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A HISTOCHEMICAL AND FINE STRUCTURAL STUDY OF EXTRACELLULAR FIBRILS IN THE DEVELOPING CHICK EMBRYO

by

James J. O'Connell

Bachelor of Arts, Carroll College 1967

A Thesis

Submitted to the Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the Degree of

Master of Arts

Grand Forks, North Dakota

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This thesis submitted by James J. O'Connell in partial fulfillment of the requirements for the Degree of Master of Arts from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

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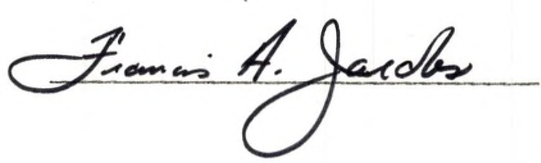

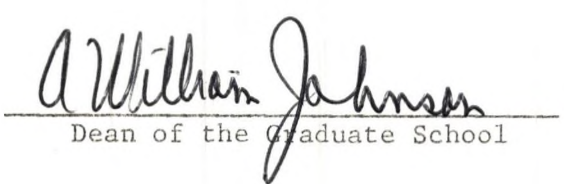

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Date July 28, 1969

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ABSTRACT

The histochemistry of developing connective tissues and its relation to early connective tissue fibrils was investigated in the chick embryo. An area well suited to correlated histochemical and electron microscopic study occurs around the notochord. Here early microfibrils later contribute to the cartilaginous model of the future vertebral body. Chick embryos were sacrificed at one, two, three, four, six and ten days of incubation and were prepared routinely for both light and electron microscopy. A series of five histochemical stains (PAS, alcian blue, Hale colloidal iron, metachromatic toluidine blue, methenamine silver) and two histological techniques (Mallory's connective tissue; Weigert's elastin) was used for the light microscopic demonstration of polysaccharides, mucoproteins, mucopolysaccharides and mature connective tissue elements.

The first positive response for all histochemical stains occurs on the third day of incubation. Moderate microfibrillar growth in electron microscopy precedes this by one day. At this time light microscopic staining patterns differ from electron microscopic fibrillar arrangements. By the sixth day, dense microfibrillar concentrations appear in the precartilage area where acid mucopolysaccharides are intensely concentrated. Staining for mature connective tissue fibrils does not occur until the tenth day.

Polysaccharides, mucoproteins and mucopolysaccharides are interfibrillar components that are closely associated with the early stages of fibrillogenesis. Microfibrils become intensely concentrated in a matrix of these substances. Here, the future cartilaginous model with its heavy population of unit collagen fibrils will form.

CHAPTER I

INTRODUCTION

The discovery in recent years of fine extracellular fibrils has shed new light on the possible origins of connective tissue. The presence of fine fibrils unknown before the advent of electron microscopy was noted at first without much speculation as to their significance. More recently increasing notice of the association of microfibrils with other extracellular connective tissue components has stimulated further interest.

The brief history of microfibrils began with the observations of Jakus in 1954 (1) who first reported fine filaments between the unit collagen fibrils of the rat corneal stroma in an electron microscopic study. Later, Robertson (2), working on the motor-end plates of reptilian skeletal muscle, described filaments of 100 Å in diameter. These were interspersed among the larger unit collagen fibrils close to the basement membrane of the muscle. Karrer (3) observed similar structures in the tunica propria of bronchioles in the rat and later (4) in the tunic media of the developing chick aorta. Low (5) observed fine fibrils with regularity in the tissue space of the human pulmonary alveolar wall and later with Battig (6) in human cardiac muscle. In a later publication he (7) named these structures *microfibrils* and interpreted them as routine components of the extracellular connective

tissues. They appeared in a loose tangle throughout the tissue space but were more thickly matted around elastic fibers and within boundary (basement) membranes. They possessed an average diameter of 80 Å with a range from 40 Å to 120 Å. More recently Low (8) discovered a zone of intense microfibrillar growth around the notochord of early chick embryos, which reached its peak of activity at incubation ages from 40 to 72 hours. His survey of developing extracellular connective tissue fibrils included chick embryos spanning ages from the time of laying to an incubation age of one week. Low concluded from this study that microfibrils were the natural precursors of the unit collagen fibrils that were already familiar to electron microscopists. This observation concurred with the opinion of numerous other investigators (2-4, 9-13) who had expressed the same view derived from non-developmental sources. The apparent developmental relationship between microfibrils and more mature connective tissue elements led to the formulation of a unitary hypothesis of extracellular connective tissue fibrils (8). This viewpoint proposes that the microfibril, called the "common denominator" by Haust (9), represents the main line of development of the fibrous connective tissues.

The area of intense fibrillogenesis around the notochord of the chick embryo occurs before the organism has developed connective tissue fibers such as characterize the mature state. This provides an interesting opportunity for correlated study of developing histochemistry and microfibrils in early connective tissues. Since it is now feasible to prepare tissue fixed for fine structure for light microscopy (14), it

is possible to choose freely from the roster of light microscopic stains in exploring the development of the connective tissues in this area. It may clarify this problem to present a brief account of the staining techniques deemed most likely to yield interesting results in this study.

The periodic acid-Schiff (PAS) technique presents interesting possibilities. Periodic acid is a selective oxidizing agent (15) which attacks 1,2-glycol groups, primary amino and secondary amino groups and 1-hydroxy-2-keto groups. Aldehydes thus formed combine with the Schiff reagent. The periodic acid-Schiff stain, according to Barka and Anderson (16), gives its characteristic color reaction (magenta to pink) in the presence of glycogen, neutral mucopolysaccharides, mucoproteins and glycoproteins.

Alcian blue and Hale colloidal iron both give a characteristic blue reaction in the presence of acid mucopolysaccharides. Alcian blue is a water soluble phthalocyanin dye which, when used at low pH (2.5 to 3.0) for a short period of time (30 minutes; ref. 17), produces a light blue color indicative of and highly selective for acid mucopolysaccharides (16). Uncertainty surrounding the actual structure of the dye has led to controversy over the actual mechanism of the staining reaction. According to Barka and Anderson (16), "Chemical forces probably play a role in the binding of the dye, but the exact mechanism of the staining reaction is not known." Less uncertainty surrounds the colloidal iron method of Hale (18), which is dependent upon the affinity of free radicals for Fe^{+++} at a pH of 1.1 to 1.3 (19,20). Subsequent treatment with the Prussian Blue reaction reveals

the sites of acid mucopolysaccharides. Mowry's modifications (21) and Müller's (22) introduction of colloidal ferric hydroxide enhances the specificity of this stain and facilitates staining procedure.

