1979

Extracellular Macromolecules in Regenerating Sabellids: A Histochemical Study

Michael R. Bigelow
Medical University of South Carolina

EXTRACELLULAR MACROMOLECULES IN REGENERATING SABELLIDS: A HISTOCHEMICAL STUDY

by

Michael R. Bigelow

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Master of Science in the College of Graduate Studies.

Department of Anatomy

1979

Approved by:

Timothy Fitzharris
Chairman, Advisory Committee

Donald R. Wilbur

TABLE OF CONTENTS

I. INTRODUCTION 1

II. LITERATURE REVIEW 3

Role of Glycosaminoglycans in Development 3
Role of Collagen in Development 10
Actions of Agents Inhibiting Extracellular Material Biosynthesis 13
Aspects of Sabellid Regeneration 15

III. MATERIALS AND METHODS 17

IV. RESULTS 21

V. DISCUSSION 26

VI. SUMMARY 33

VII. FIGURES 36

VIII. LIST OF REFERENCES 49

ABSTRACT

MICHAEL R. BIGELOW. Extracellular Macromolecules in Regenerating Sabellids: A Histochemical Study. (Under the direction of Timothy Fitzharris, Ph.D.)

Regenerating sabellid polychaetes were histochemically studied with regard to the synthesis and deposition of glycosaminoglycans (GAG) and collagen. Both of these types of macromolecules are known to be actively involved in cell migrations and differentiations of several vertebrate systems. The purpose of this study was to investigate whether or not these extracellular materials might be similarly involved in invertebrate systems. Results demonstrate for the first time that these same extracellular materials, GAG and collagen, actively participate during invertebrate development. Production of hyaluronic acid is shown to accompany mesenchymal migration and blastema formation. Deposition of sulfated GAG and collagen in the form of an epithelial basal lamina and cartilaginous matrix mark differentiation of epithelial and mesenchymal cell types.

The effects on normal sabellid development of 6-aminonicotinamide (6-AN), an inhibitor of GAG synthesis, and α,α'-dipyridyl and β-aminopropionitrile (βAPN),

inhibitors of collagen synthesis, were investigated. α,α'-Dipyridyl at 10^{-4} M and β-APN at 10^{-3} M were effective at stopping regeneration at all stages. 6-AN at 10^{-5} M was effective at blocking formation of regenerate blastemas, however no effect was observed after completion of blastema formation.

Possible mechanisms for the developmental role of these extracellular macromolecules are also discussed.

INTRODUCTION

The importance of tissue interaction during development has long been recognized. The dependence of one tissue's normal development upon another is a phenomenon whose exact mechanisms remain elusive. However, it has become increasingly evident that the extracellular materials produced by developing tissues have an important and active involvement in determining the fate of these tissues.

From the very onset of gastrulation, extracellular localization of glycosaminoglycans and collagen can be shown in association with the primitive streak, the mesenchymal cells migrating through it, and as components of the basal lamina of the epiblast and hypoblast. Deposits of these same extracellular materials are found between the chordamesoderm and the overlying neural plate during induction of neural tube formation and they subsequently accumulate around the notochord and the neural tube during the time they are thought to induce migration, condensation, and chondrification of the sclerotome into the axial skeleton. Furthermore, glycosaminoglycans and collagen are associated with the lens capsule during differentiation of the overlying corneal epithelium and their presence in the normal development of the salivary gland, kidney, heart, lung, and brain has been shown to be essential. Regeneration of limbs by lower vertebrates provides another developmental mental model which demonstrates the

involvement of glycosaminoglycans and collagen with the processes of tissue reorganization including cell proliferation, migration, and differentiation.

Similarities among these systems extend beyond the character of the extracellular materials produced, to the sequence in which they appear and the morphological events with which they are associated. Hyaluronate production can be correlated with swelling of the extracellular spaces and the movement of mesenchymal cells into these spaces. Its removal by hyaluronidase and a shift to the production of sulfated glycosaminoglycans accompanies condensation of the migrating cells and establishment of a basal lamina by the apposing epithelium. The mesenchymal contribution of collagen to the extracellular matrix as well as to the basal lamina has also been demonstrated.

This study was proposed to investigate the role of these materials in invertebrate development. The extracellular materials produced during regeneration of sabellid polychaetes Branchiomma nigromaculata and Sabella melanostigma were histochemically localized and identified. The effects of known inhibitors of glycosaminoglycan and collagen synthesis on the development of these polychaetes were also investigated. Through these methods the extracellular materials produced in this invertebrate system were characterized and compositional changes within the extracellular matrix were correlated with the different stages of regeneration. Possible roles for the involvement of these materials during development are proposed.

LITERATURE REVIEW

Role of Glycosaminoglycans in Development

Glycosaminoglycans (GAG) are polyanionic macromolecules composed of repeating disaccharide units. With the possible exception of hyaluronic acid, they exist in tissue covalently bound to a protein core in the form of proteoglycans. Their involvement in a number of developmental systems has been investigated.

In the chick embryo GAG appear early in development. Trelstad et al. (1967) demonstrated deposition of basal lamina material on the undersurface of the epiblast during gastrulation. This observation correlated with autoradiographic demonstration of S^{35} incorporation associated with the primitive groove and Hensen's node by definitive primitive streak stage (Johnston and Comar, 1957). Hay (1973) also demonstrated increased S^{35} deposition between the chordamesoderm and the overlying neural plate as the basal lamina under the epiblast becomes continuous and neurulation begins.

Kosher and Lash (1975) effectively demonstrated the role of notochordal basement membrane and its GAG components in stimulating chondrogenesis of somitic mesoderm. Although it had long been known that the embryonic notochord induces chondrogenesis of the axial skeleton (Grobstein, 1954; Lash, 1968), the inductive effect was demonstrated to be due specifically to the extracellular materials of the perinotochordal sheath. Treatment of excised notochords by enzymes which selectively degrade GAG eliminated the

inductive ability of the notochord and its inductive potential was restored concomitantly with the resynthesis and secretion of GAG into the perinotochordal sheath. This effect appears not to be specific for notochord basement membrane. O'Hare (1972) showed that various ectodermal epithelia were capable of stimulating chondrogenesis in somite explants. However, this effect was dependent upon the presence of basement membrane materials associated with the various epithelia, and removal of these materials following trypsinization eliminated their inductive capacity.

