SOMITE CHONDROGENESIS

A Structural Analysis

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ABSTRACT

Light and electron microscopy are used in this study to compare chondrogenesis in cultured somites with vertebral chondrogenesis These studies have also characterized some of the effects of inducer tissues (notochord and spinal cord), and different nutrient media, on chondrogenesis in cultured somites Somites from stage 17 (54–60 h) chick embryos were cultured, with or without inducer tissues, and were fed nutrient medium containing either horse serum (HS) and embryo extract (EE), or fetal calf serum (FCS) and F12X Amino acid analyses were also utilized to determine the collagen content of vertebral body cartilage in which the fibrils are homogeneously thin (ca. 150 Å) and unbanded. These analyses provide strong evidence that the thin unbanded fibrils in embryonic cartilage matrix are collagen. These thin unbanded collagen fibrils, and prominent 200–800 Å protein polysaccharide granules, constitute the structured matrix components of both developing vertebral cartilage and the cartilage formed in cultured somites Similar matrix components accumulate around the inducer tissues notochord and spinal cord. These matrix components are structurally distinct from those in embryonic fibrous tissue The synthesis of matrix by the inducer tissues is associated with the inductive interaction of these tissues with somitic mesenchyme. Due to the deleterious effects of tissue isolation and culture procedures many cells die in somitic mesenchyme during the first 24 h in culture. In spite of this cell death, chondrogenic areas are recognized after 12 h in induced cultures, and through the first 2 days in all cultures there are larger accumulations of structured matrix than are present in equivalently aged somitic mesenchyme in vivo. Surviving chondrogenic areas develop into nodules of hyaline cartilage in all induced cultures, and in most non-induced cultures fed medium containing FCS and F12X There is more cell death, less matrix accumulation, and less cartilage formed in cultures fed medium containing HS and EE. The inducer tissues, as well as nutrient medium containing FCS and F12X, facilitate cell survival, the synthesis and accumulation of cartilage matrix, and the formation of cartilage nodules in cultured somites.

INTRODUCTION

The aggregates of primary mesoderm known as somites undergo chondrogenic, fibrogenic, and myogenic differentiation, and the chondrogenic pathway of somite differentiation is influenced by the inducer tissues notochord and spinal cord (cf. Grobstein and Parker, 1954, Lash et al, 1957) Being easily isolated from other embryonic tissues, somites provide a convenient system for studying the effects of such environmental factors as inducer tissues, culture substrate, or nutrient media on the

chondrogenic response of embryonic mesoderm (cf. Lash, 1967; Ellison et al., 1969, Ellison and Lash, 1971).

Histological and biochemical studies of cultured somites have suggested that chondrogenic differentiation in these cultures is similar to that which occurs during vertebral chondrogenesis (Grobstein and Parker, 1954, Lash et al, 1957, Lash, 1968) The cartilage formed in cultured somites is histologically similar to vertebral cartilage, and in both cartilages the protein-bound polysaccharides are almost exclusively chondroitin-4-sulfate (CSA), and/or chondroitin-6-sulfate (CSC) (Franco-Browder et al, 1963; Marzullo and Lash, 1967, Kvist and Finnegan, 1970, Abbott et al., 1972). In cartilage matrix chains of the chondroitin sulfates are linked to a linear protein backbone (Mathews and Lozaityte, 1958, Hascall and Sajdera, 1969). These molecules form large proteoglycan (chondro-mucoprotein) complexes (Hascall and Sajdera, 1969; Rosenberg et al, 1970), and the synthesis of the chondroitin sulfates is inseparable from the synthesis of the complete protein polysaccharide molecule (de la Haba and Holtzer, 1965, Telser et al., 1965). Since detectable amounts of these chondroitin sulfates are only found in cartilage in 10-day chick embryos (Marzullo and Lash, 1967), biochemical studies of the chondrogenic response of somites have used either labeled sulfate incorporation into matrix proteoglycans, or the synthesis and accumulation of chondroitin sulfates, as criteria of chondrogenic expression in cultured somites (inter alia Lash, 1968, Ellison and Lash, 1971, Gordon, 1971) There was, however, a lack of information about collagen synthesis, and the patterns of organization of matrix components, in these cartilages.

The structural components of cartilage matrix are collagen which is present in the form of fibrils, and the large proteoglycan complexes which are present in fixed cartilage in the form of 200–700 Å protein polysaccharide matrix granules (Matukas et al, 1967; Smith, 1970; Anderson and Sajdera, 1971). This study has therefore utilized electron microscopy to compare the patterns of accumulation of these matrix components in cultured somites with that which occurs during vertebral chondrogenesis (cf. Strudel, 1971; Olson and Low, 1971). In addition, the effects of the inducer tissues (notochord and spinal cord), as well as the effects of different nutrient media, on structural differentiation in cultured somites have been examined. This comparison of in vivo and in vitro chondrogenesis has shown that structural differentiation in cultured somites is similar to vertebral chondrogenesis. The structured matrix components in both cartilages are thin unbanded collagen fibrils, and prominent protein polysaccharide granules These components have a recognizable structure, which is easily distinguished from the structured matrix of fibrous tissues derived from somites.

MATERIALS AND METHODS

Tissue Culture Preparations

All tissues were derived from 54–60 h, stage 17 (Hamburger and Hamilton, 1951), White Leghorn chick embryos. Somites, notochords, and spinal cords between the levels of somites 10 and 30 were cleanly isolated by the methods of Lash (1967). This consists of collecting and staging embryos in sterile Simms balanced salt solution (SBSS) (Simms and Sanders, 1942), dissecting away all extraembryonic membranes, and all tissues cranial to the midcervical level, caudal to the hind limb buds, lateral to the somites, and ventral to the aortas. Special care is taken to cut away all nephrogenic mesoderm The remaining tissues, called "trunks," thus consist of paired somites, aortas, notochord, spinal cord, and overlying ectoderm. These trunk tissues were cleanly separated with a 30–45 s trypsinization (Lash, 1967).

Cultures consisting of 6–10 somites, with or without a piece of spinal cord or notochord (i e, inducer tissues), were grown on a nutrient agar substrate consisting of a 1 8% solution of Bacto-Agar in the nutrient medium that was being tested (Lash, 1967). Kanamycin solution was added to give a final concentration of 0.1% in all nutrient media. The two nutrient media tested were (a) a 2:2:1 ratio of SBSS horse serum (HS) 11 day chick embryo extract (EE), and (b) a 2:2:1 ratio of SBSS· fetal calf serum (FCS) F12X (Ellison and Lash, 1971). 5–15 cultures, consisting of approximately equal numbers of cranial somites (somites 10–20), and caudal somites (somites 20–30), were placed on the nutrient agar in each culture dish, and from 60 to 120 cultures were set up in each experiment. The dishes were placed in a humidified incubator in an atmosphere of 5% CO_2 in air at 37°C. The stereomicroscopic appearance of the cultures was checked frequently, and sufficient medium to prevent spreading without submerging the cultures was maintained. At selected times (i.e., at 6, 12, and 24 h, and daily through 7 days) three cultures were removed from each dish and were transferred directly to buffered glutaraldehyde fixative for preparation for light and electron microscopy.

Embryonic Tissues

To determine the normal patterns of cytodifferentiation and accumulation of extracellular matrix components, segments of 1–10-day White Leghorn chick embryos were prepared for light and electron microscopy. Segments of trunk from the levels of somites 10, 20, and 27 were taken from embryos at each stage (Hamburger and Hamilton, 1951) from stage 10 (33–36 h) through stage 19 (3 days). After 3 days of incubation these segments were collected at 12-h intervals. All segments were transferred to buffered glutaraldehyde fixative, and all tissues ventral to the aortas and lateral to the proximal end of the lateral plate mesoderm were removed. With embryos older than 4 days the segments were halved or quartered, and transferred to individual tubes of buffered glutaraldehyde

Microscope Techniques

Tissues were fixed for 1–4 h in 4 0% glutaraldehyde buffered at pH 7.4 with either 0.1 M phosphate buffer or 0 1 M cacodylate buffer. For postfixation the glutaraldehyde solution was replaced with a 1 0% solution of buffered osmium tetroxide. For tissues fixed in phosphate-buffered glutaraldehyde the osmium was buffered with a standard salts buffer (Pepe, 1967), and for tissues fixed in cacodylate-buffered glutaraldehyde the osmium was buffered with a 0.1 M cacodylate buffer at pH 7 4. All tissues were postfixed for 20–30 min at 4°C in the buffered osmium, dehydrated in graded alcohols, and embedded in Araldite

For light microscopy 0 5–1.0 μm sections were cut with glass knives. These sections were stained with either 1.0% toluidine blue O in 1 0% borax (sodium borate), or with Ito's stain (Ito and Winchester, 1963) consisting of 4 parts of 1.0% toluidine blue O in 1 0% borax and 1 part of 1 0% pyronine Y. Of these stains Ito's stain yielded the best results. For comparison with other histologic studies glutaraldehyde-fixed tissues were also embedded in paraffin, sectioned, stained with Alcian blue, and counterstained with Delafield's hematoxylin (Ellison and Lash, 1971).

For electron microscopy gray to pale-gold sections, cut with Dupont diamond knives on an MT-2 Porter Blum ultramicrotome, were picked up on bare 75 × 300-mesh copper grids. Sections were stained for 20 min with 4.0% uranyl acetate in equal parts of methanol and 70% ethanol, and for 5 min with Reynolds' lead citrate (Pease, 1964). Stained sections were lightly coated with carbon and were examined with a Siemens Elmiskop I electron microscope.

Collagen Analysis[1]

Columns of vertebral bodies between the levels of somites 10 and 27 were dissected from 9-day embryos The blotted wet weight of each column was approximately 7 mg, and from 6 to 72 columns were utilized for each analysis The cartilaginous columns were cleaned of residual muscle tissue with a 1 min digestion in a 2 5% trypsin solution, followed by three rinses in SBSS and three rinses in 0.5 M acetic acid (HAc). The cleaned vertebral bodies were homogenized for 1 min at 4°C in 0 5 M HAc. The collagen was extracted from the whole homogenate in 0 5 M HAc at 4°C for 3 days. The extracted homogenate was centrifuged for 1 h at 15,000 g, and the supernatant was collected for further purification and analysis. The collagen in this supernatant was precipitated with 7 5% NaCl, stirred overnight at 4°C, and collected by centrifugation Amino acid analysis was performed on the whole homogenate, the first acid extract, and the salt precipitate of the first acid extract, using a Jeolco 5AHA automatic amino acid analyzer