Metachromatic toluidine blue is also useful. The currently accepted mechanism of metachromasia is based on the idea of polymerization of the dye-stuff itself, which results in a reddish-purple color as compared to the blue of the monomeric state (19). Metachromatic toluidine blue characteristically responds to acid mucopolysaccharides particularly those with sulfated ester groups (16,23).

The methenamine silver reaction is in the words of Gomori (24) "A histochemical test for glycogen and mucin based on the liberation of aldehyde groups and subsequent demonstration of the latter by a reduced silver method..." The aldehyde groups are formed by oxidation with 5% chromic acid. The methenamine silver complex is then reduced by the aldehydes leaving a jet black deposit.

In a developmental study the stains routinely used for demonstrating mature connective tissue elements should also be interesting. Mallory's connective tissue stain is commonly used to show collagen fibers which take on a deep blue color. Elastic fibers stain a dark blue-black following Weigert's resorcin-fuchsin technique (25).

The connective tissues are known to contain mucoprotein, glycogen and various mucopolysaccharides which should be amenable to demonstration by the above techniques (26). However, it is not clear when these

substances appear in the course of embryonic development. Their relationship to early fibrillogenesis is likewise unknown. It therefore seems worthwhile to employ the techniques of both light and electron microscopy on a series of early chick embryos of known ages. This approach should provide a correlation between histochemistry and fibrillogenesis among the early connective tissues. This paper describes in some detail the results of an investigation conducted along these lines.

CHAPTER II

MATERIALS AND METHODS

Freshly laid eggs of White Leghorn pullets were used in this study. Incubation was carried out at 38°C with forced-air ventilation. Embryos were sacrificed at one, two, three, four, six and ten days incubation age by techniques previously described in detail (27). Following removal of the embryo from the egg, it was fixed by immersion in cold buffered aldehydes (28) from ½ to 4 hours. Carnoy's acid-alcohol fixer (29) was used for the light microscopic preparation of the younger embryos (one and two days) since the tissue masses tended to separate after aldehyde fixation.

Each embryo was staged according to the Hamilton-Hamburger series (30). The area of the head process was used in the one day embryos. In older embryos the area investigated was restricted to the cephalo-caudal extent of the five most cephalic postotic somites. This was done in order to insure an even progression of developmental stages in the notochordal area. To facilitate comparison of light and electron microscopic preparations, some of the embryos were cut in cross section, one piece being used for light microscopy and the other for electron microscopy.

Light Microscopy

Preparations for light microscopy were dehydrated in alcohols by

drop-by-drop addition of stronger solutions to avoid damaging turbulence. Tissues were passed into chloroform and then embedded in 60 to 62°C paraffin. Serial sections were cut at 4 to 6 μ on a specially sharpened steel knife without fluid receptacle (14,31). Ribbons four to five sections long were mounted on separate slides which were subsequently stained by different techniques. Each slide contained four separate pieces of ribbon from the same embryo.

Five connective tissue stains were used routinely. Periodic acid-Schiff (31), metachromatic toluidine blue (23) and methenamine silver (24) were used without essential modification. Hale colloidal iron was used according to the methods of Mowry and Müller (21,22). Alcian blue was used according to the original method of Steedman (32) as modified by Mowry (17). The older embryos were additionally stained with Mallory's connective tissue stain and Weigert's elastin stain (25). These preparations were examined with a Zeiss Photomicroscope.

Electron Microscopy

Tissues were post-fixed in 2% OsO_4 (28) and dehydrated in alcohol by the same technique as used for light microscopy, passed into propylene oxide and embedded in Epon (33). Thin sections (< 0.1 μ) were stained with lead citrate (34) and uranyl acetate (35). These preparations were observed on a Philips EM-200 electron microscope. Thick sections (1 μ) were stained with toluidine blue (36) for purposes of orientation and comparison with electron micrographs.

CHAPTER III

OBSERVATIONS

The cephalic portion of a chick embryo of 24 hours incubation age (30,37) consists of medullary plate, head process (notochordal primordium) and the more ventrally located entoderm (figs. 10,5). The histochemical reactions are largely negative for all procedures in the immediate proximity of the head process. However, a faint color reaction for the Hale colloidal iron and alcian blue is present along the basilar surface of the ectoderm (fig. 5). Electron microscopy reveals small microfibrils between the medullary plate and the notochordal primordium. The fibrils are distinct along the boundary membrane of the medullary plate and a few fibrils span the tissue space linking the head process and the medullary plate (fig. 11).

An embryo of 40 to 44 hours incubation age possesses a closed neural tube and well delineated notochord at the cephalo-caudal level used in this study. The perinotochordal area is as yet a cell free area devoid of any sclerotomal elements (fig. 12). The PAS stain shows a faint pink color restricted to the area of contact of the notochord with the entoderm. The alcian blue and Hale colloidal iron stains give similar results (fig. 6). Methenamine silver and metachromatic toluidine blue are still negative. Fine structure in this area consists of a narrow band of moderate microfibrillar population close

to the surface of the notochord (fig. 13). Interstitial bodies are prevalent in the immediate proximity of the notochordal boundary membrane (38).

The notochordal area of the three day embryo shows strong positive histochemical reactions for all procedures (figs. 1-4, 14). PAS, colloidal iron, alcian blue, metachromatic toluidine blue and methenamine silver all give their characteristic chromatic reactions. The reactions tend to be uniform in an area restricted to the notochordal sheath (39). Extensions of color positive material radiate out from the notochord into the perinotochordal region, but have no counterpart in electron microscopy. However, a dense microfibrillar population is evident immediately around the notochord (fig. 15). Although microfibrils extend further out into the surrounding tissue space, their pattern of organization does not correspond to that of the color positive materials (figs. 7,16).