In the developing chick eye, the basal lamina of the lens, the lens capsule, has been similarly implicated as an inducer of corneal stroma production by the corneal epithelium. Hay and Meier (1974) demonstrated GAG incorporation into lens capsule at the time that it is thought to induce the overlying corneal epithelium. Meier and Hay (1974b) showed that, when added to the incubation medium, GAG (chondroitin sulfate, heparin and heparin sulfate) stimulate a twofold increase in the in vitro synthesis of similar GAG by the corneal epithelium.

This effect of GAG stimulating their own production is not unique to the corneal epithelium. The stimulatory effect of perinotochordal GAG on chondrogenesis is expressed by an increased production by the sclerotome of similar sulfated GAG. GAG production by chondrocytes in suspension or in culture can also be stimulated by addition of exogenous chondromucoprotein (Kosher et al., 1973).

The positive feedback of sulfated GAG discussed above is one observed aspect of their role in development. Another is the requirement for GAG contribution to basal lamina during epithelial-mesenchymal interactions. Although basement membrane requirements for the inductive potential of notochord and lens have been discussed, the interface between epithelium and mesenchyme has been shown to be crucial in several other developing systems (Grobstein, 1967). Although early investigations focused on the collagenous component of the basement membrane (Hay and Revel, 1963; Grobstein and Cohen, 1965, Wessells and Cohen, 1968; subsequent investigations (Kallman and Grobstein, 1966; Hay and Meier, 1974, Banerjee et al., 1977; have demonstrated sulfated GAG as intrinsic components of basement membranes and actively involved in maintaining epithelial morphology.

Evidence for the importance of sulfated GAG in developing systems has been presented. However, it should be noted that in the early chick embryo as much as 90% of the GAG can be accounted for as hyaluronic acid (Solursh, 1976). From formation of the primitive streak to the four somite stage, during formation of the primary mesenchyme, hyaluronate production has been estimated at nine times that for sulfated GAG.

Considerable evidence has been presented correlating sulfated GAG production with establishment of basement membranes, and the involvement of these basement membranes in induction. However, the resulting morphological events

including cell migrations and subsequent differentiations are dramatically correlated with hyaluronate production and subsequent removal by hyaluronidase. During axial skeleton development production of hyaluronate is twice that of chondroitin sulfate during migration of sclerotome preceding cartilage formation as determined by isotope labelling and differential enzyme degradation methods of Toole and Gross (1971). Hyaluronidase activity and deposition of cartilage matrix become detectable at the same time and hyaluronate production drops to 60% of that for chondroitin sulfate, and continues to drop through subsequent stages (Toole, 1972). These results confirmed earlier biochemical and histochemical determinations of the same region (Kvist and Finnegan, 1970). Toole (1972) also examined relative GAG production, hyaluronidase activity and their correlation with chondrogenesis in the developing limb buds of chicks. Although chondroitin sulfate was the predominant GAG produced at all stages, hyaluronate production dropped precipitously with the concomitant appearance of cartilage matrix and hyaluronidase activity in the limb buds. In vitro, hyaluronate has been shown to impair drastically nodule formation in explanted chick somite cultures (Toole, 1973). Solursh et al. (1974) further demonstrated the inhibitory effect of hyaluronate on both sulfated GAG synthesis and matrix deposition. This inhibition was found in response to both polymeric hyaluronate and oligosaccharide fragments. The finding by Toole (1976) that

the inhibition was effected when somite cells exposed to hyaluronate were washed and cultured in hyaluronate-free medium, suggests that hyaluronate may act by binding to cell surfaces.

In the case of corneal development in the chick, hyaluronate production has been correlated with swelling and mesenchymal invasion of the primary stroma and hyaluronidase activity with subsequent compaction and dehydration of the stroma and differentiation of corneal fibroblasts (Toole and Trelstad, 1971). Trelstad et al. (1974) subsequently demonstrated corneal endothelium as the sole initial source of hyaluronate at the time the primary stroma suddenly swells, with subsequent contribution from the invading mesenchymal cells.

The developing embryonic heart is another system in which GAG have been shown to play a significant role during morphogenesis. Both sulfated and unsulfated forms of GAG have been identified in this developing organ (Markwald and Adams Smith, 1972; Manasek et al., 1973). Orkin and Toole (1978) demonstrated that hyaluronate is present at concentrations similar to those found around the notochord and in the primary stroma of the cornea during mesenchymal cell invasion, and that the level of hyaluronate declines at a faster rate than that of other GAG. They further demonstrated hyaluronidase activity corresponding to the time mesenchymal cells invade the cardiac jelly.

The involvement of hyaluronate and hyaluronidase in the developing brain has also been investigated. Developing chick brains have been shown to contain increased levels of

GAG correlating with higher water content (Polansky et al., 1974; Margolis et al., 1975) and higher hyaluronidase activity (Polansky et al., 1974) when compared with mature brain. Although brain development cannot be separated into distinct phases of cell migration and cell differentiation, but consists rather of a series of overlapping waves of neuronal migrations and differentiations, hyaluronate and hyaluronidase levels as well as water levels are high during embryonic development when these series of migrations are taking place and drop sharply after hatching at which time most of these neuronal migrations have ceased.

The developing brain also demonstrates the relationship between hyaluronidase and thyroxine (see Toole, 1976, for review). Studies in hypothyroid rats correlate lack of thyroxine with abnormal differentiation of Purkinje cells and delayed migration of external granule cells in the cerebellum (Hamburgh et al., 1971; Legrand, 1971). Finding continued mitosis in these non-migrating cells, Hamburgh postulated that thyroxine triggers cessation of cell proliferation and initiation of migration. Gona (1973) later demonstrated that administration of thyroxine induces the formation and subsequent migration of the cells of the external granular layer in the cerebellum of bullfrog tadpoles. Toole (1976) reports a doubling of hyaluronidase activity in response to thyroxine in these tissues.

