GLYCOSAMINOGLYCAN SYNTHESIS BY EMBRYONIC INDUCTORS:
NEURAL TUBE, NOTOCHORD, AND LENS

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INTRODUCTION

Considerable indirect evidence (1-4) and some
direct evidence (5-8) indicate that glycosaminogly-
cans (GAG) promote certain types of morphogene-
sis and cell differentiation. Removal of basement
membrane by hyaluronidase disrupts salivary
gland branching (2) and hyaluronate interferes
with chondrocyte aggregation (5). On the positive
side, chondromucoprotein stimulates the synthesis
of extracellular matrix by chondrocytes and pre-
chondrocytes (6, 7). Chondroitin and heparan
sulfates and collagen, but not dermatan or keratan
sulfates or hyaluronate, promote production of
primary corneal stroma by corneal epithelium (8,
9), an event which in vivo is said to be induced by

FIGURE 1 Pseudobranchial mitochondria from a fish adapted to a normal photoperiod. The cristae are of
the foliate type. Cisternae of the smooth endoplasmic reticulum are in close association to the
mitochondria. × 19,900.

FIGURE 2 Mitochondria from a fish adapted to continuous darkness for 7 days. Some of the cristae are
angulate (short arrows) while others have a wavy appearance suggestive of coiling (long arrow). × 24,800.

FIGURE 3 Mitochondrion of a fish adapted to constant darkness for 28 days. Several of the cristae appear
helical (arrows). × 50,000.

FIGURE 4 A mitochondrion from the pseudobranch of a fish that was kept in constant light for 28 days.
Some of the cristae are arranged into rows which alternate with foliate cristae. In cross section the modified
(helical) cristae appear tubular (arrows). The line A—B indicates the section plane represented in Fig. 6. ×
70,000.

FIGURE 5 A row of modified (helical) cristae sectioned longitudinally from a fish adapted to continuous
light for 28 days. In several areas, the helical, twisting nature of the cristae is visible (arrows). × 56,000.

FIGURE 6 A portion of a mitochondrion cut in the plane marked A—B in Fig. 4. In this orientation, the
helical form cristae are aligned longitudinally and alternate with foliate cristae. × 56,000.
the lens (10). Kosher et al. (7) and others have postulated that the neural tube and notochord supply morphogenetically active GAG to the somites during cartilage induction in vivo, and Cohen and Hay (11) called attention to the production of collagen as well as mucopolysaccharides by neural tube during the developmentally significant period of neural tube-somite interaction. In this report, we analyze the GAG produced by embryonic neural tube and notochord during the period of somite induction, and the GAG synthesized by the lens at the time it is believed to induce primary corneal differentiation.

MATERIALS AND METHODS

For autoradiography, anterior and posterior trunk segments (8–10 somites each) and whole eyes from 3-day old chick (white leghorn) embryos were incubated for 24 h in H\textsubscript{2}\textsuperscript{35}SO\textsubscript{4} (New England Nuclear, Boston, Mass.), at a concentration of 100 μCi/ml of culture medium (Ham's F-12 medium supplemented with 10% fetal calf serum, Grand Island Biological Co., Grand Island, N. Y. 0.25% whole embryo extract and antibiotics as previously described, 8, 9). The incubation in isotope was followed by a 4-h chase in cold medium. Tissues were fixed in 0.45 μm pores), four per Falcon dish (12-day old), five per dish (5-day old) and eight per dish (3-day old). Tissues were grown for 24 h in the medium described above to which was added 10 μCi/ml of H\textsubscript{2}\textsuperscript{35}SO\textsubscript{4} (carrier free) or 5 μCi/ml of D-[6-\textsuperscript{3}H]glucosamine (7.3 μCi/mM). The medium and tissue were collected separately and treated with 500 μg/ml of pronase (Calbiochem, San Diego, Calif.) in 0.2 M Tris-HCl buffer (pH 8) overnight at 55°C. Samples were then dialyzed overnight against running tap water, heat for 5 min at 100°C, and divided into equal aliquots which were treated at 37°C for 3 h with (1) 100 μg testicular hyaluronidase (Worthington Biochemical Corp., Freehold, N.J., type HSE), or (2) 100 μg streptococcal hyaluronidase (Lederle Laboratory, Pearl River, N. Y.), or (3) 50 μg of leech hyaluronidase (a gift of Dr. Andrê Balazs), or (4) enzyme buffer alone (0.1 M Na\textsubscript{2}PO\textsubscript{4}, pH 6.5). Carrier GAG was added to the samples as previously described (14), and they were precipitated with 1% cetylpyridinium chloride (CPC). The redissolved samples (2 ml methanol each) were counted in 10 ml each of cocktail T (14). For determination of heparan sulfate, pronase-treated, dialyzed samples were degraded with nitrous acid and undegraded polysaccharides precipitated with CPC for counting as previously described (14).