At four days incubation age the secondary mesenchyme (39,40) migrates into closer relationship with the notochord (fig. 17). The histochemical reactions, although definitely positive, now diffuse into the adjoining extracellular areas. PAS, Hale colloidal iron, alcian blue, metachromatic toluidine blue and methenamine silver (fig. 17) remain strongly positive immediately adjacent to the notochord. With the exception of the PAS and methenamine silver, chromatic reactions appear interspersed among the surrounding mesenchymal elements (fig. 8). Neither Mallory's nor Weigert's stain show any indications of collagen or elastin at this stage. Examination of fine structure around the

notochord and adjacent areas reveals a dark line a short distance from the notochord made up of heavily packed microfibrils (fig. 18). The space between the notochordal boundary membrane and the intertwined microfibrillar line is relatively empty of microfibrils suggesting an outward movement of the microfibrils from the notochord (fig. 19). The extracellular spaces between the mesenchymal cells shows a moderate microfibrillar population (fig. 18).

Embryos of six days incubation age give positive staining reactions in the perinotochordal and precartilage areas. Alcian blue (fig. 9), Hale colloidal iron and metachromatic toluidine blue elicit pronounced chromatic reactions in both the perinotochordal and precartilage areas. PAS gives a weak reaction in the precartilage area. Methenamine silver responds to a limited perinotochordal region. Mallory's connective tissue stain and Weigert's elastin stain give only vague responses in the area of the common carotid artery. Electron microscopy shows a dense microfibrillar growth throughout the perinotochordal and precartilage tissue space (fig. 20). Microfibrils are seen in close relationship with chondroblasts as well as with the notochord. Areas relatively free of microfibrils completely or partially surround certain of the chondroblasts (fig. 21).

Connective tissue elements possessing the staining characteristics of mature tissue appear at ten days incubation age. Mallory's connective tissue stain and Weigert's elastin stain give definitely positive reactions for collagen and elastic fibers in the common carotid artery (figs. 22,23).

CHAPTER IV

DISCUSSION

The results of the correlated histochemical and fine structural investigation presented in this paper provide additional information about the origins of connective tissues. This study emphasizes two points: 1) the time at which polysaccharides, mucoproteins and mucopolysaccharides become demonstrable in the developing embryo and, 2) the relationship of this histochemical development to early extracellular connective tissue fibrils.

Acid mucopolysaccharides in small amounts are present during the second day of incubation, although the distribution is limited to the ventral surface of the notochord (fig. 6). At this time there is a conspicuous lack of sulfated acid mucopolysaccharides as revealed by the negative results achieved with metachromatic toluidine blue. Mucopolysaccharide concentration increases during the third day, when a definite and consistent distribution of mucoproteins and mucopolysaccharides is present close to the notochordal sheath (fig. 7).

Electron microscopy reveals that a demonstrable microfibrillar concentration precedes the histochemical response by about one day. In embryos of two days incubation, microfibrils exist in moderate numbers (8). It is not until the third day of incubation, after the microfibrils form a dense tangle, that histochemical response is

definite. It is unlikely that the discrepancy between fibrillar and histochemical development is due to insufficient sensitivity of the methods (20), since distinct differences in distribution of fibrils and chromatic responses soon become clear. By the third day conspicuous color positive radiations are noted extending from the notochord into the perinotochordal region. Electron microscopy reveals no fibrillar counterpart in comparable patterns. Later, the observed fibril-free moats or capsules (41) surrounding early chondroblasts of six day old embryos are heavily stained along with the rest of the fibril-rich matrix. Chromatically, there is no distinction between fibril-rich and fibril-free areas. Polysaccharides, mucoproteins and mucopolysaccharides therefore appear to be interfibrillar components even in the early stages of fibrillogenesis.

The close anatomical relationship between microfibrils and unit collagen fibrils as noted by Jakus (1), Karrer (3,4), Haust (9), Low (7,8), Anderson (42) and others, is indirectly supported by this study. Mucoproteins, glycogen and acid mucopolysaccharides, all of which are characteristic of adult connective tissues, are found early in embryological development. Furthermore, they exist in close association with microfibrils and small unit collagen fibrils. This occurs in the perinotochordal area in which a period of continuous growth later gives rise to the cartilaginous model of the vertebral body. It is interesting to note that the secondary mesenchyme that invades this area (40) gives rise to microfibrils which are

indistinguishable from those immediately surrounding the notochord (figs. 20,21). These fibrils are the precursors of the unit collagen fibrils that later occupy the matrix of the mature cartilage. In the six day old embryo, acid mucopolysaccharides and their sulfated derivatives, both of which are essential to developing cartilage, are concentrated in this area. This is indicated by strongly positive staining with alcian blue (fig. 9) and metachromatic toluidine blue. It is only later, at about ten days incubation age, that the precartilage model begins to react positively with Mallory's connective tissue stain. This sequence of events makes it difficult to escape the impression that microfibrils and unit collagen fibrils are related in a continuous line of development.

In summary, the evidence presented in this paper indicates that polysaccharides, mucoproteins and mucopolysaccharides play a significant part in early fibrillogenesis in the ontogeny of the chick. Their development is largely concurrent with development of microfibrils, but chromatically positive areas have been shown not to coincide exactly with the fibrillar component. The persistent correlation of histochemistry and fibrillogenesis in later embryos tends to support the unitary hypothesis of extracellular fibrillogenesis.

LEGEND TO FIGURES

A - aorta
BM - boundary membrane
CB - chondroblast
CC - common carotid artery
CF - collagen fibers
DA - dorsal aorta
EF - elastic fibers
ECT - ectoderm
ENT - entoderm
F - fibrils
FF - fibril-free
HP - head process
MES - mesenchyme
MF - microfibrils
MP - medullary plate
N - notochord
NT - neural tube
PN - perinotochordal
R - radiations
RBC - red blood cells
SC - spinal cord
SCLER - sclerotome
SM - secondary mesenchyme
V - vacuole

PLATE I

These light micrographs illustrate chromatic reactions on the third day of incubation (stage 16).

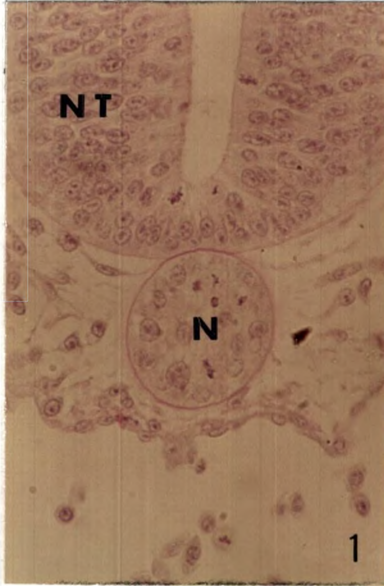
Figure 1. Periodic acid-Schiff (PAS). The pink to magenta color around the notochord (N) is indicative of the presence of mucoproteins and polysaccharides. Paraffin, counterstained with Mayer's hemalum. 450X.