Thyroxine has also been shown to promote dehydration of the corneal stroma and the onset of transparency (Coulombre and Coulombre, 1964) which has subsequently been correlated

with high hyaluronidase activity and falling hyaluronate levels (Toole and Trelstad, 1971). Toole (1973) has shown reversal of the inhibition of hyaluronate on in vitro chondrogenesis by thyroxine and other adenylate cyclase activating hormones.

Toole and Gross (1971) investigated GAG synthesis in another morphologically developing system. During the process of regeneration of amputated newt limbs, synthesis of hyaluronate, chondroitin sulfate, and hyaluronidase activity occur in familiar sequence. As dedifferentiated mesenchymal cells migrate from the initial wound site to form a loosely packed blastema core in the developing bud, hyaluronate synthesis, measured by H^3-acetate incorporation, is at a maximum. As formation of a critical blastemal mass is attained, hyaluronidase activity becomes detectable and hyaluronate synthesis drops sharply. These events are correlated with increased chondroitin sulfate synthesis, which subsequently reaches a maximum as cartilage formation becomes prominant.

Another property of GAG involves interaction with collagen, another component of cartilage matrix. Toole and Lowther (1968) demonstrated that chondroitin sulfate-proteoglycan precipitated out collagen fibrils from tropocollagen solutions. Proteoglycans, isolated from chick embryo limb buds prior to chondrogenesis, have been shown to be of smaller molecular weight and to express a lower reactivity with collagen than do those of cartilage (Goetinck et al., 1974. Toole and Linsenmayer, 1975).

The Role of Collagen in Development

Another major component of extracellular matrix which has been implicated as having a significant role in development is collagen. This universal component of connective tissue has long been known to serve as a preferred substrate for cells grown in culture (Ehrmann and Gey, 1956). However, it has become evident that the role of collagen in development is far more complex than serving simply as structural support for connective tissue or as substrate or scaffolding for cell mobility. Konigsberg (1970) demonstrated that the conditioned medium factor which allowed for muscle fiber differentiation in vitro could be replaced by collagen. This finding provided direct evidence that collagen promotes differentiation. Studies of developing epithelio-mesenchymal explants (Grobstein and Cohen, 1965; Wessels and Cohen, 1968) demonstrated the requirement for collagenous basal lamina in maintaining epithelial morphology, and the ability of mesenchyme to re-establish the morphology of the collagenase-treated epithelium. Using H^3-proline incorporation techniques, Kallman and Grobstein (1965), demonstrated mesenchymal contribution as the major source of collagen to the basal lamina of salivary gland explants. This observation was confirmed by Banerjee et al. (1977). The early patches of basal materials which form under the epiblast during migration of primary mesenchyme and the continuous basal lamina which is established preceding neurulation also contain collagen (Hay, 1973).

The inductive properties of the notochord discussed earlier can be correlated with the synthesis of collagen as well as GAG (Cohen and Hay, 1971; Trelstad et al., 1973). The collagen synthesized by the notochord at the time it induces somite chondrogenesis has further been characterized as being composed completely of α1 subunits of type II collagen (Trelstad et al., 1973). Type II collagen and to a lesser degree type I collagen have been shown to promote collagen and proteoglycan synthesis in chondrocyte cultures two to three fold (Kosher and Church, 1975). The collagenous basal lamina of lens has also been demonstrated as having inductive properties (Hay, 1973), and Meier and Hay (1974a) have demonstrated that rat tail collagen or chondrosarcoma collagen may substitute for the lens capsule in promoting differentiation of corneal epithelium as defined by corneal stroma production.

In developing limbs of chick embryos, a sequence of differential collagen synthesis has been correlated with the stages of blastema formation and cartilage differentiation described earlier. Type I collagen has been identified as the sole collagenous component of the developing limb bud prior to cartilage matrix deposition, at which time the differentiated chondrocytes switch over to synthesizing type II collagen exclusively. This observation has been confirmed both biochemically (Linsenmayer et al., 1973; Lash and Vasan, 1978) and immunohistochemically (von der Mark et al., 1976).

In embryonic rat hearts, Crow (1979) demonstrated failure of mesenchymal migration into the cardiac jelly of prospective cardiac cushions as well as aortic aneurisms in rats treated with β-aminopropionitrile, a lathyrogenic agent shown to inhibit normal collagen biosynthesis.

Actions of Agents Inhibiting Extracellular Material Biosynthesis

Another method of investigating the role of extracellular materials is to interfere with their biosynthesis and to examine the effects of such treatment.

6-Aminonicotinamide is a niacin antimetabolite, which has been shown to have teratogenic effects in rats. When administered to rats during the second or third week of pregnancy, a high frequency of neural, ocular, urogenital, skeletal, and vascular anomalies are observed (Chamberlain and Nelson, 1963). Overman and Beaudoin (1971) further demonstrated both *in vitro* and *in vivo* inhibition of S^{35} incorporation into GAG in embryonic rat hearts during that organ's most active period of cardiac jelly production. Mills (1974) subsequently demonstrated aberrant cushion tissue formation in embryonic rats exposed to 6-AN. Abnormalities included atrophy of secretory organelles in mesenchymal cells invading the cardiac jelly and the endocardium from which they arise.

β-Aminopropionitrile (βAPN) is a teratogen long known to cause skeletal and cardiovascular abnormalities in experimental animals (Ponseti and Shepard, 1954). Increased solubility and decreased tensile strength of collagen extracted from treated rats (Martin et al., 1961) and embryonic chicks (Levene and Gross, 1959) indicate

that βAPN inhibits normal cross-linking of collagen molecules without affecting their amino acid composition, i.e. synthesis. The decreased aldehyde content of this lathyritic collagen has led investigators to postulate inhibition of lysyl oxidase as one mode of βAPN's teratogenic action (Page and Benditt, 1972). Page and Benditt (1972) have also demonstrated binding of βAPN and cyanoacetaldehyde (a βAPN metabolite) to collagen.

α,α'-Dipyridyl is a ferrous chelator, which has been shown to inhibit the hydroxylation of both proline and lysine in collagen precursor peptides (Chvapil et al., 1966; Prockop et al., 1966; Hurych and Nordwig, 1967). When incubated in the presence of 1 mM α,α'-dipyridyl, embryonic chick skin and cartilage formed collagen-like proteins containing both proline and lysine but none of their hydroxlyated counterparts. Lysine, incorporated into these proteins in the presence of α,α'-dipyridyl, was subsequently hydroxylated when the chelating agent was removed by dialysis (Prockop et al., 1966).