RESULTS

Vertebral Chondrogenesis

VERTEBRAL CARTILAGE MATRIX

Through the 10th day of development the structured matrix components of embryonic chick vertebral cartilage are a homogeneous population of randomly distributed thin (100–200 Å) unbanded collagen fibrils and prominent (200–800 Å) protein polysaccharide granules (Figs. 1–2). There are no thicker (>200 Å) fibrils with the periodic staining (banding) of collagen in this cartilage matrix, and the only fibrils thinner than 100 Å are the 40 Å tails of the protein polysaccharide granules. All of the interterritorial matrix of mature (10 day) vertebral cartilage is a meshwork of both of these structured matrix components (Fig 1). In contrast, the lacunae surrounding chondrocytes contain large numbers of protein polysaccharide granules but few collagen fibrils (cf Figs. 1 and 3) At the periphery of the lacunae the number of fibrils increases sharply. This polymerization or aggregation of collagen in the matrix away from the surface of vertebral chondrocytes is similar to the patterns of fibrillogenesis demonstrated by
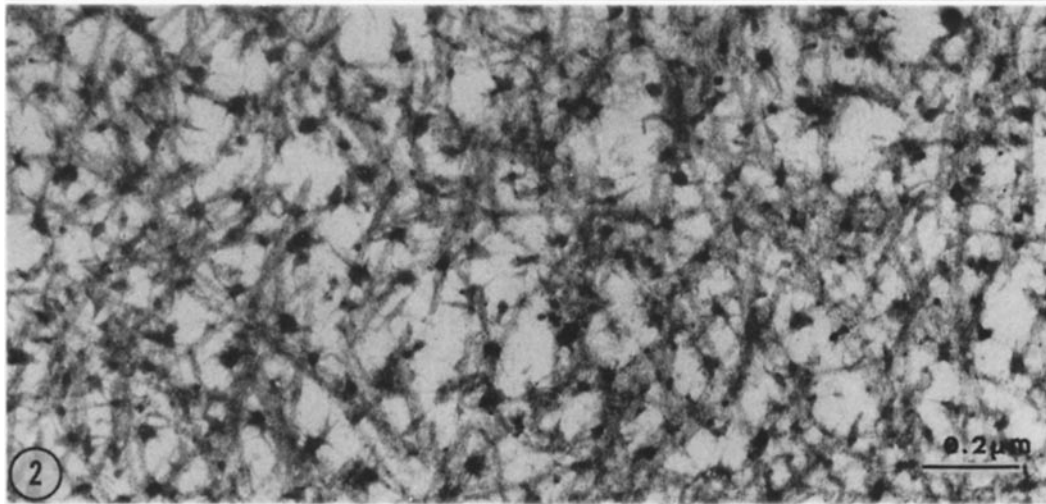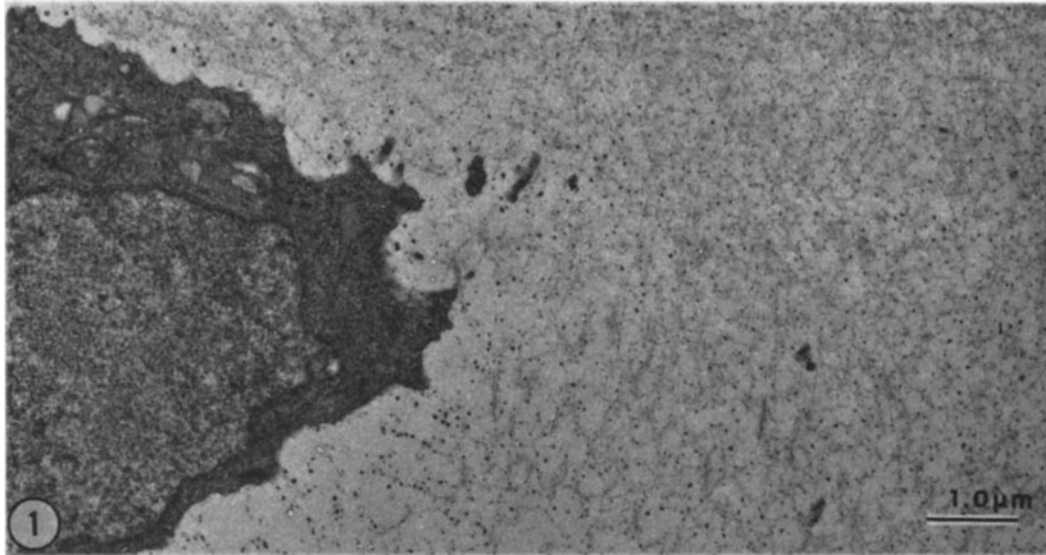

FIGURE 1 Vertebral body cartilage in a 10 day chick embryo. Note that the interterritorial matrix is a meshwork of collagen fibrils and protein polysaccharide granules, whereas the lacuna contains many granules but few fibrils. × 12,000

FIGURE 2 Dense 200+ Å protein polysaccharide granules, and thin (ca. 150 Å) unbanded collagen fibrils constituting the structured cartilage matrix components in a 9 day embryonic chick vertebral body. × 66,000.

Revel and Hay (1963) in regenerating salamander limb cartilage.

The results of the amino acid analysis of the whole homogenate, first acid extract, and salt precipitate of the first acid extract of 9 day vertebral body cartilage are shown in Table I. For comparison the amino acid compositions reported by Trelstad et al (1970) for purified native cartilage collagen, and skin collagen from 2.5–wk lathyritic chickens, are also listed in Table I. A comparison of the analysis of the whole homogenate with the acid-extracted and salt-precipitated collagen shows that collagen constitutes a large proportion of the protein in this vertebral body cartilage.

Protein polysaccharide matrix granules have been shown to be the structural form of fixed
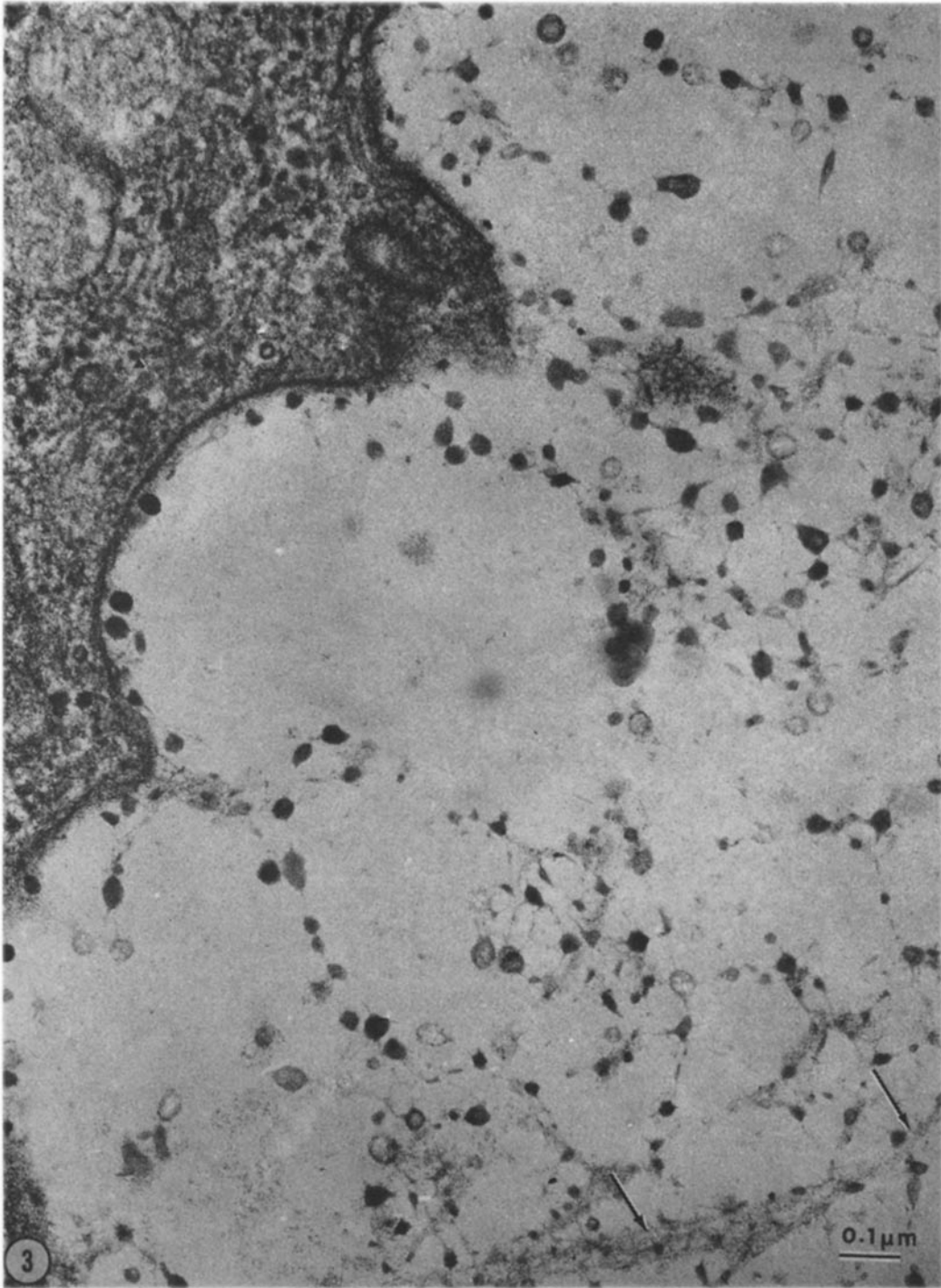
FIGURE 3 The edge of a chondrocyte and the surrounding lacuna in 10 day embryonic vertebral body cartilage. The matrix of the lacuna consists of large numbers of protein polysaccharide granules which are frequently interconnected by filamentous tails. At the periphery of the lacuna the protein polysaccharide granules become associated with thin unbanded collagen fibrils (arrows). × 90,000.

	Vertebral body cartilage, 9 day chick embryo			Trelstad et al, 1970, 2 5 wk lathyritic chicken	
	Whole homo-genate	1st acid extract	Salt ppt , 1st acid extract	Native car-tilage	Skin
Hydroxylysine	16	10	16	22	6 9
Lysine	40	32	29	17	27
Histidine	8.4	11	5 6	3.2	4.2
Arginine	45	43	47	52	51
Hydroxyproline	58	56	78	99	106
Aspartic acid	65	59	57	43	45
Threonine	36	30	32	24	19
Serine	55	52	44	24	29
Glutamic acid	99	110	100	87	73
Proline	87	122	107	117	118
Glycine	246	267	271	331	332
Alanine	89	91	91	102	116
Valine	27	18	21	21	18
Methionine	10	8.8	10	9.8	7.4
Isoleucine	21	16	16	9.1	10
Leucine	56	38	46	27	24
Tyrosine	14	13	8 5	1 5	1 6
Phenylalanine	27	24	19	14	12

Residues per 1000 total residues The amounts of cysteine were not determined.

cartilage proteoglycan complexes (Matukas et al 1967; Smith, 1970, Anderson and Sajdera, 1971). During the initial phase of vertebral chondrogenesis (4th–6th days of incubation) these granules are from 200–400 Å, and they are frequently spindle shaped or stellate. In maturing (7–10 day) vertebral cartilage the diameter of the larger granules increases to approximately 800 Å, and their shape is usually globular with short angular projections at the points of attachment of filamentous tails (cf. Figs. 2–3). The diameter of these tails is approximately 40 A, and they frequently interconnect granules or associate with the surface of collagen fibrils. Within lacunae aggregates of the granules are visible in phase-contrast micrographs, and they show a strong metachromasia when stained with toluidine blue.

TRANSITIONAL AND FIBROUS TISSUE MATRIX

At the periphery of mature vertebral cartilage there is a sharp transition in the structured matrix components. In both the perichondrium and intervertebral joints the numbers and size of the protein polysaccharide granules decrease, and thick (300–500 Å) banded collagen fibrils begin to appear (Figs 4–5). The perichondrium of 10 day embryonic vertebral body cartilage consists of four to six layers of discoidal cells with relatively little extracellular space between them In the intervertebral joints there is more extracellular space between the cells immediately surrounding the notochord (Fig. 4), but the more peripheral cells are closely packed.