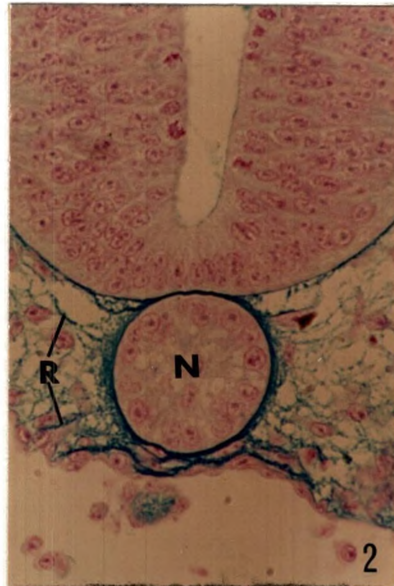
Figure 2. Hale colloidal iron. Acid mucopolysaccharides cause the dark blue stain which surrounds the notochord (N) in this section. Color positive radiations (R) extend from the notochordal sheath. These are not matched in the microfibrillar pattern (compare with figs. 15,16). Paraffin, counterstained with nuclear fast red. 450X.

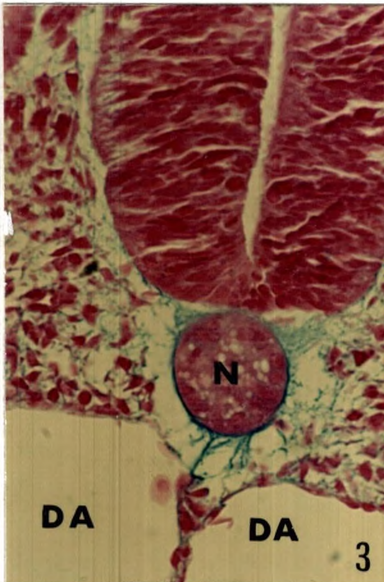
Figure 3. Alcian Blue. The light blue color in the notochordal sheath is characteristic of acid mucopolysaccharides. Paraffin, counterstained with nuclear fast red. 300X.

Figure 4. Metachromatic toluidine blue. Metachromasia is seen as a pink color surrounding the notochord (N). This response indicates the presence of sulfated acid mucopolysaccharides. Paraffin, no counterstain. 750X.

PLATE I







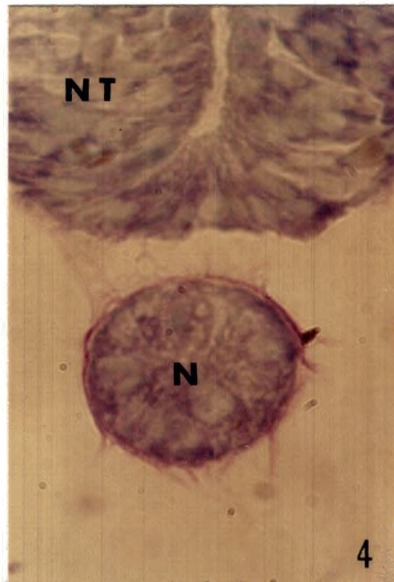


PLATE II

These light micrographs illustrate the development of mucopolysaccharides from 24 hours incubation to the sixth day as seen after staining with alcian blue. All figures are from paraffin sections counterstained with nuclear fast red.

Figure 5. Twenty-four hours of incubation; stage 7. The head process (HP), medullary plate (MP) and entoderm (ENT) are visible. Arrows indicate a faint positive reaction beneath the ectoderm (ECT). 400X.

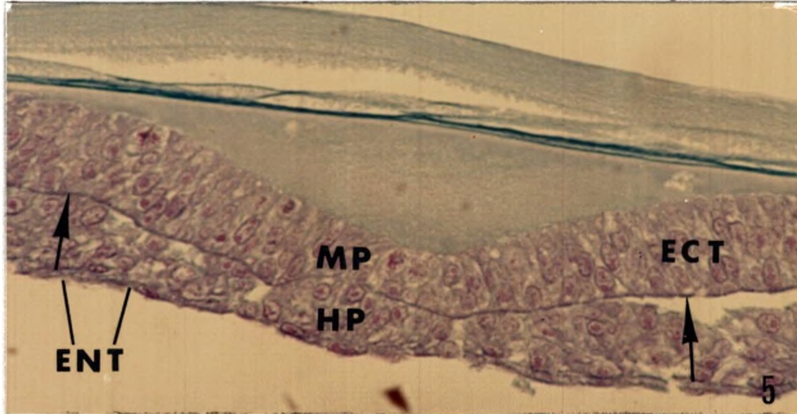
Figure 6. Forty-four hours of incubation; stage 11. The neural tube (NT) and notochord (N) are visible. The distinct positive reaction is conspicuous on the ventral surface of the notochord (arrows). 400X

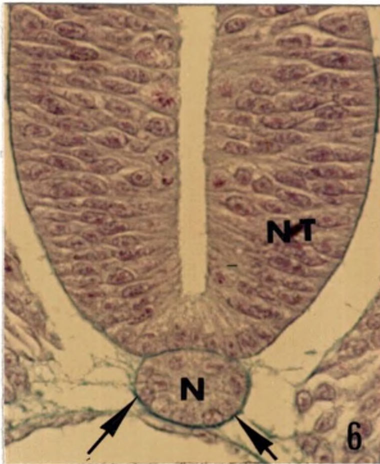
Figure 7. Third day of incubation; stage 11. A definite chromatic reaction is visible in the notochordal sheath. Color positive radiations (R) extend from the notochord (N). These are not matched in the microfibrillar pattern. (Compare with figs. 15,16). 300X

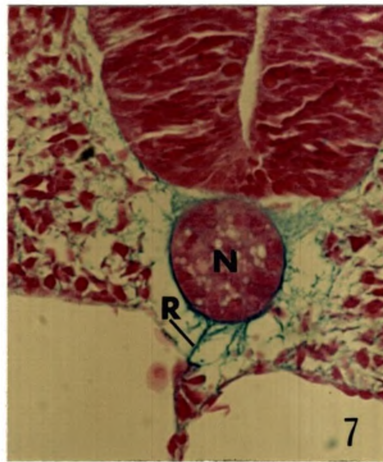
Figure 8. Fourth day of incubation; stage 20. A chromatic reaction is visible around the notochord (N), but is diffuse in the surrounding secondary mesenchyme (SM). 200X

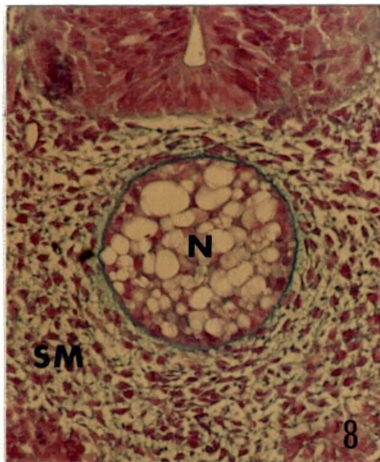
Figure 9. Sixth day of incubation; stage 29. Spinal cord (SC) and notochord (N) are visible in this cross-section. The precartilage matrix is heavily stained. 200X.

