The role of extracellular matrix in the formation of the sclerotome

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SUMMARY

The development of the sclerotome is considered as a model for the formation of mesenchyme from an epithelium. In early epithelial somites, transmission and scanning electron microscopy indicate considerable ultrastructural similarity between the future sclerotome and dermamyotomal regions. Subsequently, these two regions diverge in their development. In the forming dermamyotome, junctional complexes become more extensive and the cells become elongated, closely applied to each other, and have angular surface contours. In the forming sclerotome, there is an early reduction in apical junctions. The cells elongate, keeping their original polarity, and acquire numerous filopodia which contain punctate junctions at sites of cell-to-cell contact. Associated with cellular extension is an expansion of the intercellular spaces which do not contain any ultrastructurally recognizable material.

Evidence for a role of hyaluronic acid in the expansion of the intercellular spaces is presented. As identified by the susceptibility of cetylpyridinium chloride precipitates to Streptomyces hyaluronidase and chromatographic separation of chondroitinase ABC digestion products, as much as 64–68% of the [³H]glucosamine-labeled glycosaminoglycans synthesized by explanted somites is hyaluronic acid. In addition, hyaluronidase-sensitive label is localized in the intercellular spaces of the sclerotome, as demonstrated by autoradiography. When Streptomyces hyaluronidase is injected in ovo into living embryos, the sclerotomal mesenchyme differentiates morphologically, but intercellular spaces are drastically reduced. It is hypothesized that the sclerotomal cells produce a hyaluronate-enriched extracellular matrix which is inflated by hydration to mediate the expansion of the sclerotomal mass towards the notochord.

INTRODUCTION

One activity that occurs during numerous morphogenetic processes in embryonic development is the interconversion of epithelia and mesenchyme (Hay, 1968). For instance, during chick embryo gastrulation epithelial cells leave the epiblast at the primitive streak and migrate from the midline as primary mesenchyme (Solursh & Revel, 1978). Some of these mesenchymal cells reorganize into an epithelial endoblast, the definitive embryonic endodermal germ layer.

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In addition, much of the primary mesenchyme lateral to the axis becomes organized into epithelial structures such as pronephros, lateral plate, and vascular channels (Meier, 1979a). The interconversion of mesenchyme and epithelium occurs repeatedly during the development of the somite, where primary mesenchyme in the paraxial region first organizes into an epithelial ball, only to disperse later into mesenchymal cells once more. The additional advantage of accessibility makes the somite particularly suitable for studies concerning underlying morphogenetic mechanisms associated with these developmental processes.

The first account of the history of the somites of the chick was by Remak (1855) and there have been numerous studies since then (e.g. Williams, 1910). More recently, Trelstad, Hay & Revel (1967) reported that various types of cell contacts (gap junctions, tight junctions and macula adherens dimunata) are present during several stages of somite development. Meier (1979b) demonstrated the early appearance of segmentation of the primary mesenchyme in the paraxial mesoblast and studied its conversion into epithelial somites in posterior regions. Circular domains of paraxial mesenchyme, somitomeres, become polarized and separate from one another as they undergo development in the segmental plate. Lipton & Jacobson (1974) proposed a role of the neural folds in the early condensation of paraxial mesenchyme into epithelial somites.

The later stages of somite morphogenesis, where dermatome and sclerotome formation occur, are especially appropriate for studying the conversion of epithelia into mesenchyme. Studies so far have been largely descriptive. Mestres & Hinrichsen (1976) and Christ, Jacob & Jacob (1978) have described somite ultrastructure, emphasizing in particular the formation and origin of cells of the myotome. For sclerotomal development, changes in cell junctions (Trelstad et al. 1967) and the occurrence of filopodia have been described (Hay, 1968; Flint & Ede, 1978). In addition, lamellipodia have been reported in the sclerotome region that is adjacent to the perinotochordal extracellular matrix (Eberdal, 1977). The later stages of sclerotome development into cartilage and vertebral bodies have been studied in greater detail (Olson & Low, 1971; Minor, 1973).

The mechanisms leading to the conversion of an epithelium into mesenchyme are largely unknown. However, one mechanism that has been suggested for sclerotome formation involves the few cells that remain mesenchymal in the myocoele. Whether trapped there (Trelstad et al. 1967), or added by mitoses of cells in the adjacent epithelial wall (Langman & Nelson, 1968), it is hypothesized that the continued proliferation of cells in the myocoele creates forces which cause part of the somitic epithelium to disperse into a migratory mesenchyme. In the present study, the formation of the sclerotome is examined by transmission and scanning electron microscopy in order to characterize the early steps in the conversion of somitic epithelium into sclerotomal mesenchyme. The appearance of extensive extracellular spaces led us to identify one prevalent
Sclerotome formation component of the extracellular matrix (hyaluronic acid). As a third part of this study, the effects of enzymic removal of a portion of the extracellular matrix on specific steps in sclerotome formation are examined. A new model for mesenchyme formation is proposed here which involves (1) the early loss of some cell junctions and the formation of filopodia; (2) the secretion of an inflatable extracellular matrix, containing hyaluronic acid, that leads to cell elongation along the original apical-basal cell axis and expansion of the sclerotomal mass, in directions determined by cell contacts at the leading edge of the sclerotome and by compressive forces of adjacent structures.

