds have diverged laterally. Arrowheads, cellular inclusions; DA, dorsal aorta. ×400.

Fig. 13. Paraffin section (stained with haematoxylin) through the defective neural tube (future spinal cord) of a stage-14 embryo treated with SH at stage 9. The surface ectoderm on the left side of the micrograph (arrowheads) has extended ventrally toward the notochord. A shallow groove (asterisk) flanks the neural fold on the same side. ×390.
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Figs. 11-13
of the neural folds, convergence of the neural folds and fusion of the neural folds across the dorsal midline. It has been suggested that inflation of the hyaluronic acid component of the extracellular matrix underlying the neural folds might generate forces involved in neurulation (Morriss & Solursh, 1978a,b). If the extracellular matrix is involved in pushing the neural folds toward the dorsal midline, then two morphological relationships must be present: (1) the cells of the neural plate in the ventral midline must be 'tacked down' (e.g., to the notochord), otherwise the extracellular matrix would infiltrate the space between these two layers, elevating the midline cells (as well as the neural folds) as inflation occurred; and (2) the extracellular matrix must be constrained laterally — this could be accomplished, presumably, by the surface ectoderm, which drapes downward around the paraxial mesodermal (or head mesenchymal) cells and associated extracellular matrix. Both of these morphological relationships are present throughout the entire craniocaudal extent of the developing neural tube in chick embryos (Schoenwolf, 1982). Thus, the suggestion that the extracellular matrix is involved in the bending of the neural plate is reasonable, at least on morphological grounds. Our results demonstrate that depletion of the extracellular matrix with SH has little or no effect on the elevation of the neural folds. However, convergence of the neural folds is blocked totally by depletion. Furthermore, fusion of the converged neural folds (e.g., especially in the region of the anterior neuropore) was often inhibited in embryos treated with SH. Thus, the principal adverse effects of SH on neurulation appear to be on the processes of convergence and fusion. Similar results have been found in rat embryos treated with \( \beta \)-D-xyloside, a compound that inhibits chondroitin sulphate-proteoglycan synthesis, altering the extracellular matrix. The neural folds of such embryos likewise fail to converge and fuse (Morriss-Kay & Crutch, 1982).

There are several specific ways in which treatment of embryos with SH might block convergence of the neural folds (admittedly, SH might also act in some non-specific way, but at present there is no evidence to support this notion). The major uncertainty about convergence stems from the fact that it is unknown whether a causal relationship exists between the formation of the bilateral furrows and convergence. That is, do the bilateral furrows form because convergence occurs, or does convergence occur because the bilateral furrows form? In the former case, the bilateral furrows presumably form passively, whereas in the latter, these furrows are active participants in convergence. Below we discuss three plausible mechanisms of convergence that might be affected adversely by removal of hyaluronic acid. Other possible mechanisms might be affected by SH as well, but at the present time these three mechanisms seem to us to be the most likely ones. The first two are based on the assumption that the bilateral furrows form passively; the last, that the furrows form actively.

The first possible mechanism of convergence that might be affected by SH is an increased, localized production and/or hydration of the hyaluronic acid
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located directly beneath the neural folds. This change in the matrix would be expected to push the neural folds toward the dorsal midline. Enzymatic removal of hyaluronic acid at this time would, therefore, remove the driving force, and the neural folds would fail to converge.

A second possible mechanism of convergence that might be affected by SH is the displacement of the surface ectoderm medially, and, consequently, the pushing of the neural folds toward the dorsal midline. A structural relationship that must exist for medial displacement of the surface ectoderm to be effective in causing convergence of the neural folds, is that the surface and neural ectodermal layers of the neural folds must be attached to one another. In both treated and control embryos, these two layers are in contact on each side along the lateral one third of the neuroepithelium. In fact, in embryos treated with SH, this association was extended sometimes beyond the normal limits. In amphibian embryos, the surface ectoderm exhibits an innate ability to migrate medially as an epithelial sheet (Jacobson & Jacobson, 1973), and similar migratory forces likely occur in other organisms. A migrating, epithelial sheet must move on some substrate. In the case of the surface ectoderm this substrate is probably either an underlying basement membrane or the overlying vitelline membrane. Altering the integrity of these substrates could conceivably have an adverse effect on migration of the surface ectoderm. It is possible that the cases where we saw an extended apposition of the surface and neural ectodermal layers, represent the misdirection of epithelial migration, due to changes in the composition of the substrate. The neural folds might also be displaced medially due to growth of the surface ectoderm, rather than to the migration of an epithelial sheet. This growth could be due to mitosis and/or the ‘vacuolation’ of the surface ectoderm (i.e., the formation of an extensive network of extracellular spaces). The latter process is clearly associated with the accumulation of hyaluronic acid in cell-free spaces, and is retarded or absent in embryos treated with SH (cf. Figs. 11, 12; Solursh et al., 1979b).

The final plausible mechanism of convergence that we will consider is the possible pulling of the neural folds medially, as neuroepithelial cells undergo apical constriction. In this possible mechanism, the bilateral furrows represent localized sites of epithelial invagination (i.e., apical constriction). It is well documented that neuroepithelial cells contain apical bands of microfilaments that could, presumably, contract (reviewed by Karfunkel, 1974). But it is unknown whether more microfilaments are present, or whether they contract more vigorously, in the areas where the bilateral furrows form. Several studies have shown that invaginating epithelia are characterized by having coats of extracellular material on their apical surfaces (e.g., optic cup: Hilfer & Yang, 1980; lens vesicle: Van Rybroek & Olson, 1981; nasal placode: Smuts, 1977; Burk Sadler & Langman, 1979; and neural plate: Rice & Moran, 1977; Lee, Sheffield, Nagele & Kalmus, 1976; Mak, 1978; Sadler, 1978; Silver & Kerns, 1978). Although the precise composition of these coats and their roles in invagination are
not known, these studies do suggest that surface coats may be important in bending of epithelial sheets. In such a case, SH treatment would, presumably, affect closure of the neural groove by depleting the important surface coat materials. Furthermore, fusion of the neural folds might be inhibited by this same mechanism, since the apices of the neural folds also contain abundant surface coat materials (Rice & Moran, 1977; Lee et al., 1976; Mak, 1978; Sadler, 1978; Silver & Kerns, 1978), which are removed by SH (as determined in the present study with Alcian-blue stained sections).

The results presented here add support to the notion that neurulation is a complex process caused by the interplay of several different mechanisms. Our results suggest that the extracellular matrix plays an important part in the formation of the neural tube. Although the precise role of extracellular matrix in neurulation is not yet clear, we have presented three plausible, testable mechanisms by which the extracellular matrix might generate forces that assist in this fascinating developmental process.

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