PLATE II









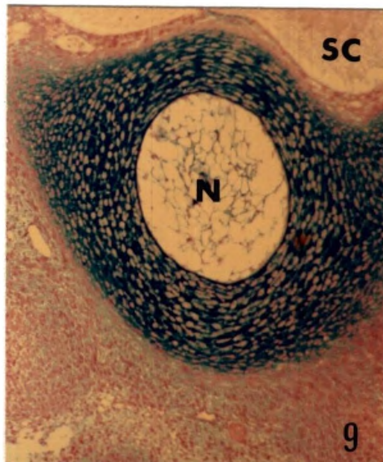


PLATE III

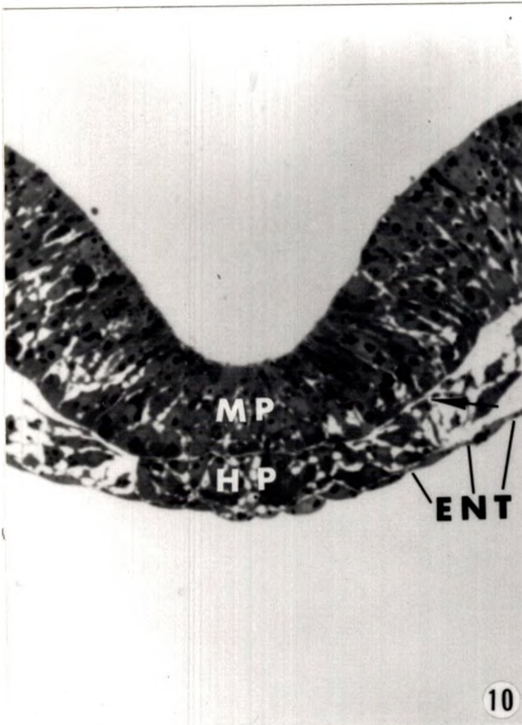
Figure 10. Twenty-four hours of incubation; stage 7. In this cross-section of a 24 hour embryo, the medullary plate (MP), head process (HP) and entoderm (ENT) are visible. The location of the field in figure 11 is indicated by an arrow. Epon, toluidine blue. 300X.

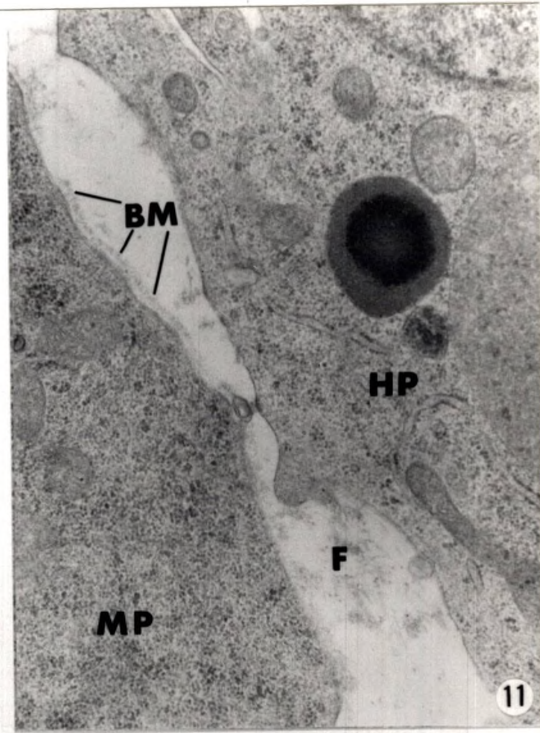
Figure 11. Medullary plate and head process (24 hours of incubation; stage 7). Fibrils (F) are present between the head process (HP) and the medullary plate (MP). This area remains largely negative for mucoproteins and mucopolysaccharide stains. Epon, lead citrate and uranyl acetate. 19,000X.

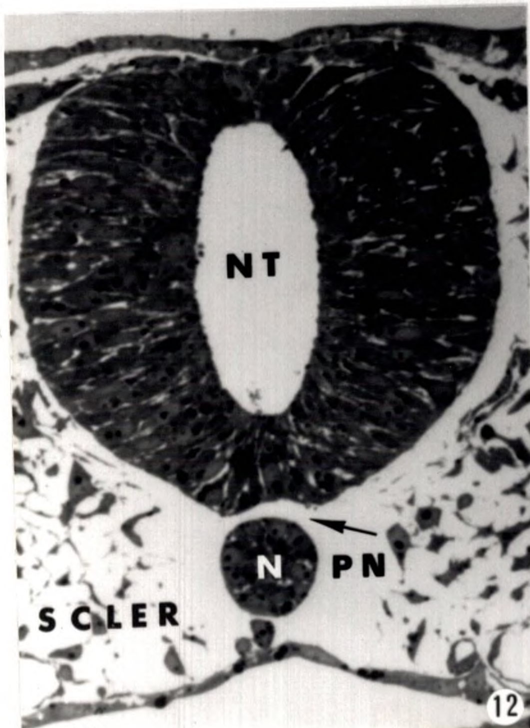
Figure 12. Forty-four hours of incubation; stage 11. The neural tube (NT) and notochord (N) are well formed. The perinotochordal area (PN) remains free of sclerotomal elements (SCLER). The location of the field in figure 13 is indicated by an arrow. Epon, toluidine blue. 300X.

Figure 13. Neural tube and notochord (44 hours of incubation; stage 11). An area of moderate microfibrillar growth (MF) is present in the perinotochordal area between the notochord (N) and the neural tube (NT). Interstitial bodies are found close to the notochordal surface (arrows). Epon, lead citrate and uranyl acetate. 5000X.