MATERIALS AND METHODS

Hyaluronidase treatment of embryos

Chick embryos (Hubbard Golden Comet, obtained from Johnson County Feed and Hatchery, Iowa City, IA, or White Leghorn) were incubated at 38 °C until stage 12–13 (Hamburger & Hamilton, 1951). For enzyme treatment, eggs were candled to determine the position of the embryo so that a hole could be cut in the shell directly above. The living embryos received a sub-blastodisc injection of either 20 µl Streptomyces hyaluronidase, 200 TR u./ml (Calbiochem), or 20 µl Howard Ringer, as described previously (Fisher & Solursh, 1977). The eggs were then resealed with Parafilem and returned to the incubator for an additional 1–6 h.

Fixation and microscopy

The embryos were explanted, rinsed in saline at 37 °C and fixed at room temperature. Some embryos were fixed for 1 h in Carnoy's fixative containing 0·5 % cetylpyridinium chloride, dehydrated through an alcohol series, and embedded in paraffin. Sections were cut at 10 µm and stained with Alcian blue (ICN Pharmaceuticals Inc.), pH 2·5 (Humason, 1972) to demonstrate glycosaminoglycan distribution. Other embryos were fixed in half-strength Karnovsky's fixative (Karnovsky, 1965), containing 2 % tannic acid (Fisher) in some cases. The embryos were rinsed well with cacodylate buffer before further processing.

Specimens for scanning electron microscopy were dissected with tungsten needles before being post-fixed at room temperature for 1 h in 1 % OsO₄ in 0·1 M-cacodylate buffer and dehydrated through a graded ethanol series. The embryos were critical-point-dried with liquid CO₂ as the transitional fluid, mounted on aluminum studs, sputter coated with 8–10 nm of gold-palladium alloy and examined in a JEOL JSM-35 scanning electron microscope at 20–25 kV.

For transmission electron microscopy embryos were dehydrated through a graded ethanol series and propylene oxide and embedded in Epon (Luft, 1961). Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM300 electron microscope. Thick Epon sections for light microscopy were stained with methylene blue and Azur II.
Enzyme controls

*Streptomyces* hyaluronidase was chosen for *in ovo* injection because of its reported specificity for hyaluronic acid (HA) (Ohya & Kaneko, 1970). The enzyme was tested for possible protease contamination (Davis & Smith, 1955) and none was found by this method. Low levels of protease were detected when C\(^{14}\)-labeled proteins were used as a substrate and then precipitated with trichloroacetic acid (TCA). However, the injection of small amounts of pronase or trypsin did not cause any of the effects described here for *Streptomyces* hyaluronidase. It is also unlikely that the effects of hyaluronidase treatment reported here are due to toxic effects of the digestion products. Hughes, Freeman & Fadem (1974) studied the teratogenic effects on chick embryos of a number of sugars including mono-, di- and trisaccharides. While all sugars tested were found to be teratogenic, the abnormalities reported did not include those reported here. In the present study some embryos were also injected with 20 \(\mu\)l of a 0·05% solution of hyaluronic acid (Sigma, grade IIP) which had been previously digested with 40 units/ml of testicular hyaluronidase (Worthington, HSEP) at 37 °C for 6 h. The digest was heat inactivated at 80 °C for 5 min before being diluted with Howard Ringer to a concentration of 2 mg/ml. No effects similar to those found after hyaluronidase injection were observed.

Biochemical identification of glycosaminoglycans (GAG) synthesized by isolated somites

Stage-13 White Leghorn chick embryos were explanted and trimmed. The embryos were cut into two pieces by making three cross-sectional slices, the first across the heart region and the second just anterior and third just posterior to the last four pairs of somites. The embryo fragments were incubated in 1 % trypsin in saline G for 45 sec at 37 °C and quickly transferred to 50% horse serum (Grand Island Biological Co.) in saline G. The somites were dissected from anterior and posterior fragments, carefully checked for the absence of any adherent non-somite tissue, and pooled separately. Approximately 20 somites were used for each group. These were transferred to 0·1 ml of F\(_{12}\) stock (Grand Island Biological Co.) containing 25 \(\mu\)g/ml of streptomycin sulfate, 50 units/ml of penicillin, 1·25 \(\mu\)g/ml of fungizone, and 200 \(\mu\)Ci of D-[6-\(^3\)H N]glucosamine hydrochloride (New England Nuclear). The somites were incubated at 37 °C in a humidified atmosphere of 5% CO\(_2\) and 95% air for 3 h and frozen. The combined somites and medium were thawed, sonicated for 15 sec and treated with 1 mg/ml pronase (Calbiochem) for 24 h as described previously (Solursh, 1976). The 5 % TCA supernatant was dialyzed against running tap water and then distilled water.