Aspects of Sabellid Regeneration

Sabellid worms have been found to have a high regenerative capacity. Segments taken from any level of the abdominal region show no difference in either their regenerative rate or potential. These annelids develop regenerate buds within three days and small radioles containing chondrocytic supportive tissue as soon as five days after fragmentation. These buds develop both anteriorly and posteriorly as bilobed blastemas packed with undifferentiated mesenchymal type cells, providing a model system in which the *in vivo* changes occuring in the composition of the extracellular matrix can be studied. Anteriorly this blastema develops into a highly organized regenerate structure, completely replacing the adult branchial crown. Posteriorly, a bilobed pygidium develops and remains. Abdominal segments arise posterior to existing segments, just anterior to the pygidium. The cells comprising the small blastemas of the pygidium remain suspended in a proliferative state and do not themselves exhibit overt differentiation as do those of the anterior regenerate buds. These invertebrates therefore provide two contrasting systems in which the associated extracellular materials can be compared and studied.

The specific purposes of this project were to:

1. identify and characterize the extracellular materials produced in the regenerating sabellids.

2. correlate changes taking place in the composition of the extracellular matrix with morphological events.
3. investigate the effects of known inhibitors of GAG and collagen synthesis on development.

MATERIALS AND METHODS

Marine polychaetes, Sabella melanostigma and Branchiomma nigromaculata, were obtained from Tropical Atlantic Marine Specimens, Big Pine Key, Florida. They were maintained in a ten gallon aquarium of Instant Ocean synthetic sea salts solution, which was equipped with an aerator and undergravel filter. Suspensions of brewers' yeast were periodically added as a diet for these filter feeders, and salinity was maintained at 1.025 sp. gr. by the occasional addition of distilled water. The adult polychaetes appeared to remain healthy and active for three months or longer under these conditions.

Fragments for regeneration were taken from the abdominal portion of healthy adult worms. Fragments of five to eight body segments were used in most cases, although regeneration was observed in fragments of as few as three segments. Regenerating fragments were kept in bowls of Instant Ocean, which was changed daily, at which time the regenerates were cleansed of excess mucus which might serve as a substrate for bacterial infection.

The effects of known teratogenic agents were investigated by allowing experimental animals to regenerate in bowls of Instant Ocean containing the teratogen. 6-Aminonicotinamide at 10^{-5} M, α,α'-dipyridyl at 10^{-4} M, and β-aminopropionitril at 10^{-3} M were used. Exposure to the

teratogens followed 24 hours in Instant Ocean to allow for recovery from trauma and initiation of wound healing.

Fixation of adult and regenerate specimens for light microscopic examination was accomplished by overnight immersion in 10% formalin, buffered with 2% calcium acetate to pH 7.2. After fixation, specimens were dehydrated through a graded ethanol series, cleared in xylene, and embedded in Paraplast II embedding medium. Sections were cut at five microns and mounted on glass slides coated with an albumin film.

I. Alcian Blue Staining

GAG were visualized by reaction with alcian blue 8GX (Allied Chemical Co., Morristown, N.J.) as described by Mowry (1963). Sections were deparaffinized in xylene and rehydrated to distilled water through a graded ethanol series. After tissue sections were exposed for three to five minutes to solutions of either 3% acetic acid (pH 2.5) or 0.1 M HCl (pH 1.0), staining proceeded for 30 minutes in the same solution containing 1% alcian blue, followed by a three-minute rinse in running tap water. Some slides were further stained by the periodic-acid-Schiff's reagent method of McManus (1948). Sections were exposed to 1% periodic acid for 15 minutes, rinsed and placed in Schiff's reagent for ten minutes. Sections were then passed through

three changes of 0.5% sodium metabisulfite for two minutes each, rinsed for five to ten minutes in tap water, dehydrated and cover slips were permanently affixed with a mounting resin.

Alcian blue at pH 2.5 reacts with the negatively charged carboxyl and sulfate radicals of GAG. Reducing the pH of the staining solution to 1.0 results in the reduction of carboxyl radicals, leaving only the sulfate groups to react with the dye. This method permits differentiation between sulfated and non-sulfated GAG.

II. Hyaluronidase Digestion

Sections were deparaffinized and rehydrated to distilled water. Experimental slides were incubated in 0.05% bovine testicular hyaluronidsse (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M phosphate buffer pH 5.5 for four hours at 37^{o} C. Control sections were incubated in buffer alone. Sections were rinsed and stained in alcian blue (pH 2.5) as previously described. Significant reduction of staining in enzyme-treated sections was attributed to the removal of specific hyaluronidase-sensitive materials by the enzyme.

III. Dialyzed Iron - Prussian Blue

The dialyzed iron - Prussian blue method of Rinehart and Abul-Haj (1951) was also utilized for demonstration of acid GAG. Deparaffinized and rehydrated sections were exposed to a concentrated solution of dialyzed colloidal

iron for ten minutes. After the slides were rinsed for five minutes, the iron which reacted with the tissue was visualized by a modified Perl's Prussian blue method. Slides were exposed to a solution of equal volumes of 2% potassium ferrocyanide and 2% HCl for ten minutes, rinsed, dehydrated and mounted. Control sections were exposed only to the HCl/potassium ferrocyanide solution.

IV. Masson's Trichrome Staining

This technique was employed as a specific stain for collagen as described by Masson (1929). Deparaffinized sections were rehydrated to distilled water and immersed in Bouin's fixative to mordant overnight. Sections were rinsed and the nuclei stained for ten minutes in Weigert's iron hematoxylin. After rinsing, sections were stained in Beibrich Scarlet - Acid Fuchsin for 2.5 minutes, rinsed, exposed to a phosphomolybdic acid - phosphotungstic acid solution for ten minutes, and placed directly into aniline blue stain for six minutes. This acid solution excludes certain acid dyes from collagen and excludes the aniline blue from other tissues so that the aniline blue staining becomes selective for collagen. Sections were rinsed twice in distilled water, placed in 1% acetic acid for five minutes., dehydrated, cleared and mounted.