PLATE III







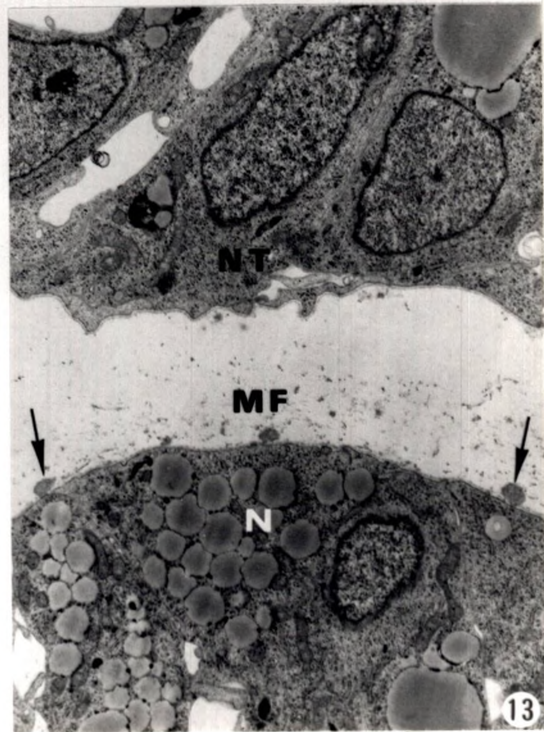


PLATE IV

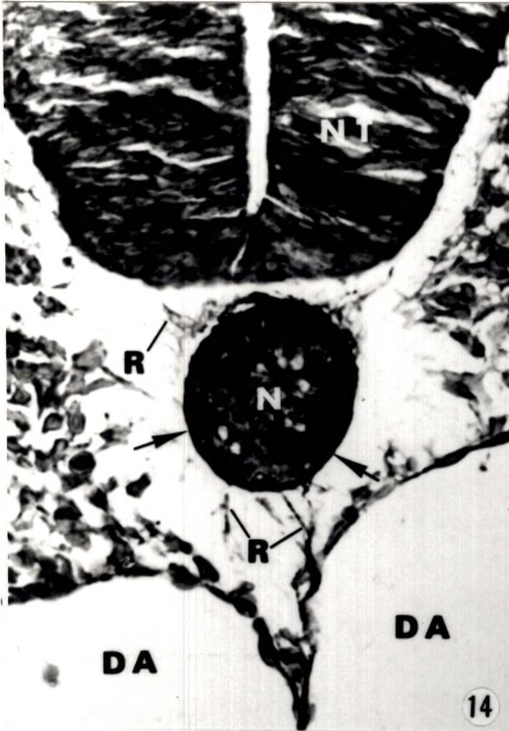
Figure 14. Third day of incubation; stage 16. The neural tube (NT), notochord (N) and dorsal aortae (DA) are visible in this light micrograph. Methenamine silver leaves its characteristic black deposit in the area of the notochordal sheath (arrows). Silver positive radiations (R) extend into the adjacent extracellular space (compare with fig. 16). Paraffin, methenamine silver and Mayer's hemalum. 250X.

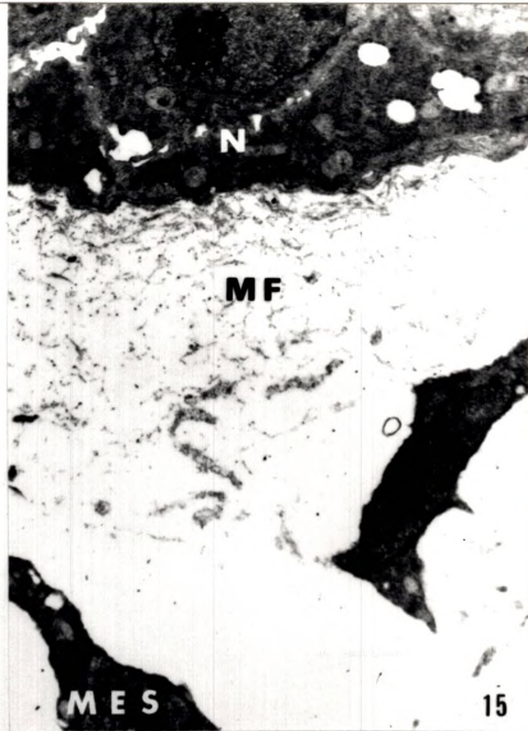
Figure 15. Notochord and mesenchymal elements (third day of incubation; stage 16). The notochord (N), microfibrils (MF) and mesenchymal cells (MES) are evident. The microfibrils (MF) exist in dense concentration throughout the perinotochordal region (compare with figs. 1-4). Mesenchymal elements are beginning to migrate toward the notochord. Epon, lead citrate and uranyl acetate. 6400X.

Figure 16. Edge of notochord (third day of incubation; stage 16). Microfibrils (MF) extend into the perinotochordal area, but they do not match the silver positive radiations (R) of figure 14. Compare also with the chromatic radiations (R) of figures 1-4. The notochordal boundary membrane (BM) is quite evident in this field. Epon, lead citrate and uranyl acetate. 270X.

Figure 17. Fourth day of incubation; stage 20. The area positive for methenamine silver is restricted to the notochordal sheath (arrows). No reaction product is to be found in the secondary mesenchyme (SM), which now completely encloses the notochord (N). Paraffin, methenamine silver and Mayer's hemalum. 10,000X.

PLATE IV





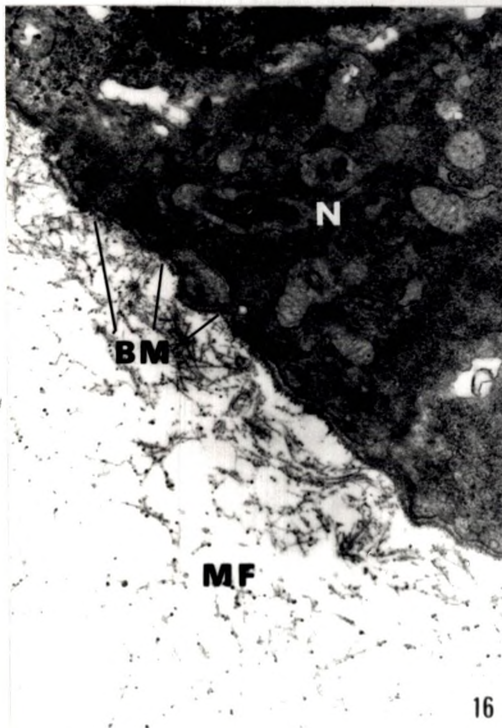


PLATE V

These electron micrographs illustrate the perinotochordal microfibrillar population at stages when light microscopy reveals distinct positives for mucopolysaccharides and mucoproteins.