The matrix in both the perichondrium and intervertebral joints is a transitional matrix consisting of thin (100–200 Å) unbanded fibrils, thick (300–500 Å) banded fibrils, and 200–400 Å protein polysaccharide granules (Figs. 4–5). Although the thick banded fibrils in the perichordal region of the intervertebral joints were previously interpreted to represent the initial appearance of banded collagen fibrils in vertebral cartilage (Minor, 1970), the fibrils within vertebral cartilage matrix remain thin and unbanded through the 10th day of development

At the periphery of the perichondrium and intervertebral joints the protein polysaccharide granules disappear, and the thick banded fibrils are predominant In contrast to the random orientation of thin unbanded fibrils in cartilage matrix, the thick banded fibrils are usually oriented in loose parallel bundles Furthermore, in both transitional matrix and fibrous tissue matrix the fibrils frequently lie immediately adjacent to cell surfaces This contrasts with the distribution of fibrils away from cell surfaces in cartilage matrix.

CHONDROGENESIS IN EARLY EMBRYOS

In stage 17 (54–60 h) embryos somites are still being formed caudally while cranial somites are reorganizing into a dermatomyotomal plate and a sclerotome (Williams, 1910; Hamilton, 1952). The control studies of somites 10, 20, and 27 showed that at stage 17 somite 10 is completely reorganized, somite 20 is in the process of reorganization, and somite 27 is an intact epithelial ball. In spite of these histoarchitectural differences, the complement of organelles is similar in the mesenchymal cells in cranial and caudal somites. Before the onset of cytodifferentiation of somitic mesoderm the ribosomes are grouped as small polysomes, and there is a paucity of Golgi components and rough endoplasmic reticulum (RER) (see Fig. 6). Large extracellular spaces are present
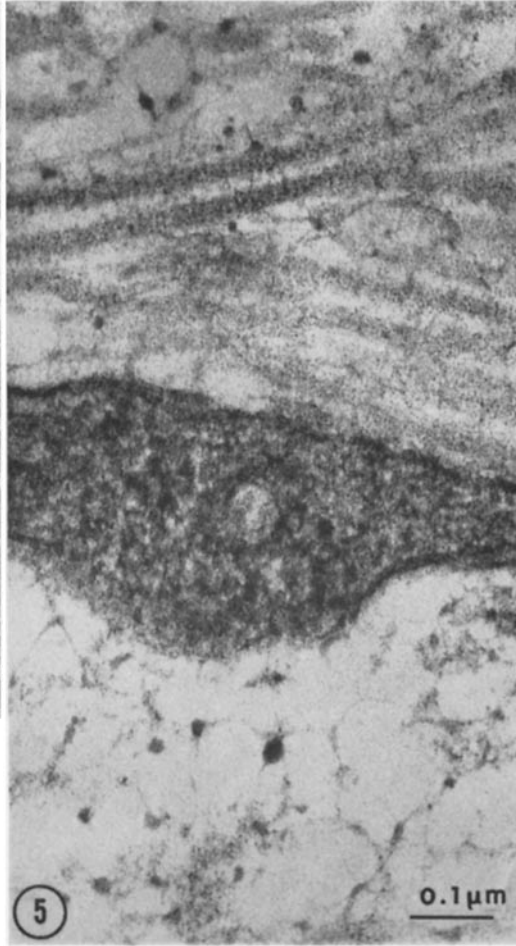
FIGURES 4 and 5 Transitional matrix (TM) in the area of an intervertebral joint surrounding the notochord of a 10 day embryo. Thick banded collagen fibrils, thin unbanded collagen fibrils, and protein polysaccharide granules are all present in this transitional matrix Fig. 4, × 13,500; Fig. 5, × 112,500.

in the sclerotome of reorganizing somites but only a few traces of strucutred matrix are present in these spaces (cf Fig 6, and Trelstad et al, 1967) Neither the complement of organelles nor the small accumulations of structured matrix change significantly until the 4th day of incubation when a gradual increase in structured matrix begins to appear between the sclerotome cells which have surrounded the notochord (cf Fig 7, and Olson and Low, 1971). This increased matrix accumulation in 4-day embryos is the first structural evidence of the onset of vertebral chondrogenesis, and it first appears between those cells which are flattened against the perichordal matrix sheath (see Fig 7)

After the 4th day the accumulation of structured cartilage matrix spreads centrifugally from the perichordal region. Simultaneously, cytodifferentiation is recognized by the appearance of an increase in the quantity of RER and Golgi components in the discoidal chondroblasts surrounding the perichordal sheath At the same time the small polysomes disappear from these cells

During the 6th day large smooth-surfaced Golgi vacuoles containing 100–300 Å dense granules begin to appear in the Golgi region of vertebral chondroblasts These large Golgi vacuoles also develop in chondroblasts in vitro (see Fig 22) At the same time a few dilated cisternae of RER begin to appear in the vertebral chondroblasts

MATRIX ASSOCIATED WITH INDUCER TISSUES IN EARLY EMBRYOS

In stage 17 (54–60 h) embryos major accumulations of structured matrix are only present around the notochord and ventrum of the spinal cord, i e, the inducer tissues (Fig. 8) Before stage 18 (when sclerotome cells begin to migrate around the notochord) this dense accumulation of matrix is

1.0 μm

0.5μm

SC

0.5μm

SC, NC labels
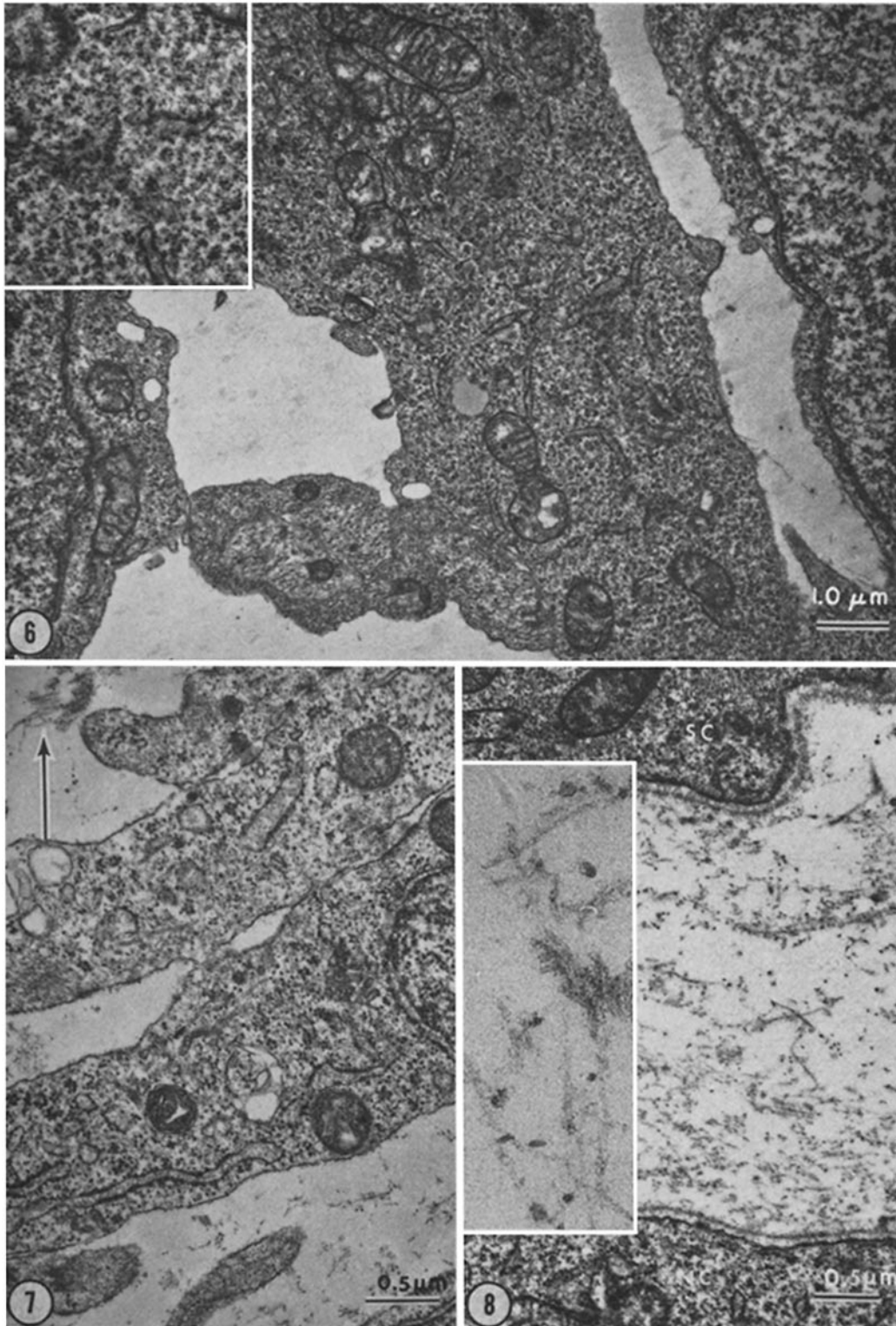
separated from the nearest sclerotome cell by a 4–10 μm extracellular space which contains almost no structured matrix This perichordal matrix begins to accumulate before the somites are formed from paraxial mesoderm in stage 10 (33–36 h) embryos, and it continues to accumulate through the 4th day of development (see Jurand, 1962). Soon after this matrix begins to accumulate, an intact basement membrane develops between the surface of the notochord and the perichordal matrix sheath (cf Fig 8, and Jurand, 1962).

The matrix around the inducer tissues is alcianophilic and stains metachromatically with toluidine blue The structured matrix in this sheath includes two populations of thin unbanded fibrils, as well as many 200–400 Å dense granules These granules are structurally similar to protein polysaccharide granules and the majority of the unbanded fibrils are similar to the unbanded collagen fibrils in developing cartilage matrix (Fig. 8, insert) Recently, Cohen and Hay (1971) showed that the inductively active spinal cord synthesizes collagen, and Frederickson and Low (1971) showed that these 150–200 Å unbanded fibrils are sensitive to collagenase The second population of fibrils in this sheath are thinner (100–120 Å), and have a tubular profile in cross-section. These thinner tubular fibrils tend to accumulate near the surface of the notochord, and Frederickson and Low (1971) showed that they are sensitive to hyaluronidase and amylase, but are insensitive to collagenase.

Even though these large accumulations of structured matrix are present when the inducer tissues are isolated for tissue culture (i e , at stage 17), this matrix and the basement membrane surrounding the inducer tissues are lost during the trypsinization process of tissue isolation for tissue culture The replacement of this matrix in tissue culture is discussed below.