RESULTS

 Autoradiographic and Histochemical Studies

 Autoradiographs of intact tissues exposed to \textsuperscript{35}SO\textsubscript{4} for 20 h, followed by 4-h chase, revealed radioactivity (sulfated GAG) over the cells and in the extracellular compartment between neural tube-notochord and somites, on the one hand, and lens and corneal epithelium, on the other. More radioactivity is associated with ventral than dorsal neural tube (15) and, in the trunk, the greatest concentration of silver grains is located around the notochord (Fig. 1). The notochord is an epithelium whose central cavity is detectable with the electron microscope (16). Radioactivity is associated with the basal surfaces (basement membranes) of both the neuroepithelium and notochord (arrows, Fig. 1) as well as with the surrounding extracellular matrix. Presumably, it is the GAG in the extracellular matrix surrounding the neural tube and
notochord that the somite cells (s, Fig. 1) contact during the period of cartilage induction. The somite cells also take up the sulfate label and synthesize GAG (17).

Autoradiographs of the developing eye at 3 days reveal sulfate incorporation by the lens, the optic cup, and the corneal epithelium (Fig. 3). The extracellular label accumulates between these epithelial tissues, especially in the area of the lens capsule, which is the basement membrane of the lens (lc, Fig. 3). Subsequently, the corneal stroma (cs, inset, Fig. 3) is laid down by the epithelium onto the anterior surface of the lens. The corneal stroma is rich in sulfated GAG (14) and is labeled by a 20-h pulse of H₂¹⁵SO₄ (inset, Fig. 3). That the label in the lens capsule is due to acid mucopolysaccharide is suggested by alcian blue staining of older lens capsules (Fig. 2).

To localize acid mucopolysaccharide in younger tissues (3-day old lens, notochord, neural tube),
ruthenium red was employed as a stain because it is readily visible in the electron microscope (13), and electron microscopy is necessary to resolve the fine connective tissue of the 3-day old chick embryo. The lens capsule at 3 days is only slightly thicker (lower right, Fig. 4) than the basement membrane (basal lamina) of the corneal epithelium (upper half of Fig. 4). In most anterior regions, the lens capsule at this stage (Fig. 5) is no thicker than an ordinary basal lamina (12). Ruthenium red is deposited in the basal lamina in the form of two layers of granules ~600 Å apart (open arrows, Figs. 4, 5). Analysing tangential sections (such as that shown at tan, Fig. 4), Trelstad et al. concluded that the distance between individual stain deposits within each layer is the same (550–600 Å) as the distance separating the two layers of stain deposits in the lamina. In addition to these two layers within the basal lamina, dense round deposits of ruthenium red occur along the thin connective tissue fibrils (18) that are forming at this time in the corneal stroma (short, solid arrows, Fig. 4). The basal plasmalemma is stained in a plaquelike fashion (curved arrows, Fig. 4). Higher magnification suggests that the intermittent densities in the plasmalemma visible in low-magnification micrographs are due to deposits of mucopolysaccharide along the outer surface of the plasmalemma (curved arrows, Fig. 5). The basal plasmalemma between these intermittent deposits does not seem to be preserved by ruthenium red-osmium tetroxide fixation (x, Fig. 5). Interestingly, the intermittent, stained membrane plaques also occur along lateral (intercellular) boundaries (y, Fig. 5), presumably due to ruthenium red staining of the polysaccharide component of the lateral cell surface coat (19). This stained component (curved arrows, Fig. 6) is not removed by testicular hyaluronidase, but the small round extracellular staining deposits are digested by this enzyme. The deposits of ruthenium red that can be identified in the basal lamina of the notochord (Fig. 7) and neural tube (Fig. 8) are similar to those in lens capsule (Fig. 5), and here again dense plaques of stain are associated with the plasmalemma.