RESULTS

1. Adult Histology

Alcian blue staining at pH 2.5 of adult organisms yields a strong positive reaction with the chondrocytic supportive tissue of the radioles. The dense fibrous band of connective tissue lying between the chondrocytic structures and the overlying epithelium, in general, stains less strongly with a more intense reaction along the epithelial boundary of this band (Figure 1). This intense staining at the base of the overlying epithelium is evident throughout the basal lamina of the organism, and at the base of the gut epithelium. The mucous coat, continuous over the animal, also reacts as do nuclei and the intestinal mucosa. Probably the most intense staining is found in sections through the ventral pads, which exude the external mucous coat and are found in pairs on the ventral aspect of each segment. Strands of alcian blue-positive material in muscle suggest reaction with cell coats and connective tissue. Certain sites within the external epithelial cells themselves exhibit intense staining. This reaction is especially prevalent in the attenuated epithelium of the pygidia. In fact, the entire posterior structure of the still elongating animal shows an increased reaction to the dye (Figure 2). At the tips of the pygidium no alcian blue-positive boundary is seen between the attenuated epithelium and the loosely packed alcian blue positive cells which it covers.

Reducing the pH of the staining solution to 1.0 extinguishes much of the reaction, leaving only the chondrocytic supportive tissue, basal lamina, and some of the epithelial inclusions in the pygidia. Some of the material of the ventral pads also stains at pH 1.0.

II. Alcian Blue Staining of Regenerate Structures

In the early stages of bud formation both epithelium and the underlying blastema exhibit reaction to the stain at pH 2.5. In sharp contrast to the epithelium covering adult anterior structures, large amounts of alcian blue-positive material are seen associated with the epithelium covering the anterior buds (Figure 3). In addition to the mesenchymal cells of the blastema, large amounts of positive reacting extracellular material appears, often in the form of intercellular strands. In these early stages, no basal lamina is yet evident and the underlying cells of the blastema and the extracellular material are seen in direct association with the overlying epithelium.

Lowering the pH of the alcian blue stain to pH 1.0 almost entirely eliminates staining in the early regenerate. Some sites of staining are evident in the epithelium, apparently intracellular.

As the regenerate buds attain their full size, several changes are noticed in the composition of the extracellular materials. There appears to be less intercellular matrix in the blastema, some strands of which now react positively with pH 1.0 alcian blue stain (Figure 5). It is also at

this time that we first see the deposition of pH 1.0 alcian blue positive material in the form of a basal lamina lying between the epithelium and blastema.

These concomitant events of matrix condensation and deposition of sulfated GAG in the form of a basal lamina and chondrocytic matrix mark the end of the proliferative stage of anterior blastema formation and the onset of cellular differentiation. Epithelial branching of each lobe of the anterior buds gives rise to the developing radioles. Chondrocytic supportive tissue forms in association with the basal lamina of an area of thickened epithelium in each developing radiole. A positive reaction with alcian blue at pH 1.0 demonstrates incorporation of sulfate into this material (Figures 6 and 7).

III. Hyaluronidase Treatment

Treatment of deparaffinized sections with bovine testicular hyaluronidase virtually eliminates alcian blue staining, at pH 2.5, of the extracellular materials of the blastema (Figures 4a and 4b). Cell nuclei continue to stain as do materials associated with the attenuated epithelium and the external mucous coat. The absence of staining in the enzyme-treated sections was assumed to be due to removal of the pH 2.5 alcian blue reactive material by the enzyme.

IV. Dialyzed Iron - Prussian Blue Staining

The distribution of staining following the Prussian blue reaction with the colloidal dialyzed iron taken up by

deparaffinized tissue sections closely resembles that seen in alcian blue staining at pH 2.5 (Figure 10). In both anterior and posterior early regenerate structures the cells and extracellular materials of the blastema react positively, as do the overlying epithelium and external mucous coat. In contrast to alcian blue, no nuclear staining is observed. Control deparaffinized sections exposed to the Prussian blue stain without previous exposure to the dialysed iron showed no reaction whatsoever to the dye, confirming that staining is indicative of the presence of iron.

V. Masson's Trichrome Staining

The Masson's trichrome staining procedure allows for the specific staining of collagen by aniline blue. Collagen in quantities detectable by this method is not apparent until after blastema formation has been completed and branching of the anterior buds has begun. This appearance of collagen is associated with the earliest detectable presence of chondrocytic tissue. Faint blue staining is apparent at the junction of the chondrocytes and overlying epithelium of the developing radioles and as strands through the cells of the blastema from which these radioles arise (Figure 9). Further development results in strong blue staining of the band of dense fibrous tissue which comes to lie between the cartilaginous tissue of the radioles and the epithelium (Figure 8).

No reaction to the stain is detectable in the proliferating posterior regenerate structures. Faint blue strands associated with the adventitia of the gut and the intersegmental membranes are the only other evidence of collagen deposition in the organism.

VI. Effects of Teratogens on Development

Exposure of regenerating worms to 6-AN at 10^{-5} M 24 hours after fragmentation, stops bud formation in all cases. The teratogenic effect diminishes with time until, at three days, no effect is seen except in those organisms already exhibiting delayed development. Although 6-AN blocks bud formation and arrests bud formation once it has begun, it has no effect after blastema development has been completed. The effect is reversible, in most cases, after a 24 hour exposure but only occasionally after a 48 hour exposure. Three days' exposure completely extinguishes the organism's regenerative capacity.

Both α,α'-dipyridyl at 10^{-4} M and βAPN at 10^{-3} M stop regeneration in all cases and at all points during development. Once radiole development has begun, the effect is reversible for as long as the animals remain healthy. If blastema formation is arrested, the capacity for further differentiation is lost by the end of the third day following fragmentation. Although some animals may survive for several days when replaced in the normal environment, no further development occurs.

DISCUSSION

Regeneration in sabellid polychaetes is a complex phenomenon involving dedifferentiation of established tissues as well as the processes of cell migration, differentiation, and tissue organization exhibited during embryogenesis. Results obtained from this study indicate that regeneration in sabellids exhibits a pattern of extracellular matrix production similar to developing vertebrate systems. Cell migrations in several previously investigated vertebrate systems have been shown to occur in hyaluronate - rich environments. Removal by endogenous hyaluronidase has been shown to precede subsequent differentiation of these cells. The present finding that blastema formation in sabellids also occurs in a matrix of hyaluronate serves to underline the significance of this material in development. The requirement for hyaluronate as a matrix for cell migration and blastema formation across such a wide phylogenetic range suggests a role basic to the processes of cell migration and establishment of tissue primordia.