Chondrogenesis in Induced Somite Cultures

The cartilage matrix components which accumulate in cultured somites are thin (150–300 Å) unbanded collagen fibrils, and prominent protein polysaccharide granules (see Figs. 9–10). The average diameter of the fibrils in these cultures is approximately 200 Å, which is 50 Å thicker than those in vertebral cartilage Although these fibrils usually appear in the matrix away from cell surfaces, they frequently lie in loose parallel bundles (cf Figs 9–10), and they sometimes lie immediately adjacent to the surface of chondroblasts There are, however, few of the thicker (300–500 Å) banded fibrils (i.e., fibrous tissue-type fibrils) in these cultures

The protein polysaccharide granules with accumulate during in vitro chondrogenesis are structurally indistinguishable from those in vertebral cartilage (cf. Figs 3 and 9–10). In the matrix synthesized during the first few days in culture these granules are from 200 to 400 Å By the 4th day the largest have increased to approximately 800 Å, and the globular form becomes predominant These granules also have 40 Å tails which frequently interconnect granules or associate with the surface of collagen fibrils (Fig 9) Some of these tails are as long as 2000 Å (Fig 10) In mature cartilage in 7-day cultures aggregates of these granules are visible in phase-contrast micrographs (see Fig 21) These aggregates are strongly alcianophilic and also stain metachromatically with toludine blue.

CULTURE DAYS 1–2, REPLACEMENT OF MATRIX AROUND INDUCER TISSUES

The matrix components and basement membranes surrounding the inducer tissues are rapidly replaced in induced somite cultures. At the end of 6 h in culture only a few thin fibrils and small

FIGURE 6 Sclerotome cells of somite 10 migrating toward the notochord in a stage 17 embryo The ribosomes are grouped as small polysomes (inset, × 25,000), and the complement of rough endoplasmic reticulum is small. Note the paucity of structured matrix in the extracellular spaces × 11,250

FIGURE 7 Chondroblasts adjacent to the notochord sheath (arrow) at the level of somite 10 of a 4 day embryo. Increasing quantities of cartilage matrix are accumulating in the surrounding extracellular spaces. × 22,500.

FIGURE 8 Cartilage-type matrix (inset, × 80,000) between the notochord (NC) and spinal cord (SC) at the level of somite 10 of a stage 17 embryo. Note that a basement membrane surrounds both of these tissues × 20,000.
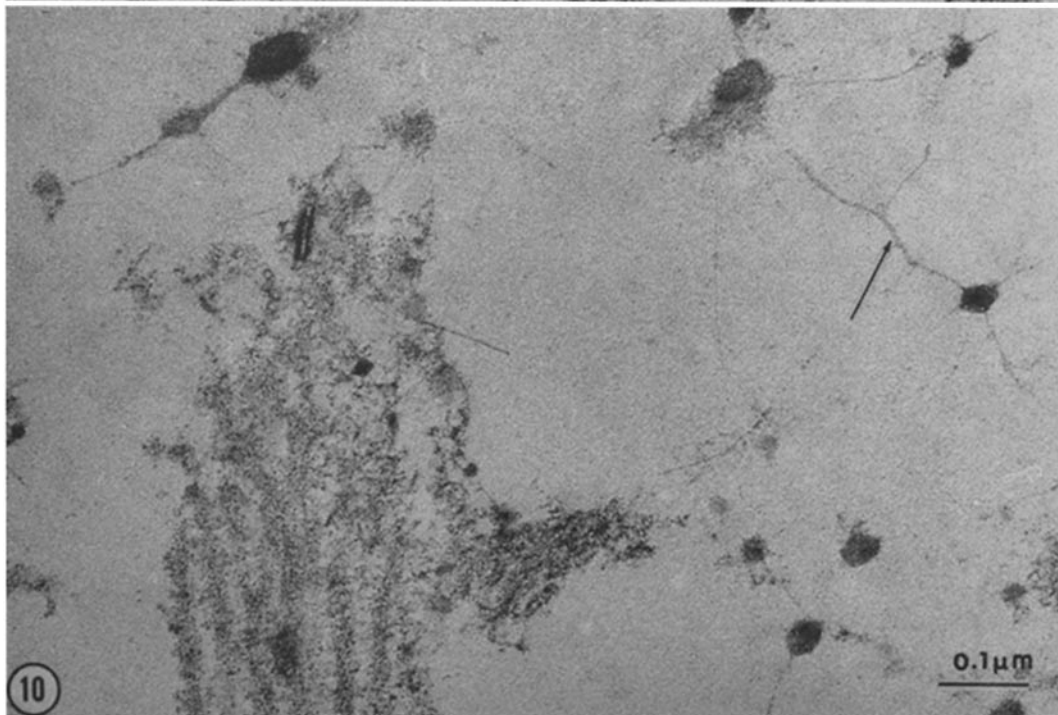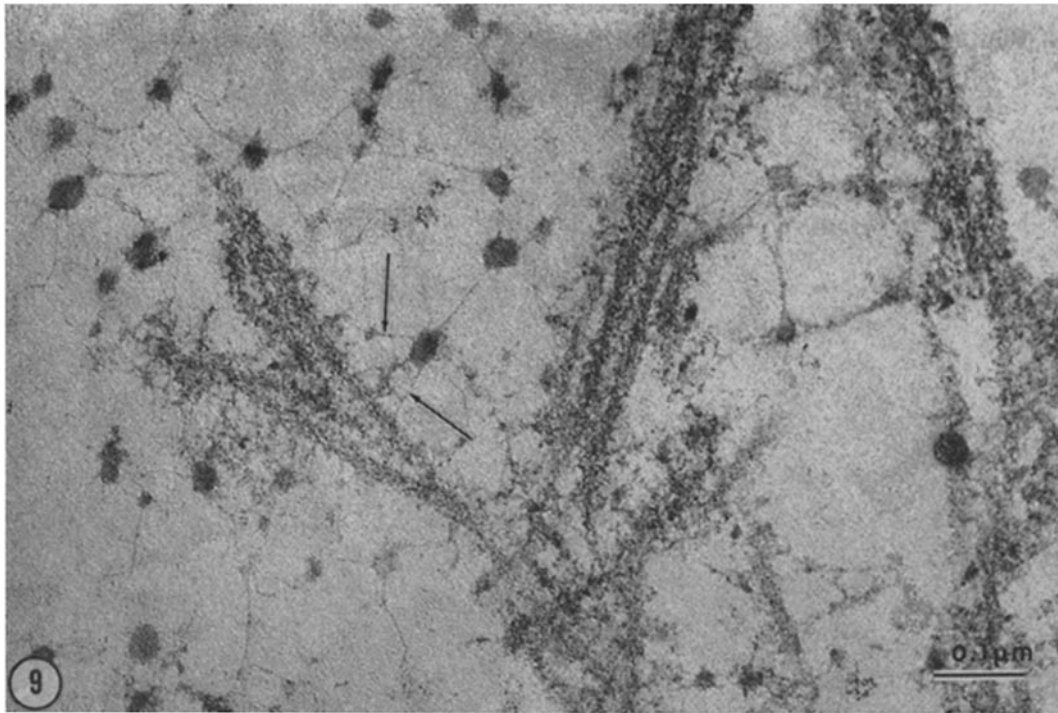
FIGURES 9 and 10 Structured cartilage matrix components in 4-day notochord-somite cultures fed nutrient medium containing FCS and F12X. In both figures prominent 40 Å tails interconnect many of the protein polysaccharide granules. In Fig. 9 some of these tails (arrows, Fig. 9) associate with the surface of collagen fibrils. In Fig. 10 one of these tails (arrow, Fig. 10) is approximately 2000 Å long. × 120,000.

dense granules are present around the inducer tissues (Fig 11) This matrix accumulation is noticeably greater by 12 h (Fig 12), and it continues to increase rapidly through the first 2 days of culture (Fig. 13) This matrix has the same histologic staining characteristics as cartilage matrix, and by 24 h in culture the perichordal matrix in notochord-somite (NS) cultures consists of thin (ca 150 Å) unbanded fibrils and 200–400 Å granules. The density of this perichordal matrix in 2-day cultures is directly comparable to that in 2–4-day embryos, and the density of this matrix
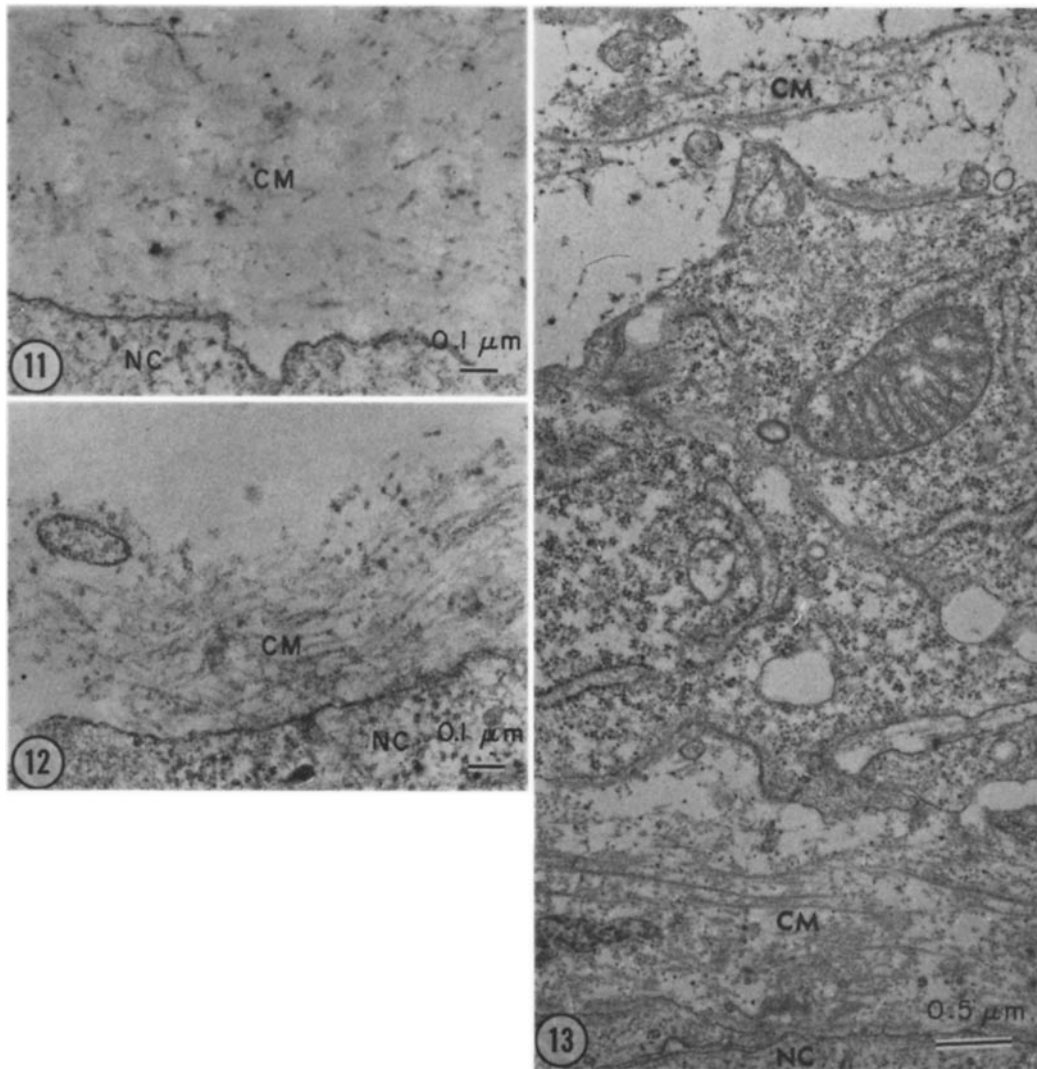
Figures 11 and 12 Cartilage matrix (CM) adjacent to the notochord (NC) in 6-h (Fig. 14) and 12-h (Fig. 15) notochord-somite cultures fed nutrient medium containing HS and EE. Note the paucity of matrix in the 6 h culture, and the increase in the 12 h culture. Also note the absence of the perichordal basement membrane. × 50,000.