The labeled GAG were identified by two different methods. First, a modification of the method of Toole & Gross (1971), as described previously (Solursh, 1976) was used. Equal aliquots of labeled sample were incubated for 3 h at
Sclerotome formation

37 °C in 0.5 ml volume containing either (1) buffer only, consisting of 0.15 M-NaCl, 0.02 M-Na acetate at pH 5, (2) buffer containing *Streptomyces* hyaluronidase, or (3) buffer containing testicular hyaluronidase. At the end of the incubation samples were brought to a boil, mixed with carrier hyaluronate and chondroitin sulfate and the cetylpyridinium chloride (CPC) precipitable counts measured.

The second method was that of Saito, Yamagata & Suzuki (1968) as modified by Cohn, Cassiman & Bernfield (1976). Samples were treated with chondroitinase ABC (Miles Laboratories) in buffer (pH 7.4) for 3 h at 37 °C. Fresh enzyme was added after 1.5 h. The samples were chromatographed on Whatman 3MM papers for 19–24 h in 1-butanol-acetic acid-1 N-NH₄OH (2:3:1, by volume) with standards obtained from Miles Laboratories or by chondroitinase treatment of hyaluronic acid (Sigma, grade I). The chromatograms were cut into 1 cm pieces, corresponding to known spots, eluted with 0.5 ml water and counted in Biofluor (New England Nuclear).

[³H]Glucosamine labeling and autoradiography

Chick embryos incubated to stage 13 were removed from the egg and most of the area opaca was trimmed away. The embryos were then placed into 1 ml Ham’s F₁₂ stock (Grand Island Biological Co.) containing antibiotics and 200 μCi [³H]glucosamine (D-[6-³H N] glucosamine hydrochloride) (New England Nuclear). The embryos were incubated at 37 °C for 45–180 min. Some embryos were labeled for 45 min, then rinsed three times with Tyrode’s saline and incubated for an additional 2 h in fresh, unlabeled medium. The embryos were rinsed briefly in saline and then fixed in Carnoy’s containing 0.5% cetylpyridinium chloride for 1 h, dehydrated through alcohol and embedded in paraffin. Adjacent 8 μm sections were mounted on slides and treated for 3 h at 37 °C with either 0.1 M-PO₄ buffer (pH 5.6), 100 TR u./ml *Streptomyces* hyaluronidase or 400 u./mL testicular hyaluronidase (Worthington, HSEP) in buffer. The sections were then stained with Alcian blue at pH 2.5 (Humason, 1972), dehydrated to 100% ETOH, dipped in 1% celloidin in equal parts of ethanol and ether, and dried at room temperature overnight before being coated with Kodak NTB-2 (diluted 1:1 with water). Coated sections were incubated for 3 weeks at 4 °C before being developed.

While glucosamine may be incorporated into many molecules besides HA, it is demonstrated here that about 68% of the glucosamine-derived label in GAG from explanted somites is found in HA. Glucosamine may also become incorporated into other macromolecules including glycoproteins, but this material would not be directly hyaluronidase-sensitive. Effects of testicular hyaluronidase, which degrades chondroitin and chondroitin sulfates as well as HA, are illustrated here because there were no detectable differences between autoradiographs prepared after either testicular hyaluronidase or *Streptomyces* hyaluronidase pretreatments.
RESULTS

Normal development of the somite

By comparing anterior and posterior somites in the same embryo, it is possible to reconstruct some of the major features of somite morphogenesis (Williams, 1910). Of particular interest in the present context are early differences in the morphogenesis of the dorsal and ventral-medial regions of the somite, since differences here might reflect underlying features in the later differentiation of the sclerotome and dermamyotome. Fig. 1 shows a dorsal overview of a string of somites undergoing morphogenesis in the paraxial region. From this perspective, it is obvious that the somites decrease in length (along the anterior-posterior axis) as they expand laterally. If the most recently formed somite (1 in Fig. 1) is compared to the tenth somite, the width increases by a factor of two, while the length decreases by half. These values are representative of measurements made from 15 similarly staged embryos. Fig. 2 shows a similar view of somitic development from the medial side. From this perspective, it can be seen that the somite height increases progressively. There is approximately a 50% increase in height between somites 1 and 10. If one assumes that the somite is approximately rectangular and that shrinkage during critical-point drying is uniform in shape (Packard & Jacobson, 1979), the combined change in dimensions suggests that somite 10 is about 50% larger in volume than the most recently formed somite. From this medial view, effects of the neural tube on somite shape are apparent. There is a depression in the dorsal-medial portion of the somites where they are associated with the neural tube. The increased somite height can be seen to occur in both the dorsal-medial, neural tube-associated region, as well as in the ventral-medial region. The ventral-medial somitic surface which lies adjacent to perinotochordal extracellular matrix is more vertical to the embryonic axis. In addition, the sclerotomal fissure (Fig. 2, small arrow) divides the somites into cranial and caudal halves. This morphological marker reflects the site of resegmentation of the sclerotome into the vertebral bodies at a later stage of development (Fig. 3). However, the fissure can be observed even in the most recently formed somites.