Anterior and posterior regenerate buds develop as bilobed blastemas of mesenchymal cells, enmeshed in a matrix of GAG. The epithelial cells covering the developing blastemas appear attenuated or stretched along their vertical axis, and contain large amounts of alcianophilic material. Elimination of most of the reactivity with alcian

blue by lowering the pH to 1.0, indicates that this material is predominantly hyaluronate. An epithelial origin of the basal lamina in later stages has been demonstrated by Fitzharris (1976) and it seems likely that some of these earlier epithelial products are transported to the basal surface. At this stage mesenchymal cells of both anterior and posterior blastemas exist in a hyaluronate-rich matrix, and are in direct association with the attenuated epithelium. This condition persists in the pygidium as new growth continues to form at the caudal end of the organism.

As anterior regeneration progresses, a blood vessel forms within each anterior bud and epithelial branching begins, giving rise to the developing radioles. Sulfated GAG appear as components of both the epithelial basal lamina and the chondrocytic extracellular matrix. As in previously described vertebrate models of developing limbs and axial skeleton, deposition of sulfated GAG in a cartilaginous matrix by these cells represents an expression of their differentiated state. Likewise, basal lamina formation can be viewed as an expression of epithelial differentiation. However, a review of the literature indicates that, in many instances, the epithelial basal lamina has an inductive effect. The basal lamina of the notochord and the developing lens effectively demonstrate such inductive capabilities. No direct evidence for an inductive role for epithelial basal lamina can be demonstrated by this histological study. However, certain observations can be made suggesting a

relationship between the appearance of a basal lamina and the fate of the underlying mesenchymal cells. The first is that establishment of a basal lamina always precedes differentiation. In the pygidium no basal lamina forms and the blastema cells retain their mesenchymal form in a hyaluronate-rich matrix. The second is that the earliest appearance of distinguishable chondrocytes occurs in direct association with the epithelial basal lamina. Mesenchymal migration and chondrification in developing radioles occurs under an area of thickened epithelium reminiscent of the apical cap of regenerating newt limbs. Studies by Thornton (1954) of the newt limb demonstrate neural invasion to be a prerequisite for apical cap formation and continued differentiation of the developing limb. In regenerating sabellids, outgrowth of nerve into developing radioles has been demonstrated to occur along the basal portion of the epithelium, proximal to the developing chondrocytes (Fitzharris and Lesh, 1969; Fitzharris, 1976). It is not unreasonable to postulate an epithelial response to neural invasion, which in turn provides a preferential zone for mesenchymal migration and differentiation.

Two contrasting systems exist in a single regenerating organism. Anterior differentiation is expressed by formation of chondrocytic tissue associated with basal lamina formation. Posterior pygidia form no basal lamina and their mesenchymal cells remain in an undifferentiated state.

Posterior tissues do, however, possess the potential to differentiate into anterior structures as demonstrated by

Fitzharris (1970). Colchicine treatment can result in formation of head structures at both ends of the regenerate. The effect appears due to the disruption of the microtubule transport system of the ventral nerves thereby eliminating the anterior-posterior gradient of neurosecretory material. This proposal is supported by the observation that head structures can be induced to form from any segment of the polychaete Spirographis when implanted with the reflected anterior ends of the severed nerves (Kiortsis and Moraitou, 1965).

Establishment of the anterior-posterior polarity of the organism, therefore, appears to be determined very early during the regeneration process. This results from the first round of tissue interactions or inductions, with each subsequent interaction further delineating the fate of these tissues. The reaction capacity of many developing tissues has been shown to vary with time (see Holtfretter, 1968 for review). Neurula ectoderm can be induced to form neural tissue in vitro. This potential is lost by the end of the neurula stage and the tissue expresses capacities to differentiate into other ectodermal derivatives. Such a sequence of co-ordinated transient potentials in developing tissues greatly reduces the need for highly specific inductive stimuli. A single stimulus, such as the basal lamina of the notochord, can induce neurulation of overlying ectoderm and chondrogenesis of the sclerotome due to differing potentials of the reacting tissues. In such a scheme, compositional changes in the extracellular matrix of developing tissues aquire a new significance.

Several possible actions have been postulated for hyaluronate in developmental sytems. Hyaluronate has been shown, upon hydration, to form an extended random coil, occupying a molecular domain 10^4 times larger than the space occupied by the dehydrated molecule. At physiological concentrations, hyaluronate forms a viscous solution able to bind large amounts of water, exert an osmotic pressure, and exclude macromolecules from its meshwork. Hyaluronate's association with water could cause swelling of extracellular spaces, thereby facilitating or directing cell migrations. Strong correlations between hyaluronate and water content in several developing organs have previously been discussed. The ability of this GAG to exclude macromolecules from its domain and its ability to inhibit chondrocyte differentiation in vitro supports a possible role in preventing premature differentiation of migrating cells prior to completion of blastema formation. The production of a specific enzyme, hyaluronidase, to remove this material prior to the expression of a certain pathway of differentiation, suggests that hyaluronate's required role has been completed and that its removal is necessary for the next phase of development.

The ability of GAG to bind cations, especially calcium, and the requirement for this cation in microtubule assembly led Lippman (1968) to suggest another possible role for this material in development. According to her model, cell surface-associated GAG could act as cationic traps, prohibiting Ca^{+2} influx and thereby blocking mitotic spindle

assembly. Removal or conformational changes of GAG could subsequently allow for restoration of Ca^{+2} flux and mitotic activity.

Treatment of regenerating sabellids with inhibitors of GAG and of collagen synthesis further demonstrates the requirement for these macromolecules in their normal development. Exposure of regenerates to βAPN or α,α'-dipyridyl stops further regeneration at all stages. Both of these agents have been demonstrated to block the extracellular assembly of collagen fibrils, and their ability to block regeneration at all stages indicates an absolute requirement for mature cross linked collagen in the regeneration process.