Figure 13 Cartilage matrix (CM) in a 2 day notochord-somite culture fed nutrient medium containing FCS and F12X. Note that the density of the matrix adjacent of the notochord (NC) at the bottom of the figure is much greater than in the chondrogenic area at the top of the figure. Also note the presence of a perichordal basement membrane. × 20,000.

accumulation is even greater than that in chondrogenic areas in somitic mesenchyme in these cultures. (see Fig 13) One exception is where the surface of the notochord is cut so that the notochord cells are dispersed in somitic tissues. In these areas the increased matrix accumulation is not present around the dispersed notochord cells.

Although the accumulation of matrix around the spinal cord in spinal cord-somite (SCS) cultures is similar to that around the notochord in NS cultures, there are more fibrils in the matrix around the spinal cord, and more granules in the matrix around the notochord With the stereomicroscope, this area of matrix accumulation initially appears as a translucent zone and then as a hyaline zone around the inducer tissues in living cultures However, the appearance of this zone is sometimes masked by the accumulation of cell debris.

CULTURE DAYS 1–2, CHONDROGENESIS IN SOMITIC MESENCHYME

Within minutes after six to eight somites are placed on nutrient agar they begin to reorganize into a compact cellular mass. By the end of 6 h in both NS and SCS cultures structured matrix components begin to accumulate in small extracellular spaces in somitic mesenchyme (Fig. 14–15). These mesenchymal areas are referred to as "prechondrogenic areas " As matrix accumulation increases, the amount of extracellular space enlarges considerably, and chondrogenic cytodifferentiation begins. The term "chondrogenic area" is then applied (cf Figs. 16–17). Although the 200 Å unbanded collagen fibrils are predominant in the chondrogenic areas at the end of 12 h in culture, some of the fibrils in the prechondrogenic areas are thinner (100–120 Å) and appear to be tubular in cross-section (cf Figs. 15 and 17). As the 200 Å fibrils become predominant, there is also a notable increase in the numbers of protein polysaccharide granules in the developing chondrogenic areas.

Since the cultured somites are randomly oriented, and the chondrogenic areas develop in presumptive sclerotome, the chondrogenic areas are randomly oriented in the cultures. Some develop near the inducer tissues (Fig. 16), whereas others develop at the periphery of the culture. Contiguous chondrogenic areas usually fuse to form a single area With the stereomicroscope the chondrogenic areas are frequently seen as translucent areas in living cultures.

By the end of 12 h (Figs 16–17) there are well-defined chondrogenic areas in all of the induced cultures, and the accumulations of structured matrix in these areas are larger than those seen in equivalently aged somitic tissues in vivo Initially, these matrix accumulations are widely scattered in areas of extracellular space which contain no structured matrix (see Fig. 14) However, from 12 h (Fig 17) through 2 days (Fig. 18) in culture, the accumulations of structured matrix in both NS and SCS cultures are larger than those in equivalently aged somitic tissues in vivo (see Fig 7). This rapid accumulation of structured matrix corresponds directly to a rapid accumulation of labeled sulfate ($^{35}SO_4$) into chondroitin sulfates during the initial period in induced somite cultures (see Ellison and Lash, 1971).

Since many mesenchymal cells die during the initial period in culture, many chondrogenic areas do not survive to form cartilage nodules. Furthermore, those which survive do not have the histological characteristics of hyaline cartilage until the 5th or 6th day in culture.

CULTURE DAYS 1–2, CELL DEATH

In 6-h cultures there are many degenerating cells, and many cells containing one or more phagosomes filled with cell debris (Fig 14). The accumulation of phagosomes increases sharply during the first 24 h, and this corresponds to a rapid loss of total DNA during this period in NS cultures (cf. Ellison and Lash, 1971; Gordon, 1971). In all of these cultures the cell death is most evident in the structurally undifferentiated mesenchyme and in chondrogenic areas farthest from the inducer tissues (see Fig. 16). There are few signs of cell death in structurally differentiated muscle or in the inducer tissues (Fig. 16).

After the first 24 h in NS and SCS cultures there are fewer signs of cell death After 2 days some presumptive chondroblasts contain phagosomes, but there are many more of these cells without phagosomes (Fig. 18). This is especially true of the chondrogenic areas near the inducer tissues In contrast, many of the structurally undifferentiated mesenchymal cells contain phagosomes throughout the 7 day culture period

CULTURE DAYS 1–2, CHONDROGENIC CYTODIFFERENTIATION

Structural cytodifferentiation is only recognized after the onset of structured matrix accumulation.
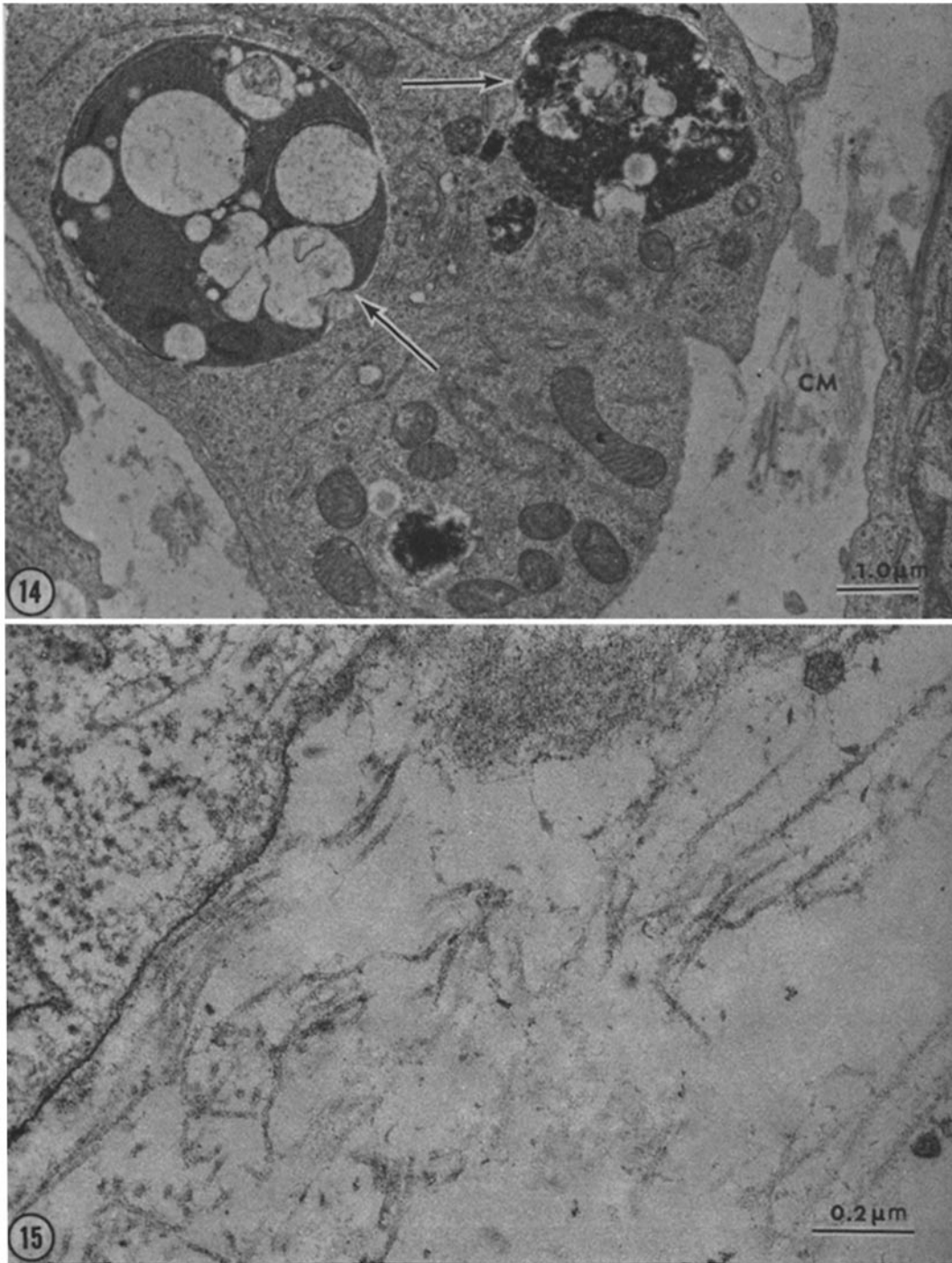
FIGURES 14 and 15   A prechondrogenic area in a 6 h notochord-somite culture fed nutrient medium containing HS and EE Phagosomes (arrows) are present in the presumptive chondroblast in Fig 14 ×
12,000. Cartilage-matrix (*CM*) which has accumulated in these prechondrogenic areas is also shown in
Fig. 15. × 75,000.
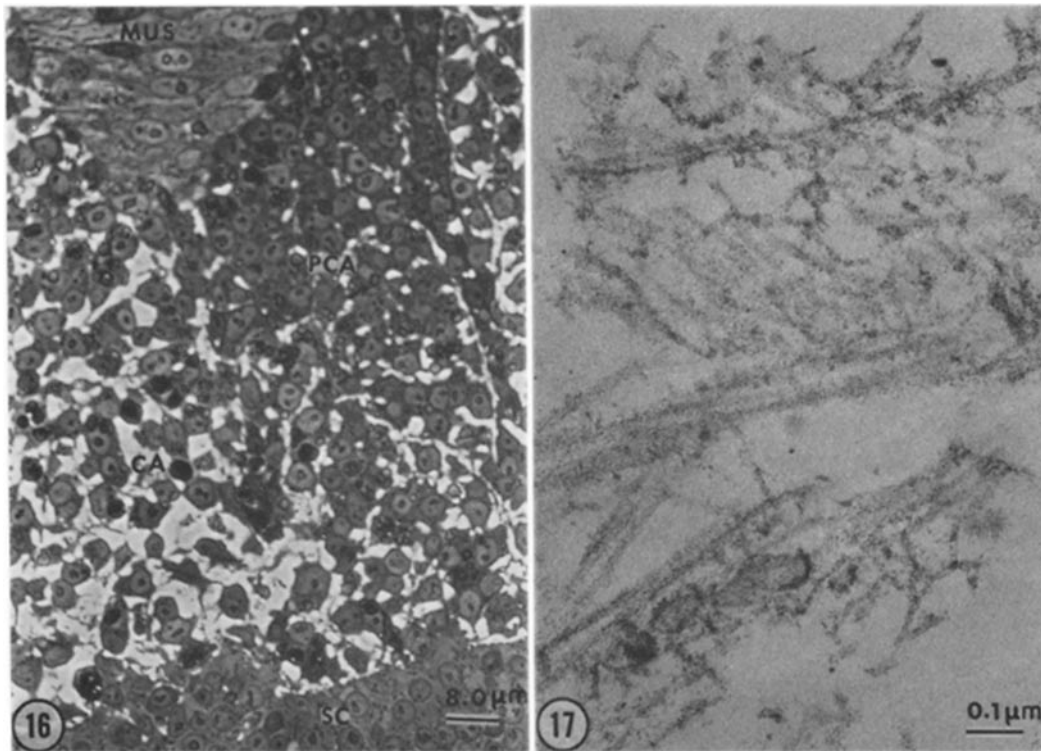
FIGURE 16 A phase-contrast micrograph of a stained (Ito's stain) 1 μm section of a 12 h spinal cord somite culture fed nutrient medium containing FCS and F12X. A large chondrogenic area (*CA*) and a prechondrogenic area (*PCA*) are present between the spinal cord (*SC*) and muscle (*MUS*) tissue. The dense inclusions in many of the somite cells are phagosomes filled with cell debris. × 875.