**Biochemical Identification of GAG**

After labeling tissues with $^{35}$SO$_4$$_2$, one can determine the relative percentage of sulfated GAG which is chondroitin 4- and 6-sulfate by digestion with testicular hyaluronidase, because these are the principal sulfated GAG susceptible to this enzyme (4). The relative amount of testicular hyaluronidase-resistant sulfated GAG which is heparin and/or heparan sulfate can be determined by nitrous acid degradation. When residue resistant to both testicular hyaluronidase and nitrous acid is present, it is assumed to be keratan sulfate and dermatan sulfate (20, pp. 102-103). In this report, testicular hyaluronidase-sensitive sulfated GAG will be referred to as chondroitin sulfate and nitrous acid-sensitive materials will be collectively called heparan sulfate. Since $[^{3}H]$glucosamine will be incorporated into nonsulfated as well as sulfated GAG, after labeling with this isotope the relative

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**Figure 4** Electron micrograph of a region between the corneal epithelium (upper) and lens epithelium (lower) from a 3-day old chick eye fixed in ruthenium red-OsO$_4$. Small round stain-GAG precipitates occur in the basal lamina (small open arrows) and larger precipitates on connective tissue filaments and fibrils (small solid arrows). At the * a filament connects to a GAG precipitate in the lamina. Curved arrows call attention to intermittent staining of the plasma-membrane. The lens basal lamina is cut tangentially at tan. × 66,000.

**Figure 5** Higher magnification micrograph of the lens basal lamina in another region of the preparation illustrated in Fig. 4. Open arrows point to ruthenium red localized on the inner and outer surfaces of the lamina and curved arrows indicate stained plaques of basal plasmalemma. Where stain-GAG precipitate is not present, the plasmalemma is not well preserved (x). The stained plaques occur on the lateral (y) as well as the basal plasmalemma. × 100,000.

**Figure 6** Electron micrograph of lens capsule from a 3-day old chick eye incubated in testicular hyaluronidase before processing with ruthenium red in the same experiment illustrated in Figs. 4 and 5. Only the stain associated with the plasmalemma persists (curved arrows). × 100,000.
percentages of nonsulfated and sulfated GAG can be ascertained by employing two additional enzymes: streptococcal hyaluronidase, which is specific for nonsulfated GAG (hyaluronate and chondroitin), and leech hyaluronidase, which is specific for hyaluronate.

Notochord and neural tube from 3-day old chick embryos were labeled with $^{35}$SO$_4$ for 24 h in order to examine the relative amounts of sulfated GAG being synthesized. Table I indicates that all of the new sulfated GAG accumulated by notochord or neural tube can be accounted for as chondroitin sulfate and heparan sulfate. Explanted notochord produces more chondroitin sulfate than heparan sulfate, and the relative percentage of sulfated GAG found in the medium is similar to that associated with the tissue. For neural tube, however, more heparan sulfate is associated with the tissue than chondroitin sulfate. The relative amount of sulfated GAG found in the medium with cultured neural tube is similar to that of notochord.