Masson's trichrome staining clearly demonstrates deposition of a dense fibrillar collagenous matrix between the epithelium and the chondrocytic tissue. However, collagen in quantities undetectable by this method is almost certainly present as components of the cartilage matrix and the epithelial basal lamina of the organism. Inability to form these extracellular structures as a result of βAPN or α,α'-dipyridyl treatment could prevent further development. However these effects do not explain the ability of these agents to block blastema formation, which occurs prior to the appearance of a basal lamina or of chondrocytic tissue. The results of Crow (1979) demonstrating that βAPN inhibits mesenchymal migration into prospective endocardial cushions of embryonic rats and the present demonstration of failure of mesenchymal blastemas to form in βAPN-treated sabellid

regenerates suggest that collagen synthesis is necessary for mesenchymal migrations. Both collagen and hyaluronate have been shown to promote spreading of mesenchymal cells in culture (Lash and Vasan, 1978).

Exposure to 6-AN at 10^{5}M blocks blastema formation in regenerates. Treatment at this concentration was ineffective in stopping further regeneration during post-blastema stages when the epithelial basal lamina had been established and deposition of cartilage matrix had begun. The inhibitory effect of this niacin antimetabolite on GAG synthesis, while not affecting protein synthesis, makes it attractive to postulate that depressed hyaluronate synthesis during these formative stages is solely responsible for the failure of the blastema to form. However, other effects of 6-AN treatment cannot be ruled out. Depressed levels of ATP as well as DNA have been observed in rat embryos from 6-AN-treated mothers. These effects could interfere with the proliferation of epithelial and mesenchymal cells as well as deplete energy stores available for cell migration.

Blocking blastema formation by treatment with any of these agents also demonstrates the transient nature of the developing tissues' potential for differentiation. In the normal regenerate, blastema formation is completed by the end of the third day following fragmentation, and histologically detectable chondrocytes usually appear the following day. If blastema formation is arrested, the capacity for further development is lost by the end of the third day. The capacity for dedifferentiation of tissues

and establishment of a blastema, resulting from the initial round of inductive events is transitory.

SUMMARY

Production of specific extracellular materials has been shown to accompany the two distinct phases of regeneration in sabellids. An initial phase of migration of mesenchymal cells, resulting in formation of anterior and posterior blastemas, occurs in a matrix rich in hyaluronate. Anteriorly, the second phase is characterized by deposition of a basal lamina comprised of collagen and GAG, epithelial branching, and differentiation of mesenchymal cells into chondrocytes which produce a typical cartilaginous matrix rich in sulfated GAG. Posterior blastemas do not progress beyond the first stage, exhibiting neither basal lamina formation nor mesenchymal differentiation, and maintaining a hyaluronate matrix.

Hyaluronate production is associated with migrating mesenchyme in several vertebrate systems. Demonstration of this association for the first time in invertebrates, supports the proposal that hyaluronate plays an essential role in regulating mesenchymal migration, differentiation, and establishment of tissue primordia. Several possible mechanisms for its action have been discussed. Failure of blastemas to form in response to 6-AN lends further support to this proposal.

Evidence for the requirement of GAG and collagen during normal epithelial-mesenchymal interactions has been reviewed. Both of these materials in the form of a basal

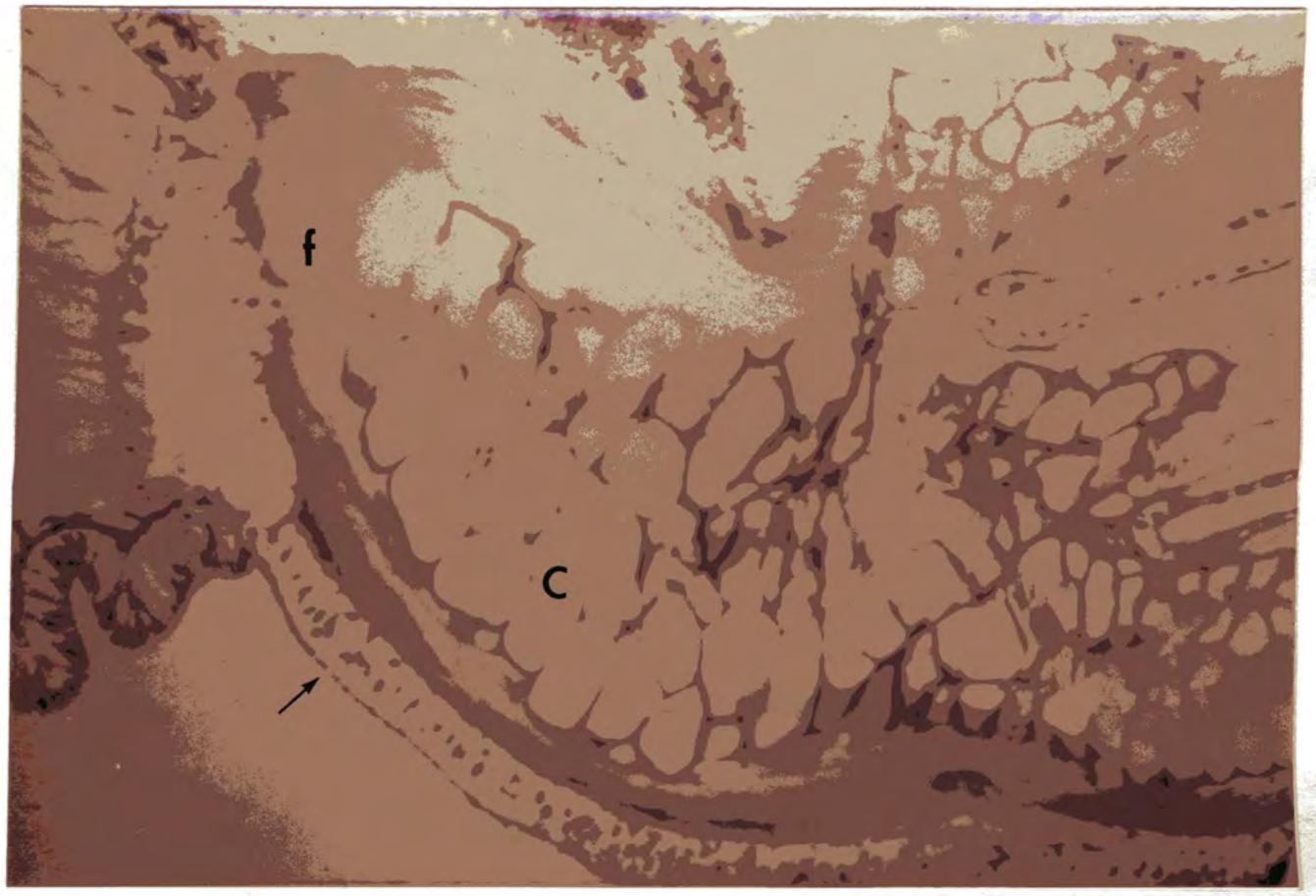
lamina have been demonstrated to promote cell differentiation in many vertebrate systems. Observations made as part of this study show that basal lamina formation precedes chondrocytic differentiation in all cases and that chondrocytes first appear directly under this epithelial basal lamina. These observations and evidence from the reviewed literature support the proposal that the extracellular materials present in the form of a basal lamina promote differentiation of chondrocytes from mesenchymal cells of the blastema. The ability of α,α'-dipyridyl and βAPN to block regeneration at all stages indicates that normal collagen synthesis is necessary not only for elaboration of the extracellular structures of basal lamina and cartilage matrix by well-differentiated tissues, but is also necessary for mesenchymal migration and blastema formation.