FIGURE 17 Cartilage matrix in a chondrogenic area in a 12 h notochord-somite culture fed nutrient medium containing HS and EE. × 75,000.

Except for the presence of phagosomes, the presumptive chondroblasts after 6 h of culture are structurally comparable to in vivo sclerotome cells. By the end of 12 h small polysomes are still present but there is an increase in the numbers of free ribosomes, lamellar and small vesicular Golgi components, and profiles of RER. The increase in these cytoplasmic organelles continues at such a pace that by the end of 2 days (see Fig. 18) some of the cells in chondrogenic areas are structurally comparable to 6- or 7-day in vivo chondroblasts Although the accumulated age of these somites is 4.5 days (Fig. 18), many of the chondroblasts have extensive Golgi areas with large vacuoles containing 100–300 Å granules (cf. Figs. 18 and 22, insert) Comparable Golgi vacuoles are not seen in vertebral chondroblasts until 6 or 7 days. Similarly, some of the chondroblasts in 2-day induced cultures contain dilated profiles of RER

Culture Days 3–4

Structural cytodifferentiation and structured matrix accumulation continue during days 3 and 4 in culture, but the rate of these changes is slower than that in 5–7 day vertebral cartilage. By the end of 4 days (Figs 9–10) the amount of structured matrix in some chondrogenic areas is similar to that in 6–7 day vertebral cartilage, whereas in other areas there is less matrix accumulation than in vivo Although the matrix in these areas is alcianophilic, stains metachromatically with toluidine blue, and contains moderate amounts of structured matrix, the chondrogenic areas do not develop the other histological characteristics of hyaline cartilage until the 5th or 6th day in culture. In 4-day cultures the chondroblasts have not yet developed into polygonal hypertrophied chondrocytes, and a perichondrium has not developed.
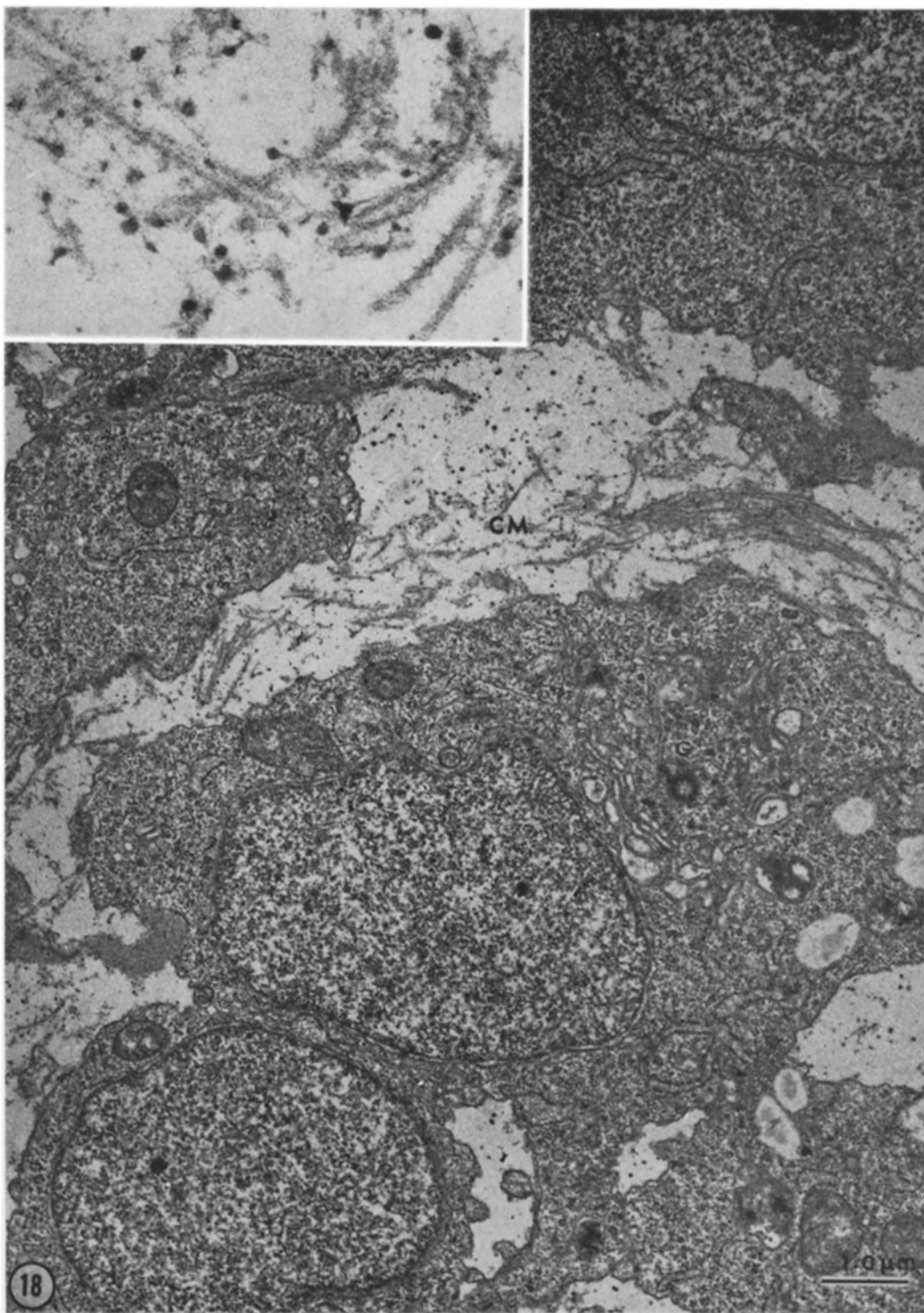
FIGURE 18   A chondrogenic area in a 2 day notochord-somite culture fed nutrient medium containing FCS and F12X Note that large vacuoles are present in the Golgi complex (G) in the chondroblasts. Also note the considerable quantities of cartilage matrix (CM) which have accumulated. × 13,000; inset, × 75,000.
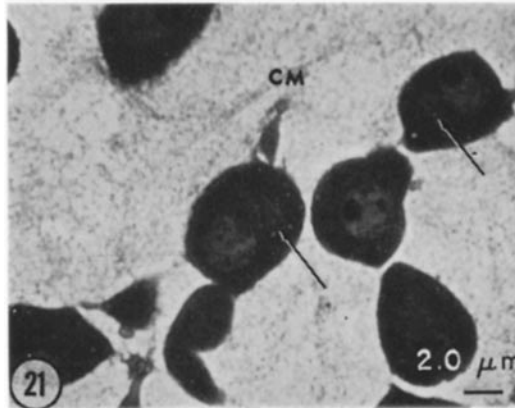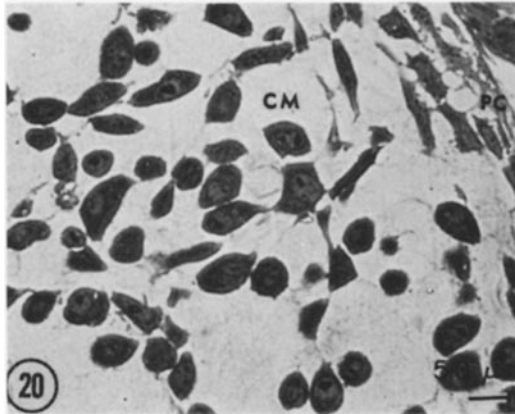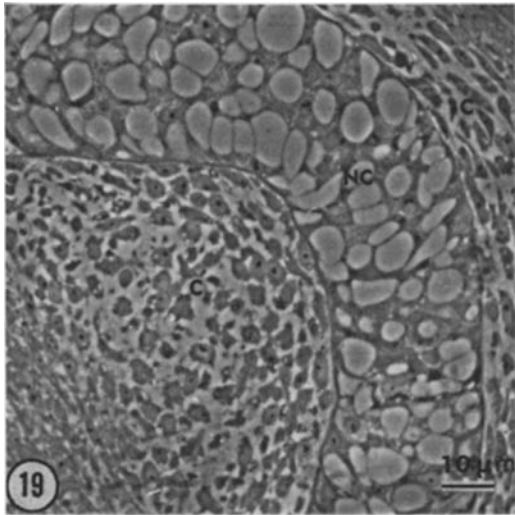
41

FIGURE 19 A phase-contrast micrograph of an unstained 1 μm section of an immature cartilage nodule (C) surrounding the notochord (NC) in a 6 day notochord-somite culture fed nutrient medium containing HS and EE. × 700.

FIGURES 20 and 21 Phase-contrast micrographs of a

CULTURE DAYS 5–7, THE APPEARANCE OF HYALINE CARTILAGE

LIGHT MICROSCOPY During days 5–7 chondrogenic areas in all induced somite cultures develop into nodules of hyaline cartilage (Figs. 19–21). Usually, the chondrogenic areas first develop into nodules of immature cartilage in which stellate chondroblasts are isolated in matrix, and the nodule itself is surrounded by a condensed mesenchyme (Fig. 19). With continued matrix accumulation, the chondroblasts become polygonal, hypertrophied chondrocytes (Figs. 20–21) As this cartilage matures the cells in the condensed mesenchyme become flattened and discoidal, so as to form a well-defined perichondrium (Fig. 20). This cartilage is histologically comparable to 10 day vertebral cartilage, and the nodules are then referred to as mature cartilage nodules (Figs. 20–21).

Occasionally, a small region of structurally undifferentiated somitic mesenchyme, or a small part of a large chondrogenic area, will develop directly into a mature cartilage nodule. In these cases discoidal cells become arranged circumferentially in a small, compact spherical nodule. There is then a rapid accumulation of matrix between these cells, the nodule expands, the centrally located cells become polygonal hypertrophied chondrocytes, and the discoidal cells around the periphery form a well-defined perichondrium.