In order to determine the relative amount of nonsulfated GAG synthesized by neural tube and notochord, cultures were labeled for 24 h with $[^3H]$glucosamine. Table II indicates that the neural tube produces hyaluronic acid since $[^3H]$glucosamine-labeled material sensitive to both streptococcal and leech hyaluronidase is present. The notochord synthesizes little, if any, nonsulfated GAG. Because the amount of streptococcal hyaluronidase-sensitive material (hyaluronate and chondroitin) is nearly equal to the amount of leech

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**FIGURE 7** Electron micrograph of the basal surface of 3-day old notochord in a trunk segment fixed in ruthenium red-Os$_4$. Open arrows point to stain-GAG precipitates in the basal lamina and curved arrows, to precipitates in the plasmalemma. A particularly large GAG precipitate is associated with the plasmalemma at x, × 100,000.

**FIGURE 8** Electron micrograph of the basal surface of 3-day old neural tube in the same trunk segment illustrated in Fig. 7. Ruthenium red deposits are again seen on the inner and outer surfaces of the basal lamina (open arrows) and on connective tissue filaments (solid arrow). At the curved arrow, a stained plaque on the plasmalemma is well shown. The mitochondrion shown here contains a characteristic dense granule (g) similar to that seen in controls. × 100,000.
### TABLE I

**Identification of Sulfated Glycosaminoglycans Synthesized by Notochord, Neural Tube, and Lens***

<table>
<thead>
<tr>
<th>Tissue explanted</th>
<th>Sample source</th>
<th>CPC precipitable</th>
<th>Testicular hyaluronidase sensitive</th>
<th>Nitrous acid sensitive</th>
</tr>
</thead>
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<tr>
<td>Notochord Medium</td>
<td>697</td>
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<td>36.5 ± 2.4</td>
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<td>Cells</td>
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<td>Lens 12 day Medium</td>
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<td>80.4 ± 2.4</td>
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* Cultures were labeled for 24 h in 10 μCi/ml of $^{35}$SO$_4$. CPC precipitable counts are given for one experiment typical of six, while the values for enzyme- and nitrous acid-sensitive material are expressed as the mean ± the standard deviation about the mean for all six experiments.

### TABLE II

**Identification of Nonsulfated and Sulfated Glycosaminoglycans Synthesized by Notochord, Neural Tube, and Lens***

<table>
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<tr>
<th>Tissue explanted</th>
<th>Sample source</th>
<th>CPC precipitable</th>
<th>Testicular hyaluronidase sensitive</th>
<th>Streptococcal hyaluronidase sensitive</th>
<th>Leech hyaluronidase sensitive</th>
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<td>Notochord Medium</td>
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<td>65.3 ± 2.4</td>
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<td>Cells</td>
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<td>Neural tube Medium</td>
<td>825</td>
<td>76.9 ± 2.2</td>
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<td>Cells</td>
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<td>17.2 ± 1.0</td>
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<td>Lens 3 day Medium</td>
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<td>Cells</td>
<td>824</td>
<td>25.8 ± 1.8</td>
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<td>625</td>
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<td>0.0 ± 2.2</td>
<td>1.0 ± 2.0</td>
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<tr>
<td>Lens 12 day Medium</td>
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<tr>
<td>Cells</td>
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<td>20.8 ± 2.2</td>
<td>1.2 ± 2.0</td>
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* Cultures were labeled for 24 h in 5 μCi/ml of $[^{3}H]$glucosamine. CPC precipitable counts are given for one experiment typical of six. Enzyme-sensitive counts are expressed as the mean ± the standard deviation about the mean for all six experiments.
Identification of Nonsulfated and Sulfated Glycosaminoglycans Synthesized by Notochord and Neural Tube Cultured on Lens Capsule or Millipore Filter