The outer surfaces of the somites are covered by considerable extracellular matrix. Fig. 4 shows the matrix which lies along the dorsal somitic surface. It appears as a fibrillar network associated with granules. The fibrils are often oriented along the anterior-posterior axis coursing in straps. Extra-cellular matrix is observed on all somite surfaces and there are no obvious differences between dorsal and ventral-medial faces. All somitic cell surfaces which are adjacent to matrix are covered with a patchy basal lamina, as reported earlier by Trelstad et al. (1967).

By comparing the internal organization of representative somites with the scanning and transmission electron microscope, it is possible to detect ultrastructural changes in the divergence into sclerotome and dermamyotome.
Fig. 1. A scanning electron micrograph of a dorsal view of the left half of the trunk region of a chick embryo at stage 12 of development (ectoderm removed). The somites can be observed in developmental sequence as they emerge from the segmental plate (1) and progressively undergo morphogenesis (5, 10). From this view, the somite width increase two-fold from somite 1 to somite 10. At the same time their length decreases by 50%. × 115.
Sclerotome formation

Comparisons are made between the most recently formed somite (1 in Fig. 1), the fifth somite and the tenth somite. Somite 1 is illustrated in Figs. 5 and 6 in cross-section. Viewed medially, the most recently formed somite to emerge from the segmental plate is a uniform epithelial ball which encases a central core of mesenchymal cells. Cells of the epithelial wall are rather columnar or bottle-shaped, and closely applied to one another. As is typical of thickened embryonic epithelia, mitoses are observed to occur preferentially along the apical or lumenal border. However, mitotic cells are never seen dislodging and entering the myocoele, as suggested by Langman & Nelson (1968). A patchy basal lamina covers much of the external somitic surface, but frequently cell processes emerge and contact adjacent extracellular matrix fibers. Zonular junctional complexes are found surrounding the apical ends of most epithelial cells bordering the myocoele, including the ventral-medial region, but excluding a small portion of the lateral wall. In the lateral walls the cells are more loosely associated (not shown), reminiscent of the morphology of amphibians and fish somites where the myocoele is continuous with the nephrocoel. In all other regions, the apical surfaces are narrow and tapered, often projecting folds.

Figures 2–6

Fig. 2. A scanning electron micrograph of the somitic region of stage-12 chick embryo from the point of view of the neural tube (removed). The ventralmost portion of each somite is almost vertical to the midline and represents that region which borders the notochord. In the more posterior somites, a vertical cleft is already visible in the ventral-medial portion of the somite (small arrow). The dorsal portion of each somite is concave and reflects the expansion of the neural tube from the midline. Anteriorly, this surface of the somite is covered with neural crest cells (large arrow). The height of the somite increases by nearly 50% when the most recently formed and tenth somite (star) are compared. × 1480.

Fig. 3. A scanning electron micrograph of the ventral-medial region of an anterior somite at stage 12 of development. Viewed medially, the sclerotome is already divided into anterior and posterior halves by the sclerotomal fissure (curved arrows). The large arrow points to an intersomitic cleft. × 470.

Fig. 4. A scanning electron micrograph of the extracellular matrix accumulated between the surface ectoderm (removed) and dermamyotome at stage 12 of development. Many of the fibrillar elements, studded with granules, course along in small bands primarily in an axial direction (arrows). In transmission electron micrographs, faintly periodic fibrils, 10–15 nm in diameter, are seen. Occasionally the basal cell processes of somitic cells appear trapped in matrix. × 1390.

Fig. 5. A scanning electron micrograph of the most recently formed somite, similar to that labeled ‘1’ in Fig. 1, which has been cross-fractured. Viewed medially, individual cells are bottle-shaped, tapered inward toward the somite center. The centralmost cells are less oriented and more rounded. The cells of the surface ectoderm, overlying the somite, border considerable amounts of intercellular space, creating caverns and channels throughout the epithelium. × 615.

Fig. 6. A transmission electron micrograph of a thin section taken through the most recently formed somite. The neural tube (n.t.) is at the upper left. The somitic wall is rather uniform in thickness and typical of pseudostratified embryonic epithelia in that mitoses occur preferentially at the lumenal (myocoele) surface (arrows). × 535.
Fig. 7. A transmission electron micrograph of a thin section taken through the myocele surface of the dermamyotome of the third somite (stage 12). The apical ends of somitic cells show profiles of zonular junctional complexes, and cilia and basal bodies are often present. At this stage, the apical surface still has numerous filopodia. Subsequently, this surface becomes less tortuous and flattens. × 9750.

Fig. 8. A scanning electron micrograph of the cells comprising the wall of the dermamyotome of the tenth somite of a stage-12 embryo. Cells are spiny, elongate and tapered at their ends. × 2745.

Fig. 9. A stereo scanning electron micrograph (tilt angle 12°, mounted for stereo viewer) of a cross-fracture of the fifth somite (see Fig. 1) of a chick embryo at stage 12 of development. Viewed medially, the cells of the ventral (lower) portion of the somite are elongate, flattened and surrounded by considerable extracellular space. Cells of the dorsal (upper) half of the somite are columnar and closely applied to one another. × 555.
with numerous filopodia, causing the myocoele lining to appear ragged. It is from this morphological starting point that the subsequent development of the dorsal and ventral-medial regions diverge.