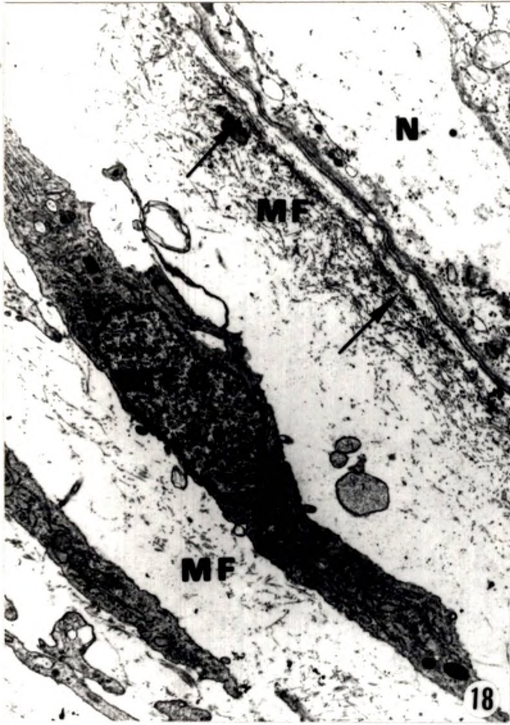
Figure 18. Notochordal edge and secondary mesenchyme (fourth day of incubation; stage 20). Microfibrils (MF) appear at the edge of the notochord (N) forming a dark line (arrows). The microfibrils, well distributed between mesenchymal elements, indicate diffuse fibrillogenesis (compare with fig. 8). Epon, lead citrate and uranyl acetate. 6600X.

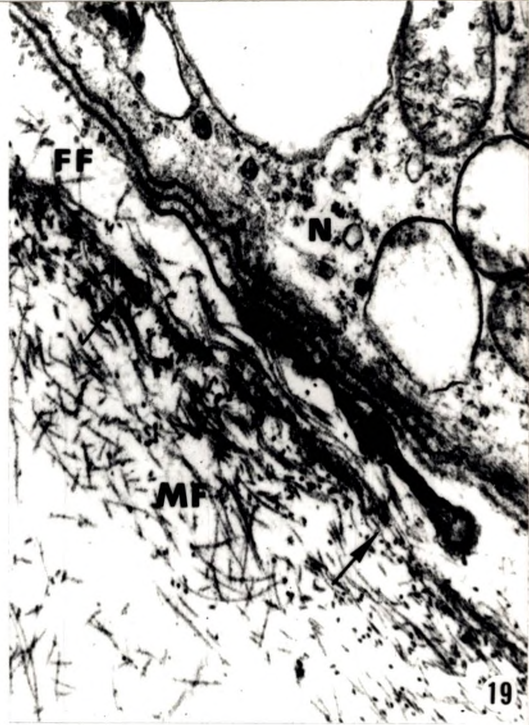
Figure 19. Fibril-free space at notochordal edge (fourth day of incubation; stage 20). A dense tangle of microfibrils (MF) appears along a line a short distance from the notochord (arrows). The relatively fibril-free space (FF) may be due to an outward movement of older microfibrils. Epon, lead citrate and uranyl acetate. 28,000X.

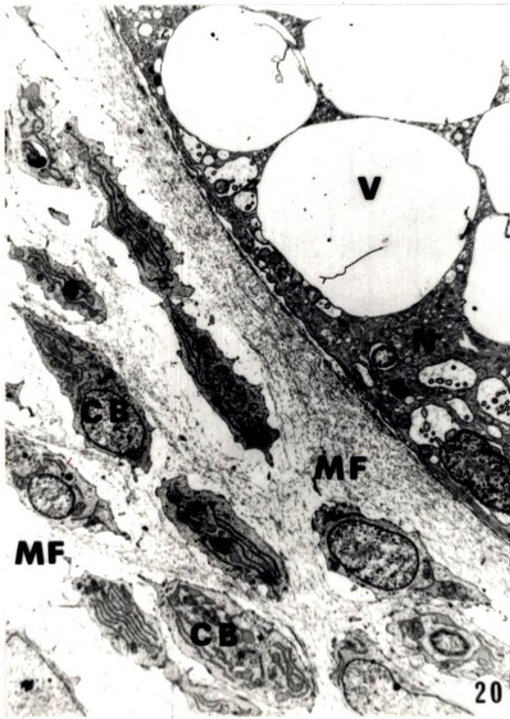
Figure 20. Sixth day of incubation; stage 29. Chondroblasts (CB) and microfibrils (MF) are the principal inhabitants of the precartilage area. By this time large degenerative vacuoles (V) are conspicuous in the notochord (N). Epon, lead citrate and uranyl acetate. 3800X.

Figure 21. Capsules free of microfibrils around chondroblasts (sixth day of incubation; stage 29). Chondroblasts (CB) throughout the precartilage matrix are in close relationship with microfibrils (MF). There are frequently capsules devoid of microfibrils either completely or partially surrounding these cells (arrows). A fibril-free (FF) area around the notochord corresponding to that noted in figures 18 and 19 is conspicous. The area peripheral to this is heavily populated with microfibrils (MF) and closely resembles the fibrillar population existing between the chondroblasts. Epon, lead citrate and uranyl acetate. 5300X.