<table>
<thead>
<tr>
<th>Tissue Exploited</th>
<th>Substratum</th>
<th>Sample Source</th>
<th>CPC Precipitable</th>
<th>Testicular Hyaluronidase Sensitive</th>
<th>Leech Hyaluronidase Sensitive</th>
<th>Nitrous Acid Sensitive</th>
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<tbody>
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<td>Notochord</td>
<td>Lens Capsule</td>
<td>Medium</td>
<td>147</td>
<td>59.1 ± 3.4</td>
<td>1.0 ± 2.6</td>
<td>40.8 ± 2.8</td>
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<td>Cells</td>
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<td>629</td>
<td>58.4 ± 2.8</td>
<td>1.6 ± 2.2</td>
<td>41.8 ± 2.0</td>
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<td>Millipore</td>
<td>Medium</td>
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<td>254</td>
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<td>1.8 ± 2.2</td>
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<td>Cells</td>
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<td>505</td>
<td>61.2 ± 2.0</td>
<td>2.4 ± 2.0</td>
<td>39.4 ± 2.6</td>
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<tr>
<td>Neural tube</td>
<td>Lens Capsule</td>
<td>Medium</td>
<td>156</td>
<td>67.8 ± 2.4</td>
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<td>47.6 ± 2.2</td>
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<td>48.5 ± 2.4</td>
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<td>Millipore</td>
<td>Medium</td>
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<td>500</td>
<td>40.0 ± 2.2</td>
<td>15.0 ± 3.2</td>
<td>58.7 ± 3.4</td>
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<td>Cells</td>
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<td>2,247</td>
<td>41.5 ± 1.8</td>
<td>7.8 ± 1.5</td>
<td>58.4 ± 2.0</td>
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* Cultures were labeled for 24 h in 7.5 μCi/ml of [3H]glucosamine. CPC precipitable counts are given for one experiment typical of three. Enzyme- and nitrous acid-sensitive values are expressed as the mean ± the standard deviation about the mean for all three experiments.

hyaluronidase-sensitive material, little, if any, nonsulfated chondroitin is produced by neural tube cultures. Interestingly, notochord and neural tube grown on Millipore filter synthesize the same GAG types as when they are grown on lens capsule substratum (Table III). However, neural tube produces twice as much GAG when Millipore filter is the substratum as when lens capsule is the substratum (Table III).

The GAG product of the 12-day old lens is 20% chondroitin sulfate and 80% heparan sulfate (Tables I and II). Less than 10% of the total new GAG accumulated in a 24-h period in vitro is lost to the medium. The 3- and 5-day old lenses seem to make slightly more chondroitin sulfate and to lose somewhat more of the total newly accumulated GAG to the medium. As in other short term studies of GAG production (4, 18, 19) it is assumed that the labeled product which accumulates in the tissue and medium is representative of the total GAG synthesized in this period. If part of the new product is degraded in this period, it is likely to be small (7, 9).

DISCUSSION

We show here that isolated neural tube, notochord, and lens produce the same types of GAG as do the tissues (precartilage somites, corneal epithelium) on which they exert an inductive (10, 17) effect in vivo. The discussion considers the distribution and possible functions of these epithelial GAG during morphogenesis.

Ultrastructural localization of GAG in basement membranes by ruthenium red staining was reported by Bernfield et al. (2), and it is also apparent by autoradiography that the general region of the basement membrane of salivary epithelium (2), neural tube, and notochord (15) contains GAG, if only in transit. In the case of the lens, it can be said that the GAG in the basement membrane is likely to be a structural component, since 80-90% of the newly synthesized GAG remains associated with the tissue in the cultures described here. The lens capsule is also an excellent basement membrane for localization of GAG by staining because of its thickness. We show here that the embryonic chick lens capsule stains with alcian blue and ruthenium red, and Gifford (21) has shown that adult human lens capsule stains with colloidal iron for acid mucopolysaccharide. Other adult basement membranes may also contain acid mucopolysaccharide, but in amounts too small to be detected by light microscopy.²

of label in the adjacent extracellular matrix, as well as in the area of the basement membrane. The remarkable regularity of the ruthenium red-stained mucopolysaccharide loci on either side of the lamina strongly supports the idea (2) that GAG is a true structural component of the basal lamina of these tissues, even though some of the mucopolysaccharide visualized in the basal lamina of the neural tube, notochord, and corneal epithelium is probably in transit since it appears subsequently in the adjacent matrix. Interestingly, the pattern of staining of acid mucopolysaccharide along forming connective tissue fibrils in the extracellular matrix is not as regular as it is along mature collagen fibrils (footnote 1; references 18, 22).