There are two main changes that occur in the dorsal somitic region or presumptive dermamyotome. As seen in Fig. 7, which illustrates somite 3, filopodia become less numerous and junctional complexes become more extensive along the apical surface. Therefore, lumenal surfaces progressively flatten and smooth out as a distinctive cap is formed. Secondly, the cells of the forming dermamyotome elongate further and acquire a distinctive morphology (Fig. 8). As seen in the tenth somite, they are rather angular in that their surface contours are more planar than curved, often creating membranous ridges and shelves. However, the cells still contact each other by numerous short filopodia, which project across the intercellular space.

In the fifth somite, early steps in the formation of the sclerotomal mesenchyme are becoming apparent (Fig. 9). Viewed in stereo, many cells in the ventral-medial region undergo extensive elongation along their original apical-basal axes. Individual somitic cells first appear bulbous basally because the major portion of the cell volume is situated nearer the basal surface of the somite (Fig. 10). Thin cytoplasmic necks taper toward the lumenal surface. Apical junctional complexes of the forming sclerotome are already becoming reduced even at this early stage, and extensive intercellular spaces appear between the dispersing sclerotome cells. The core cells of the myocoele still appear mesenchymal and intermingle with their ventral-medial neighbors at this time. Occasionally, faintly periodic collagen fibrils of small caliber (10–15 nm diameter) are found in the intercellular spaces of the myocoele (not shown), but no other extracellular matrix material is visualized.

In the tenth somite, sclerotome formation is well advanced and the entire ventral-medial portion of the somite is obviously mesenchymal (Fig. 11 and Fig. 19). As examined with the scanning electron microscope, the sclerotomal mass appears expanded and highly cellular, sitting under a well-developed dermamyotomal cap. Viewed at higher magnification (Fig. 12), individual sclerotomal cells are spiny and have angular contours. Flattened cells contact one another with filopodia and by short expanses of shelf-like processes. However, intercellular space borders most of the cell surfaces. Viewed in stereo from the notochordal point of view (Fig. 13), sclerotomal cells appear extended toward the embryonic axis. However, these extensions are primarily shelf-like and bear short filopodia. Occasionally, lamellipodia can be seen, but they are usually entangled in strands of fibrous extracellular matrix. The overwhelming majority of cells observed here lacked entirely the broad, furled lamellipodia thought to be locomotory appendages. However, many of the cells forming the leading edge adjacent to the embryonic axis contact the perinotochordal extracellular matrix. Here, the cells present broad and flat lamellipodia which become applied to the notochordal sheath (Fig. 16).
Examination of thin sections taken through the sclerotomal mass indicates that some mesenchymal surfaces bear a patch of basal lamina (Fig. 15). It is generally more prominent on cell processes that protrude away from the somite center. As seen in Fig. 14, intercellular spaces are elaborate and tortuous in the sclerotome but no identifiable matrix components are visualized. Apical junctional complexes are no longer found. Instead, the mesenchymal cells contact one another by slender filopodia which are the site of focal cell junctions (inset, Fig. 14). They appear as short expanses of membrane density and are sometimes underlain by microfilamentous material. The unequivocal identification of these junctions awaits freeze-fracture studies.

Glycosaminoglycan synthesis by explanted somites

While intercellular spaces become more extensive during the formation of the sclerotome, little extracellular matrix is found between these cells. Because hyaluronic acid is a common extracellular constituent in early embryos and is not readily preserved by routine preparative procedures for electron microscopy, it is of interest to determine whether the somite may synthesize hyaluronate during the formation of the sclerotome. Somites were carefully isolated and labeled \textit{in vitro} with $[^3H]$glucosamine. The GAGs were isolated and characterized by two different procedures. Based either on the sensitivity to \textit{Streptomyces} hyaluronidase (Table 1) or on the isolation of chondroitinase ABC digestion products (Table 2), about 64–68\% of the labeled GAG is determined to be hyaluronic acid. About 13–20\% of the label is found in chondroitin-4-sulfate, chondroitin-6-sulfate, and chondroitin. Only 12–14\% of the label is resistant to both testicular hyaluronidase and chondroitinase ABC and is likely to be heparin sulfate, as demonstrated recently for 48 h cultures of quail somites (Pintar, 1978). When the posterior-most four pairs of somites are isolated and labeled separately, a similar composition of labeled GAG is synthesized as compared to anterior ones. However, the posterior epithelial somites incor-

\textbf{Figures 10–12
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Fig. 10. A scanning electron micrograph of the sclerotomal cells of the fifth somite at higher magnification. Cells are elongate, bulbous at their basal end, and taper apically. Filopodia are also seen mostly along lateral surfaces. $\times$ 3215.

Fig. 11. A scanning electron micrograph of a cross-fracture through the tenth somite of a chick embryo at stage 12 of development (see Fig. 1). The dorsal half of the somite has remained epithelial, while the entire ventral portion is mesenchymal. $\times$ 500.