ELECTRON MICROSCOPY: With the electron microscope the chondrocytes in cartilage nodules (Fig. 22) are indistinguishable from those in maturing vertebral cartilage. Their complement of organelles includes large amounts of RER with both dilated and nondilated cisternae, and a large Golgi complement with stacked lamellar profiles, small fuzzy coated vesicles, and large smooth-surfaced vacuoles containing 100–400 Å granules (Fig. 22). These granules are structurally comparable to small protein polysaccharide granules (see Fig. 22, insert), and these Golgi

stained (Ito's stain) 1 μm section of a mature cartilage nodule in a 7 day spinal cord-somite culture fed nutrient medium containing FCS and F12X. With close inspection aggregates of metachromatically stained components can be seen in the cartilage matrix (CM). Note the well-defined perichondrium (PC) at the upper right in Fig. 20. × 1000. Golgi areas (arrows), which also stain metachromatically, are seen in the chondrocytes in Fig. 21. × 2500.

regions show a strong metachromasia when stained with toluidine blue (Fig. 21)

The structured matrix in cartilage nodules consists of thin unbanded collagen fibrils and prominent protein polysaccharide granules, but the density of accumulation of these components is less than that in mature vertebral cartilage (cf Figs 23 and 2). In the matrix in immature nodules, and around the periphery of mature nodules, the protein polysaccharide granules are randomly distributed, and they often lie in contact with unbanded collagen fibrils (Fig 23) The fibrils, however, are frequently grouped in loose bundles (Fig. 23).

Another difference in the distribution of matrix components is that collagen fibrils are not seen in the center of mature cartilage nodules (Fig 24) In these areas the structured matrix consists entirely of aggregates of protein polysaccharide granules and their filamentous tails (Fig 24). The aggregates of granules, and the absence of collagen fibrils, are similar to that in the matrix in lacunae surrounding 10-day vertebral chondrocytes (see Fig 3). Whether this absence of fibrils in the center of mature nodules is due to decreased collagen synthesis, increased collagen turnover, or a failure of collagen fibrillogenesis is not known It is in sharp contrast, however, to the quantities of both components which are present in the matrix around the periphery of mature nodules and throughout immature nodules (Fig 23)

EFFECTS OF NUTRIENT MEDIA ON CHONDROGENESIS IN INDUCED SOMITE CULTURES

In both NS and SCS cultures, with both SBSS:HS:EE and SBSS:FCS:F12X nutrient media, prechondrogenic areas are present after 6 h, and chondrogenic areas are present after 12 h in culture. Structured cartilage matrix components are accumulating in large extracellular spaces in all of the chondrogenic areas However, after 24 h there are large accumulations of both matrix components in the chondrogenic areas in cultures fed the medium containing FCS and F12X. This increased rate of structured matrix accumulation corresponds to an increased rate of labeled sulfate ($^{35}SO_4$) incorporation into chondroitin sulfates in NS cultures fed the medium containing FCS and F12X (see Ellison and Lash, 1971). In addition to this difference, there are fewer signs of cell death

in the cultures fed the medium containing FCS and F12X.

Chondrogenesis in Noninduced Somite Cultures

Although this study is primarily concerned with induced chondrogenesis, comparable S and NS cultures, fed either SBSS:HS:EE or SBSS:FCS: F12X nutrient media, were examined after 1, 2, 5, and 7 days in culture The most prominent difference between these induced (NS) and non-induced (S) cultures is that there is considerably more cell death in S cultures In both types of culture most of the cell death is in the areas of structurally undifferentiated mesenchyme This cell death is more prevalent in S cultures throughout the 7 day culture period, and it is most prevalent in S cultures fed nutrient medium containing HS and EE (Fig 25).

Another prominent difference between these cultures is that while the accumulation of structured matrix in somitic mesenchyme is comparable in S and NS cultures, the 24- and 48-h S cultures do not have the chondrogenic areas which are present after 12 h in all NS cultures As in induced cultures the accumulation of structured matrix in 24-h S cultures (Fig 25) are larger than those in equivalently aged somitic mesenchyme in vivo These mesenchymal areas are present in S cultures throughout the 7 day culture period.

By the end of 5 days in culture chondrogenic areas appear in most of the S cultures fed the SBSS:FCS:F12X medium, and in a smaller number of those fed the SBSS:HS:EE medium During the 6th and 7th days many of these areas develop into mature cartilage nodules which are structurally identical to those in NS cultures. This identity includes the absence of collagen fibrils from the matrix in the center of mature cartilage nodules. In those S cultures in which cartilage nodules fail to develop, there are more signs of cell death in the mesenchymal tissues.

Muscle and Other Tissues in Somite Cultures

Although this study is primarily concerned with chondrogenic differentiation, there are a number of other structurally differentiated tissues which develop in cultured somites For example, skeletal muscle tissue develops in all of these cultures By the end of the 2nd day the muscle tissue consists of elongated myoblasts with large glycogen de-

posits, large elongated polysomes, and both oriented and disoriented myofibrils with dense Z lines (see Allen and Pepe, 1965). In most cases the muscle is separated from the chondrogenic areas by a well-formed basement membrane During the 2nd day many of these cultures become contractile, and pulsate with an irregular rhythm After the 2nd or 3rd day muscle constitutes the majority of the nonchondrogenic tissues in these cultures.

Even though the somitic mesoderm undergoes chondrogenic and myogenic differentiation, there is a notable absence of structurally distinguishable fibrogenic differentiation in these cultures There are more fibrils than granules in the matrix surrounding the spinal cord, and in the perichondrium and structurally undifferentiated mesenchyme, but these cultures contain few of the thick (300–500 Å) banded collagen fibrils, like those found in fibrous tissues in vivo Similarly, these cultures do not appear to contain the "matrix vesicles" which would be indicative of osteogenic differentiation (see Anderson, 1969, Ali et al, 1970).

In addition to the aforementioned mesodermal tissues, nearly all of these cultures also contain isolated and grouped neurons (presumably of neural crest origin), and endothelial remnants of aorta that adhere to explanted somites The endothelial vesicles are usually surrounded by matrix containing large quantities of thin unbanded fibrils and relatively few granules The isolated and grouped neurons frequently have long processes containing neurofilaments and neurotubules, and their endbulbs are often interposed between myoblasts Nevertheless, differentiated junctional complexes have not been found where these endbulbs contact myoblasts (see Shimada et al., 1969).

DISCUSSION

Previous studies of chondrogenic differentiation of somitic mesoderm utilized histological methods (primarily staining acid mucopolysaccharides), as well as biochemical analyses of the synthesis of the chondroitin sulfates (inter alia Lash et al, 1957; Marzullo and Lash, 1967, Ellison et al, 1969, Kvist and Finnegan, 1970; Ellison and Lash, 1971) These histological and biochemical studies have been confirmed and extended by the structural analysis of somite chondrogenesis reported here In addition, these structural studies have characterized some of the effects of inducer tissues and nutrient supplementation on chondrogenic differentiation in cultured somites The correlation of these studies with the biochemical studies of Ellison and Lash (1971) have also permitted the recognition of chondrogenic differentiation within the first few hours after somites are placed in culture

Structured Matrix Components

The distinct structural differences between the matrix components of vertebral cartilage, and those of the transitional and fibrous tissue matrix of perichondrium and intevertebral joints, show that cartilage matrix has a distinct recognizable structure. Protein polysaccharide matrix granules were previously shown to be the structural form of fixed cartilage proteoglycan complexes (Matukas, et al, 1967; Smith, 1970; Anderson and Sajdera, 1971), and the presence of these granules in both

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FIGURE 22 A chondrocyte in a mature cartilage nodule in a 7 day notochord-somite culture fed nutrient medium containing FCS and F12X. The rough endoplasmic reticulum (RER) has dilated cisternae containing a fine granular material, and the Golgi complex has many large vacuoles (V) containing dense granules (inset, X 60,000). The granules in the Golgi vacuoles are structurally similar to smaller protein polysaccharide granules. X 25,000.

FIGURE 23 Cartilage matrix in an immature cartilage nodule in a 7 day notochord-somite culture fed nutrient medium containing FCS and F12X. Note the accumulation of both cartilage matrix components X 27,500

FIGURE 24 Aggregates of protein polysaccharide granules constituting the structured matrix in the center of a mature cartilage nodule in a 7 day notochord-somite culture fed nutrient medium containing FCS and F12X. Aggregates such as these correspond to the metachromatically stained components in the matrix in Figs. 20–21. Note the absence of collagen fibrils. X 82,500.
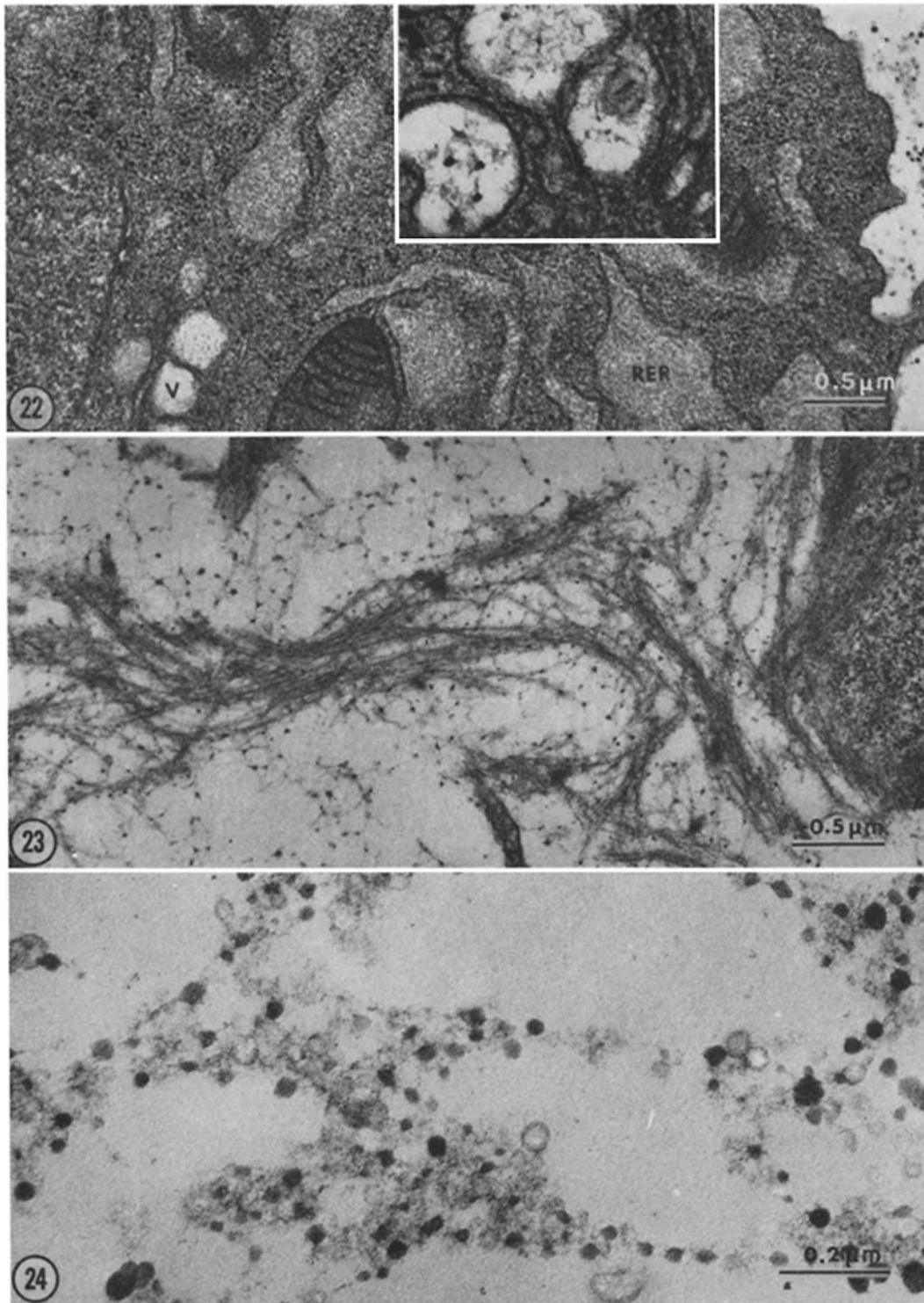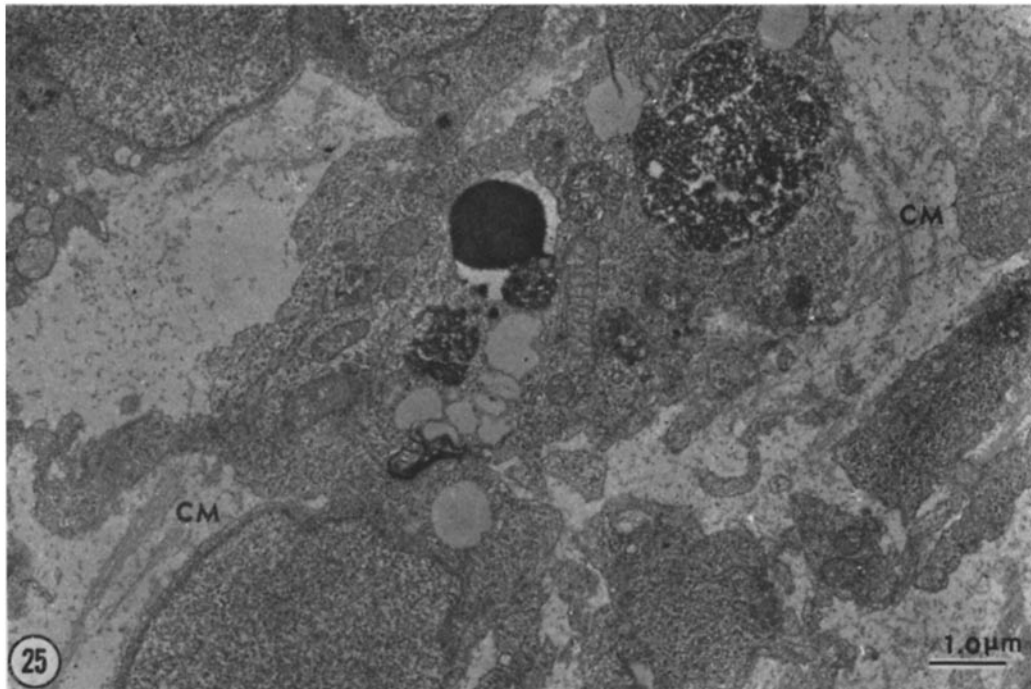
Figure 25   A prechondrogenic area in a 24 h noninduced somite culture fed nutrient medium containing HS and EE   Note the large accumulations of cartilage matrix (*CM*). Also note the phagosomes in the presumptive chondroblasts. ✕ 10,000.