The punctate mucopolysaccharide loci on either side of the basal lamina and at intervals along connective tissue fibrils are undoubtedly composed of chondroitin sulfate. We show here that they are digested by testicular hyaluronidase which removes only chondroitin sulfate, chondroitin, and hyaluronate; neither notochord nor lens capsule produces chondroitin or hyaluronate. Moreover, Trelstad et al. have shown that similar particles in the basal lamina of corneal epithelium are susceptible to chondroitinase ABC. Corneal epithelium (14), notochord, neural tube, and lens produce heparan sulfate as well as chondroitin sulfate, but heparan sulfate is not digested by testicular hyaluronidase. It could be that heparan sulfate is the mucopolysaccharide component of the hyaluronidase-resistant membrane plaques which stain with ruthenium red (23).

It is interesting to note that neural tube, but not notochord, makes a small amount of hyaluronate because 65% of the GAG produced by whole 5-day axial segments is hyaluronate (4), some of which therefore must be contributed by other trunk tissues. In the case of the chick cornea, the outer epithelium synthesizes chondroitin sulfate and heparan sulfate, but hyaluronate is produced by the endothelium which migrates into place under the primary corneal stroma between 4 and 5 days of development (references 13, footnote 1). Young heart, composed of myocardium and endothelium, also produces hyaluronate as well as chondroitin sulfate (24) as does embryonic cartilage, which lacks endothelium (25).

The evidence at hand, then, indicates that different embryonic tissues make different relative amounts of sulfated GAG and hyaluronate during embryogenesis. It is tempting to think that these differences have morphogenetic significance. Both sulfated GAG and collagen have been shown to have a stimulatory effect on cell differentiation which mimics the inductive effect of one embryonic tissue on another in vitro (see Introduction), and both collagen and sulfated GAG synthesis increase markedly at gastrulation when the first tissue interactions begin (26, 27). If we define embryonic induction as a developmentally significant interaction between tissues of dissimilar origin (28, 29), rather than as a process which changes the state of “determination” of cells (30), it may be possible to explain tissue interaction in the early embryo in part by differences in amounts and distribution of extracellular matrix (ECM) produced by epithelial inductors. The ECM hypothesis predicts that GAG and collagen interact with the cell surface (2, 5–9, 16, 28, 29) to promote or inhibit particular metabolic pathways which particular cells at particular times are programmed to pursue; it does not explain how these programs came into existence. Chondroitin sulfate and chondromucoprotein, for example, stimulate corneal epithelium, somites, or chondrocytes to produce more of the same GAG they had already begun to make (6–8). That some specificity may be involved in the interaction of ECM with differentiating cells is suggested by the fact that collagen stimulates both collagen and GAG synthesis by corneal epithelium, whereas chondroitin and heparan sulfates stimulate only GAG synthesis (8, 9); hyaluronate, on the other hand, has either no effect or an inhibitory effect on GAG synthesis by corneal epithelial cells (8) and chondrocytes (6) in the concentrations that have been tested. It should be of considerable interest in the future to examine further the relation of reacting tissues, such as somite and corneal epithelium, to the extracellular matrix produced by the embryonic inductors discussed here.

SUMMARY
In this study, we analyze the GAG produced by embryonic neural tube and notochord during the period of somite induction, and the GAG synthesized by the lens at the time it is believed to induce primary corneal differentiation. Autoradiographs suggest that much of the sulfated GAG produced by the neural tube and notochord is deposited in the extracellular compartment surrounding the epithelia, but that the lens accumulates sulfated GAG primarily in its basement membrane (lens
capsule). Alcian blue and ruthenium red stains show that the basement membranes of lens, neural tube, and notochord contain acid mucopolysaccharide. Enzymatic characterization of sulfated GAG produced by 3-day old chick notochord and neural tube and 3–12-day old lens indicates it to be exclusively chondroitin sulfate and heparan sulfate. Neural tube, but not lens or notochord, produces hyaluronate in addition. The results are discussed in terms of the distribution and possible functions of epithelial GAG during early embryogenesis.

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