Fig. 12. A scanning electron micrograph of the sclerotomal cells of the 10th somite lying adjacent to the endothelium of the dorsal aorta. Somitic cells are distinctly mesenchymal in that their surface contours are planar and meet at angles, and border primarily intercellular space. Filopodia are numerous, contacting fellow mesenchymal cells and ensnaring in the extracellular matrix adjacent to the endothelium of the dorsal aorta. $\times$ 2570.
Fig. 13. A stereo pair scanning electron micrograph (tilt angle 12°, mounted for stereo viewer) of a medial view of the sclerotome facing the notochord (removed) of the tenth somite. The cells are separated by considerable intercellular space and often appear stellate, projecting shelf-like processes which contact each other via small filopodia and occasional lamellipodia. The majority of cells are oriented towards the periphery of the somite. ×1430.

Fig. 14. A transmission electron micrograph of a thin section taken through the sclerotome of the tenth somite. Elongate filopodia make several contacts with other cells and occasionally cell junctions are found here (arrow and inset). ×17000, inset ×43750.
Fig. 15. A transmission electron micrograph of a thin section taken through the basal region of the sclerotome in the tenth somite. Somitic cells are still covered with a patchy basal lamina (arrow). × 27820.

Fig. 16. A scanning electron micrograph of axial sclerotomal cells. Cells present broad lamellipodia (arrows) as they contact the extracellular matrix sheath surrounding the notochord. × 660.

Fig. 17. Autoradiographs of the axial region of a chick embryo labeled for 45 min with [3H]glucosamine and chased for 105 min prior to fixation. Grains are localized in the extracellular spaces surrounding the somite, in basement membranes of neural tube (N) and dorsal ectoderm (E), as well as in the intercellular spaces of the dispersed sclerotomal mass (S). (B) An adjacent section, which was treated with testicular hyaluronidase prior to staining and autoradiographic development, shows fewer grains overall. Grains are considerably reduced in the extracellular regions, particularly in the sclerotomal mass. × 205.
Table 1. Identification of [3H]glucosamine-labeled GAG by CPC precipitation*

<table>
<thead>
<tr>
<th>Treatment of equal aliquots of [3H]GAG</th>
<th>CPC-precipitable CPM</th>
<th>% of sensitive CPM</th>
</tr>
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<tbody>
<tr>
<td>Buffer</td>
<td>791</td>
<td>—</td>
</tr>
<tr>
<td>Testicular hyaluronidase</td>
<td>94</td>
<td>88</td>
</tr>
<tr>
<td>* Streptomyces hyaluronidase</td>
<td>253</td>
<td>68</td>
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</table>

* The results are typical of those obtained in four independent experiments.

Table 2. Identification of [3H]glucosamine-labeled GAG by isolation of chondroitinase ABC digestion products

<table>
<thead>
<tr>
<th>Chondroitinase ABC-resistant counts</th>
<th>CPM</th>
<th>% of counts in GAG*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin-6-SO$_4$</td>
<td>261</td>
<td>14</td>
</tr>
<tr>
<td>Chondroitin-4-SO$_4$</td>
<td>152</td>
<td>8</td>
</tr>
<tr>
<td>Chondroitin</td>
<td>55</td>
<td>3</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>1233</td>
<td>64</td>
</tr>
</tbody>
</table>

* 90% of the recovered counts co-chromatographed with one of the standard disaccharides or remained at the origin. 82% of the recovered counts remained at the origin in samples treated with buffer only.

 Autoradiographic localization of hyaluronate synthesis

Microscopic inspection of autoradiograms of sections from embryos labeled with [3H]glucosamine for 45 min and chased for 105 min revealed that the dispersing sclerotome of somite 10 incorporates label into hyaluronidase-sensitive material (Fig. 17 and 17 B). Epithelial somites appear to produce some hyaluronidase-sensitive material as well (not shown). Similar results are obtained with both testicular and Streptomyces hyaluronidase. Examination of these sections and of sections from embryos which were labeled longer (3 h) showed also that [3H]glucosamine lies in all the same places where Alcian blue-stainable material was previously observed (Fisher & Solursh, 1977). Label accumulates around the somites in cell-free spaces, including the somite basement membrane, and in intercellular spaces between sclerotome cells themselves. It is of interest to note that even with the briefer, 45 min labeling period, enzyme-sensitive grains accumulated in the intercellular spaces of the sclerotome. Based on earlier studies (Kvist & Finnegan, 1970; Toole, 1972; Solursh, 1976; Fisher & Solursh, 1977)
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Fig. 18. Light micrograph of a paraxial sagittal section taken through somites on the anterior end of the embryo (somite 10 is indicated). The sclerotome is dispersed under a dermamyotomal cap. (B) Light micrograph of a similarly sectioned embryo treated in ovo with *Streptomyces* hyaluronidase. Somites are individually condensed and closer together. In addition, the overlying ectoderm and underlying endothelium of the dorsal aorta are closely applied. Sclerotomal cells are no longer dispersed and appear clumped under the dermamyotomal cap. × 305, (B) × 305.

and the present biochemical data, it is likely that much of the hyaluronidase-sensitive labeled material is in fact hyaluronic acid. Unfortunately, it is not possible to make quantitative comparisons of HA synthetic rates without information about pool sizes and degradation rates.