in vivo and in vitro tissues has consistently coincided with the demonstrable presence of proteoglycans containing chondroitin sulfates A and/or C in these embryonic chick tissues (see Franco-Browder et al, 1963; Marzullo and Lash, 1967; Kvist and Finnegan, 1970, Manasek, 1970; Abbott et al, 1972). Similarly, the synthesis and accumulation of protein polysaccharide granules consistently coincides with the demonstrable presence of the enzymatic activities required for chondroitin sulfate synthesis (Glick et al., 1964; Lash et al, 1964; Marzullo and Lash, 1967, Gordon, 1971). This correlation of the accumulation of protein polysaccharide granules with the synthesis of biochemically demonstrable chondroitin sulfates show that these structural and biochemical criteria are both sensitive indicators of cartilage-type matrix synthesis.

These structural studies have also shown that thin unbanded collagen fibrils constitute the second structured component of both in vivo and in vitro cartilage matrix. These fibrils are distinct from the thick banded collagen fibrils in transi-

tional and fibrous tissue matrix, as well as from the limited number of 100–120 Å tubular fibrils seen in the perichordal matrix and in the initial accumulations of matrix in cultured somites (see Fig. 15). These 100–120 Å fibrils may be comparable to the tubular microfibrils associated with the differentiation of elastic fibers (see Greenlee et al, 1966; Ross and Bornstein, 1969).

Although it is well known that considerable amounts of collagen are present in cartilage, the collagen content of embryonic cartilage containing a homogeneous population of thin unbanded fibrils has not been determined previously. The amino acid analyses (Table I) of cleaned strips of embryonic cartilage which contains a homogeneous population of thin (ca 150 Å) fibrils, therefore provide more direct evidence of the collagenous nature of the thin unbanded fibrils in this embryonic cartilage. The weight percent of hydroxyproline in the whole homogenate (Table I) suggests that collagen constitutes approximately 50% of the protein in this cartilage, and the amount of contamination of this cartilage

with transitional matrix is so small that thick banded fibrils could not represent more than 1% of the fibrils in these preparations Nevertheless, there is contamination of the first acid extract with noncollagenous proteins, and this contamination is only partially reduced by salt precipitation (Table I). Recent studies (Minor and Rosenbloom, in progress) suggest that the use of lathyrogens during the time of vertebral chondrogenesis will be required for the purification and separation of the chains of these collagen molecules.

Since the chains of these cartilage collagen molecules have not yet been separated and analyzed, it is not known whether the thin unbanded fibrils contain the $\alpha 1$ (II) cartilage-type collagen chain described by Miller and Matukas (1969) and by Trelstad et al (1970). It is therefore not known whether the structural differences in the collagen fibrils, or the differences in their distribution in the in vitro cartilage, are due to primary differences in the composition of the collagen chains, differences in the carbohydrate prosthetic groups linked to the collagen (see Stark et al, 1972), or to an interaction of collagen with cartilage proteoglycans (see Mathews, 1965).

Factors Affecting Somite Chondrogenesis

Even though electron microscope techniques do not permit an accurate quantitation of matrix accumulation, or cell division and cell death, there is a direct correlation of these structural observations with biochemical analyses of total DNA, DNA synthesis, cartilage-type enzymatic activities, and chondroitin sulfate synthesis in comparable somite cultures (see Ellison and Lash, 1971, Gordon, 1971) In both induced and non-induced cultures there is a direct correlation of the identification of a notable increase in structured matrix accumulation with an increasing rate of labeled sulfate incorporation into chondroitin sulfates in cultured somites (Ellison and Lash, 1971, Minor and Lash, in preparation) Similarly, the structural signs of cell death correlate with the biochemical comparison of the total amount of DNA per culture and the amount of DNA synthesis per culture (Ellison and Lash, 1971, Gordon, 1971).

By improving the culture conditions with the addition of inducer tissues, or the use of nutrient medium containing FCS and F12X instead of HS and EE, the structural evidence of cell death is decreased or minimized in cultured somites These observations coincide with biochemical studies which show that the total DNA per culture drops sharply during the first day, and then levels off or begins to increase during the second day, yet at the same time the rate of DNA synthesis is constant in both S and NS cultures (Ellison and Lash, 1971, Gordon, 1971) Since notable signs of cell death are absent during in vivo somite chondrogenesis, the cell death which occurs in cultured somites is obviously due to the deleterious effects of the tissue isolation and culture procedures. In cultured somites, however, these structural and biochemical observations suggest that the addition of inducer tissues and nutrient supplementation both have the effect of minimizing cell death This maintenance of somitic mesenchyme is undoubtedly important for the formation of cartilage in cultured somites The determination of the amount of cell death, and the identification of the types of cells which are dying, are therefore important considerations in all in vitro studies of mesodermal differentiation.

Factors Facilitating Somite Chondrogenesis

The studies reported here suggest that increased cartilage matrix synthesis in somitic mesoderm is associated with the exposure of this mesoderm to an environment containing larger quantities of cartilage-type matrix (e g, the perichordal matrix). In vivo, the increased synthesis and accumulation of matrix in somitic mesenchyme does not occur until these cells become associated with the matrix surrounding the inducer tissues. Chondrogenesis then spreads centrifugally from this initial zone of matrix accumulation. In addition, when the inducer tissues are transplanted to the lateral portion of the somite, supernumerary "vertebral" cartilages form in the surrounding somitic mesenchyme (Holtzer and Detwiler, 1953; Strudel, 1953, Watterson et al, 1954) In vitro, the inducer tissues synthesize a similar matrix, and this is also associated with an increase in matrix synthesis, as well as an increase in cell survival, in somitic mesenchyme. Lash (unpublished data) has shown that the notochord incorporates labeled sulfate into chondroitin sulfates, and Cohen and Hay (1971) have recently shown that the embryonic chick spinal cord synthesizes collagen at the time when it is inductively active Avery et al (1956) showed that dispersed notochord cells are not inductively active, and this study has shown that the increased

synthesis and accumulation of matrix does not occur when notochord cells are dispersed in somitic mesenchyme. Cooper (1965) showed that hypertrophying cartilage is the only other tissue which has an inductive effect on cartilage formation in cultured somites, and he suggested that "tissues which induce cartilage and bone formation do so only when the competent cells synthesize and secrete matrix at a high rate."

Since an increase in matrix synthesis during the first 2 days in culture is associated with an increased incidence of cartilage nodule formation, it would be expected that the inhibition of this initial phase of matrix synthesis would have an inhibitory effect on subsequent cartilage nodule formation. That such inhibitors do prevent cartilage formation in somite cultures has recently been demonstrated by Abbott et al. (1972) who showed that the presence of 5-bromodeoxyuridine (BudR) during the first 3 days in culture prevented the subsequent formation of cartilage in cultured somites. Chacko et al (1969) have shown that BudR inhibits chondroitin sulfate synthesis in cultured chondrocytes, and Mayne et al. (1971) have shown that it has a similar effect on cultured amnion cells. It is therefore suggested that the early exposure of cultured somites to BudR may inhibit the synthesis of a matrix which is necessary for somite cells to undergo chondrogenic differentiation and cartilage nodule formation.

An increase in the incidence of cell survival and cartilage nodule formation has also been shown to be associated with a high rate of synthesis and accumulation of cartilage-type matrix during the first 2 days in noninduced somite cultures. In noninduced cultures fed nutrient medium containing FCS and F12X the somitic mesenchyme synthesizes increased quantities of matrix during the initial culture period, and this is associated with continued matrix synthesis, increased cell survival, and an increase in the incidence of cartilage nodule formation Thus, the inducer tissues and nutrient supplementation may have a similar role in facilitating chondrogenic differentiation in cultured somites. It is not known, however, whether the matrix components actually have a stimulatory effect on the synthetic activities of the surviving somite cells. That this may be occurring is suggested by the observation that the addition of exogenous chondromucoprotein (cartilage proteoglycans) to suspension cultures of embryonic chick epiphyseal chondrocytes causes an increase in the synthesis of chondromucopro-

tein by these chondrocytes (Nevo and Dorfman, 1971).

The suggestion that extracellular matrix components have a role in inductive tissue interactions is not new. Grobstein (1967) proposed that macromolecular complexing at the interface of interacting tissues is an important factor in embryonic induction. From the observations reported here it is suggested that the accumulation of matrix containing both collagen and the proteoglycans has an important role in the so-called "induction" of somite chondrogenesis.

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