PLATE V







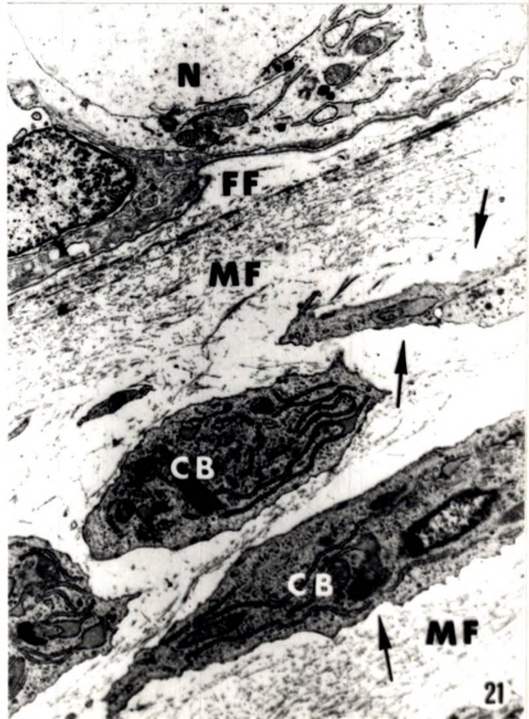
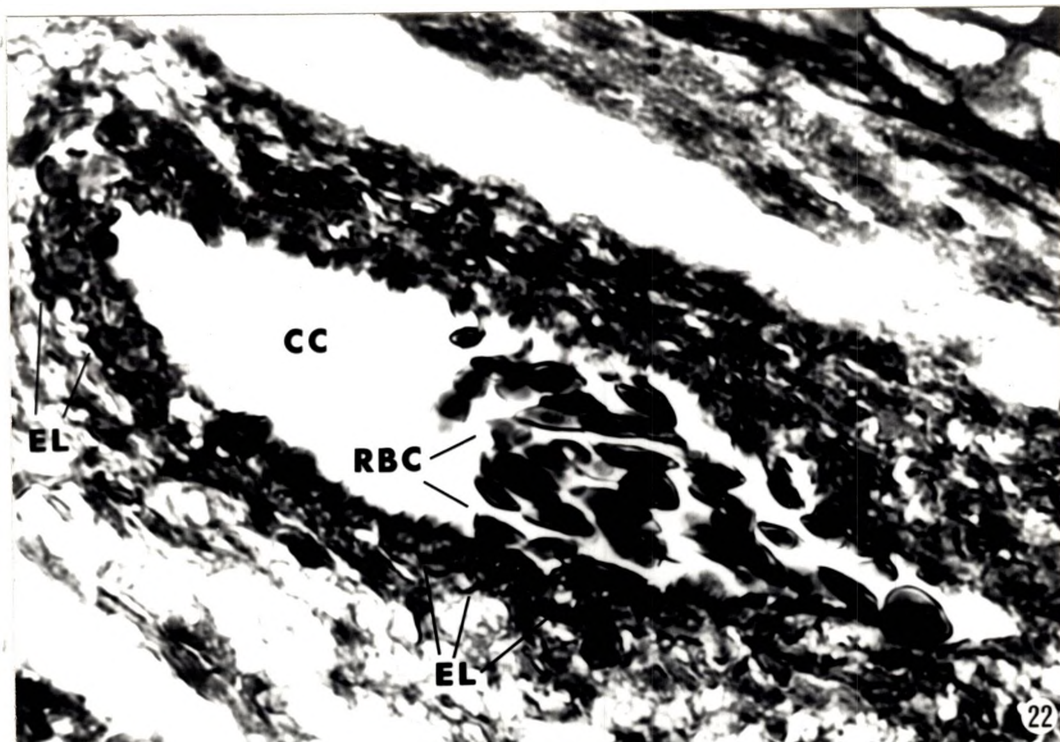


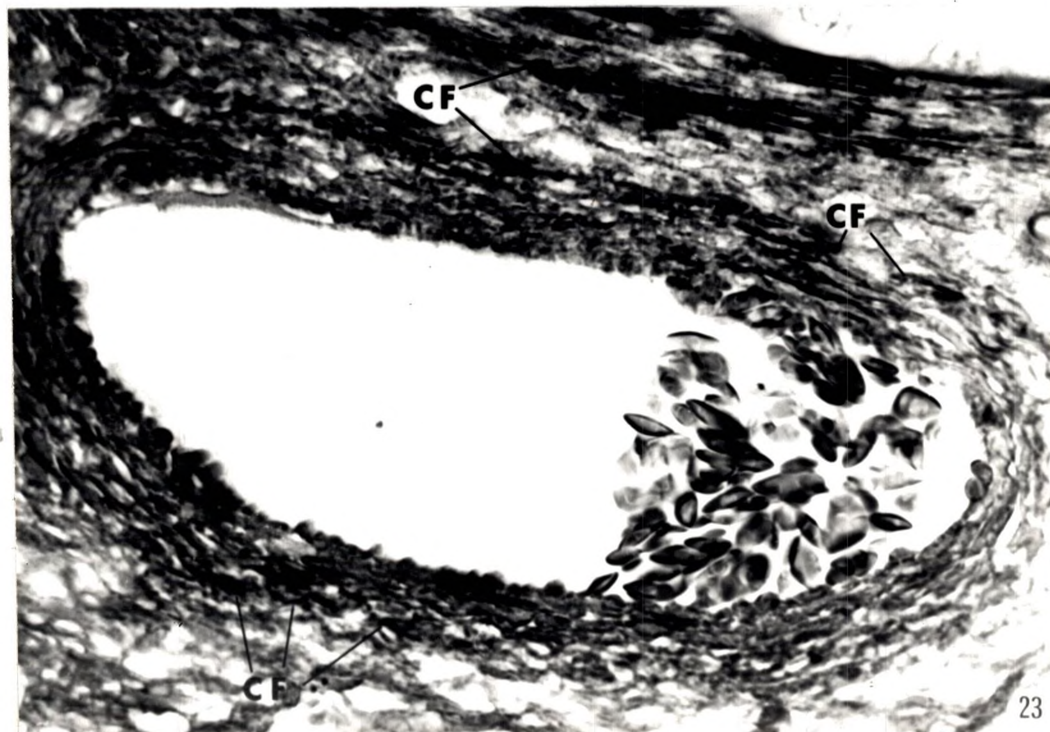
PLATE VI

Figure 22. Elastic Fibers (tenth day of incubation; stage 36). Elastic fibers (EF) stain positively with Weigert's resorcin-fuchsin in the wall of the common carotid artery (CC). This is the first demonstrable indication of mature connective tissue elements in this area. Red blood cells (RBC) are visible in the vessel lumen. Paraffin. 600X.

Figure 23. Collagen fibers (tenth day of incubation; stage 36). Small collagen fibers (CF) stain light blue in the outer layers of the arterial wall. This represents the earliest positive collagen stain in these areas. Small wisps of positive material may be observed at this stage in between the chondroblasts and along the edge of the future vertebral body. Paraffin, Mallory's collagen stain. 500X.

PLATE VI





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