**Effects of in ovo hyaluronidase treatment on sclerotome**

In order to assess the possible morphogenetic function of the accumulation of hyaluronate in the developing somite, *Streptomyces* hyaluronidase was injected under the blastodisc of living embryos that had 16–19 pairs of somites.
During the 6 h incubation after enzyme injection at least an additional six pairs of somites formed. The number of somites in control and enzyme-injected embryos did not differ at the time of fixation, suggesting that the appearance of additional somites from the segmental plate was not altered by enzyme treatment. While the time for enzyme penetration into the embryos is not precisely known, it was observed that material staining with Alcian blue at pH 2.5 was depleted from the vicinity of both epithelial and sclerotomal somites as early as 1½ h after enzyme injection. Since there was little stainable matrix visible at the light microscopic level, it is reasoned that treatment must release both the sulfated and non-sulfated glycosaminoglycans.

The morphological effects of enzyme treatment on somite development were examined at the light and electron microscopic levels. Enzyme treatment reduced some extracellular matrix constituents around the somites but basal lamina and banded collagen appeared unaltered. In addition, cell junctions appeared unaffected. However, the most striking effect of exposure of embryos to hyaluronidase was a general loss of extracellular space relative to the controls (compare Figs. 18 and 18B). The spaces between the ectoderm and somites as well as between the somites themselves are drastically reduced. In Fig. 18 the tenth pair of somites are compared, since sclerotome formation is morphologically pronounced at this level. Sclerotome cells are markedly clumped together in the treated embryos. This clumping is seen even in the more anterior somites, where
Fig. 20. A transmission electron micrograph of a thin section taken through the sclerotome region of somite 10 in a *Streptomyces* hyaluronidase-treated embryo similar to that in Fig. 19B. Filopodial processes and contacts persist, but the amount of intercellular space is greatly reduced. × 25 500.

cell dispersion was already well advanced prior to enzyme injection. In the segmental region of the trunk there is also a dramatic decrease in the cell-free space surrounding the notochord and neural tube (compare Fig. 19 and 19B). As illustrated in Fig. 20, intercellular spaces between sclerotome cells are greatly reduced, but the cells continue to display numerous filopodial processes.

**DISCUSSION**

While the dorsal-ventral polarity of somites is established very early during the formation of the epithelial somite (Jacob, Christ & Jacob, 1974), no obvious morphological differences are apparent between the ventral-medial and dorsal
regions at this time. Individual bottle-shaped cells are radially arranged about
the somite center. They exhibit epithelial polarity in that cells are bound apically
by zonular junctional complexes and their basal ends are coated with patches
of basal lamina. In the dorsal or prospective dermamyotome region, the epithe-
lial nature of the cells becomes reinforced. Junctional complexes are more
extensive as the luminal surface uniformly flattens. Cells are more closely applied
to one another and extracellular matrix fibers as well as basal lamina accumulate
over basal cell surfaces. On the other hand, in the presumptive sclerotome region,
epithelial features are not maintained. Profiles of junctional complexes appear
less frequently as individual cells become separated from one another by an
increased amount of intercellular space. Cell-to-cell contact is accomplished by
a network of filopodia which bridge these spaces. While macular or punctate
junctions are found at filopodial contacts, it is impossible to determine whether
they are newly formed or whether they are remnants of junctions formed in their
previously epithelial condition. The dispersal of the ventral-medial somitic
epithelium results in an expanded mass of cells which are individually mesen-
chymal in morphology.

The developing sclerotome is intimately associated with its extracellular matrix
environment. Fibrillar ECM accumulates over all six sides of the somite, but
is oriented primarily in an axial direction along the dorsal, ventral and medial
faces. The axial orientation of the collagenous fibers accounts for its apparent
paucity in cross-sectional views. In addition, several lines of evidence presented
here suggest that hyaluronic acid is a principal constituent of the sclerotomal
matrix. First, explanted somites synthesize hyaluronic acid, the anterior ones
being more active than the posterior ones. Later stage somites also have been
shown to contain hyaluronate (Kvist & Finnegan, 1970; Toole, 1972). Although
little ultrastructurally identifiable matrix is found within the somite, hyaluronic
acid is not readily preserved with aldehydes. Secondly, autoradiographic evi-
dence utilizing $[^3H]$glucosamine and hyaluronidase sensitivity localizes the
sclerotome as a site of synthesis and accumulation of hyaluronate. Finally,
the dramatic reduction of intercellular spaces between sclerotomal cells exposed
in ovo to Streptomyces hyaluronidase strongly suggests that hyaluronic acid
resides in the intercellular spaces which permeate the developing somitic
mesenchyme. Similar loss of extracellular space was reported earlier for
enzyme-treated primary mesenchyme (Fisher & Solursh, 1977). This report
confirms and extends the report of Pintar (1978), who estimated that 50\%
of the Alcian blue staining in quail somites could be attributed to hyaluronate
accumulation.

It is clear that somites enlarge during their early development. While they
become narrower along the anterior–posterior axis, they increase in width and
height, increasing in volume by approximately 50\%. Since regions of the somites
adjacent to both the neural tube and notochord increase in height, somite
expansion must not be dependent on the neural tube, as suggested by Packard
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& Jacobson (1979). It is more likely that increased volume of the somites results from forces within the somites, rather than from extrinsic influences. No doubt cell division contributes to the differential growth of the somite. In addition, it is likely that a portion of the volume increase is a direct result of the formation of expanded intercellular spaces. The location of hyaluronate in these spaces is consistent with the idea that because of its great capacity for hydration, it can serve as an inflatable matrix which fills the intercellular spaces. Hyaluronate is capable of expanding in volume 10,000-fold upon hydration (Laurent, 1970). The accumulation of hyaluronate in the intercellular spaces may generate forces from within the somite, resulting in the expansion of the sclerotomal mass in three dimensions. The observation that hyaluronate is a poor substrate for mesenchyme cell attachment in vitro (Fisher & Solursh, 1979a) is consistent with a role of hyaluronic acid in separating cells from each other.

The underlying mechanisms that lead to the onset of sclerotome formation are not known. Earlier, Trelstad et al. (1967) suggested that proliferation of cells trapped in the core of the epithelial somites might generate outwardly directed mitotic pressure which aids mesenchyme formation. However, Langman & Nelson (1968) found little DNA synthesis among the core cells and suggested that it was cells of the adjacent somite epithelium that were sloughed into the core. It is now known that the cells of pseudostratified embryonic epithelia normally undergo mitosis preferentially at the apical surface (Sidman & Rakic, 1973; Zwann & Pearce, 1971). After mitosis, the nuclei of recently divided cells return to a basal location as a result of interkinetic nuclear migration. However, even as cells undergo mitosis at the myocoele surface, zonular junctional complexes persist. It is therefore unlikely that cells are added to the core by apical sloughing, since such a phenomenon has not actually been observed in the somite.

The results presented here suggest that once the mesenchymal cells form, they move as an expanding mass towards the neural tube and notochord, but not by individual cell migration as is generally believed (Balinsky, 1970; Langman, 1975; Moore, 1977). Within the sclerotome, one fails to observe cellular appendages that are typical of migrating cells. Newly formed sclerotomal cells have a bulbous basal end and are highly elongated along their previous apical-basal axis. Contacts with adjacent cells are mainly through filopodia. This morphology contrasts with the shape of cells migrating in vivo (Speidel, 1932; Bard & Hay, 1975; Solursh & Revel, 1978; Tosney, 1978). Measurements of the distances between sclerotome and adjacent axial structures also suggest the lack of active somite cell migration (Gasser, 1979). It is only at the leading edge of the sclerotomal mass that possible locomotory appendages, like lamellipodia, are observed. The processes are usually seen in association with the fibrous perinotochordal extracellular matrix (Ebendal, 1977). It is possible that the direction of the expanding sclerotomal mass is determined by compressive forces provided by adjacent somites (Packard & Jacobson, 1979) and by cells at the leading
(notochordal) edge as they become associated with perinotochordal extracellular matrix. Epithelial spreading has been shown to be dependent on contact of the lead edge with extracellular matrix (Dipasquale, 1975). In addition, it is possible that these cells at the leading edge serve to anchor the sclerotomal mass to the notochordal ECM.

It has been shown that the extracellular matrix adjacent to the sclerotome is able to influence the expansion of several mesenchymes in vivo (Fisher & Solursh, 1979b). In vitro, basal laminae, as well as collagen, can promote the spreading of somite and limb mesenchyme (Fisher & Solursh, 1979a). However, it is unlikely that the bifurcation of the sclerotome into cranial and caudal halves is caused by the availability of suitable substrata for spreading. The sclerotomal fissure is already present in very early somites, and, in fact, anterior and posterior segments can even be recognized in presomite masses (somitomere) in the segmental plate (Meier, 1979b). It is likely that the somite cells retain their original orientation through later stages leading to the formation of vertebral bodies (Trelstad, 1977). This view contrasts with that proposed recently by Flint (1977), suggesting that sclerotomal resegmentation is initiated by spinal ganglia formation.

It is apparent that the formation of the sclerotome involves a progression of cellular events. Cells which are initially epithelial undergo a variety of morphological changes in becoming mesenchymal. While these ultrastructural alterations occur during sclerotomal hyaluronate synthesis, accumulation of this extracellular matrix material is not a requirement for the acquisition of mesenchymal morphology. Since sclerotome cells separate from each other and sprout filopodia even in the presence of Streptomyces hyaluronidase, it is likely that the accumulation of extracellular matrix is only one step in the conversion of the somitic epithelium into mesenchyme. The underlying mechanisms of these early changes in mesenchyme formation are still unknown, but occur independently of hyaluronate accumulation.

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