The results of this study indicate that the extracellular materials, GAG and collagen which are known to be involved in many developing vertebrate systems are active in invertebrate development as well. Their production may be a common mechanism whereby an embryo or developing organism regulates its genotypic expression.

FIGURES

Figure 1 A longitudinal section through the base of one lobe of an adult branchial crown. Alcian blue staining (pH 2.5) demonstrates GAG associated with the external mucous coat (arrow), dense fibrous connective tissue (f), and chondrocytic supportive tissue (c). Nuclei also stain at pH 2.5 (alcian blue (pH 2.5), 870X).

Figure 2 A longitudinal section through a normal pygidium. The attenuated epithelium (e), mesenchymal blastema cells (m), gut (g), and other tissues react with alcian blue (pH 2.5). (alcian blue (pH 2.5), 870X).


f
C

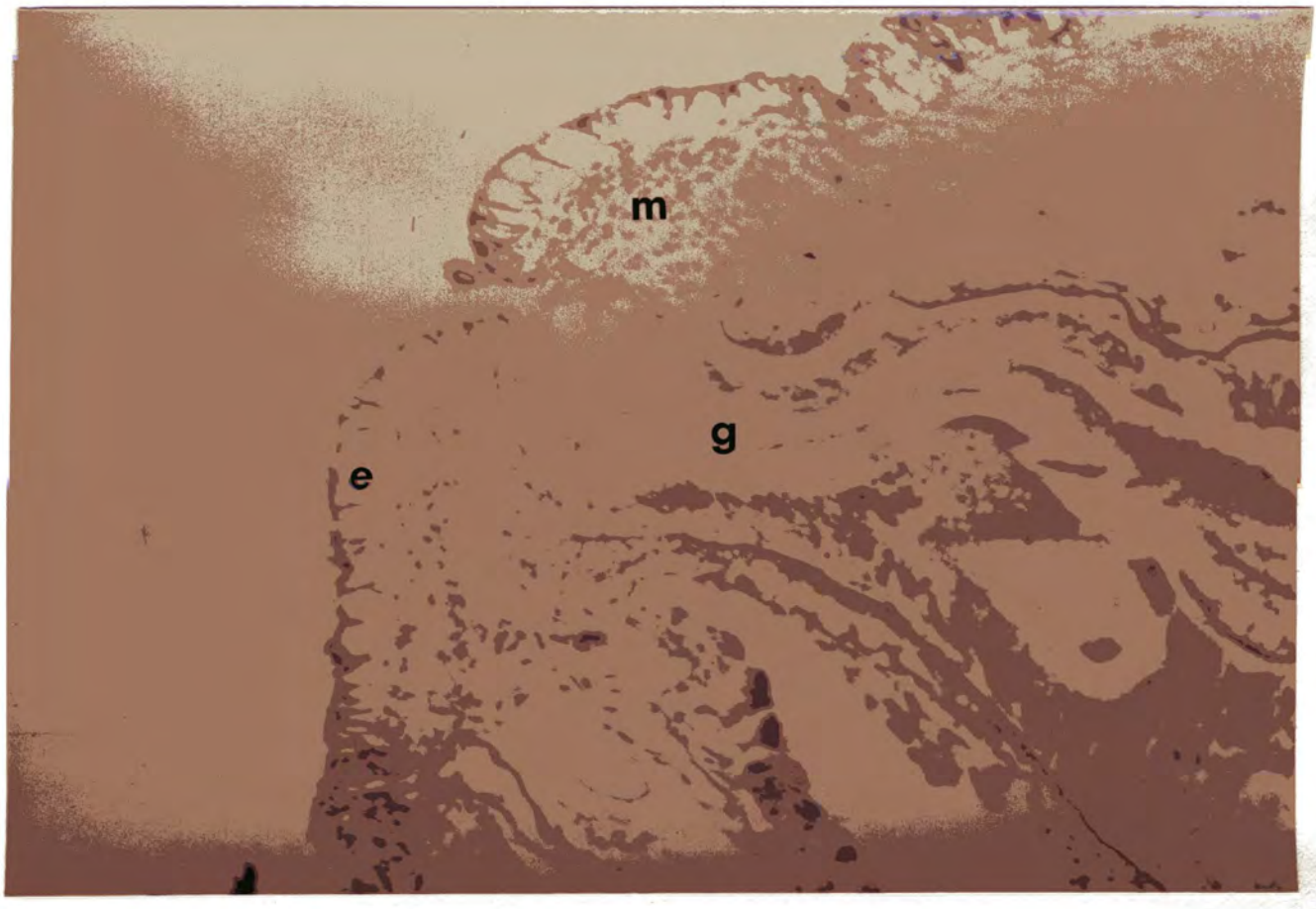
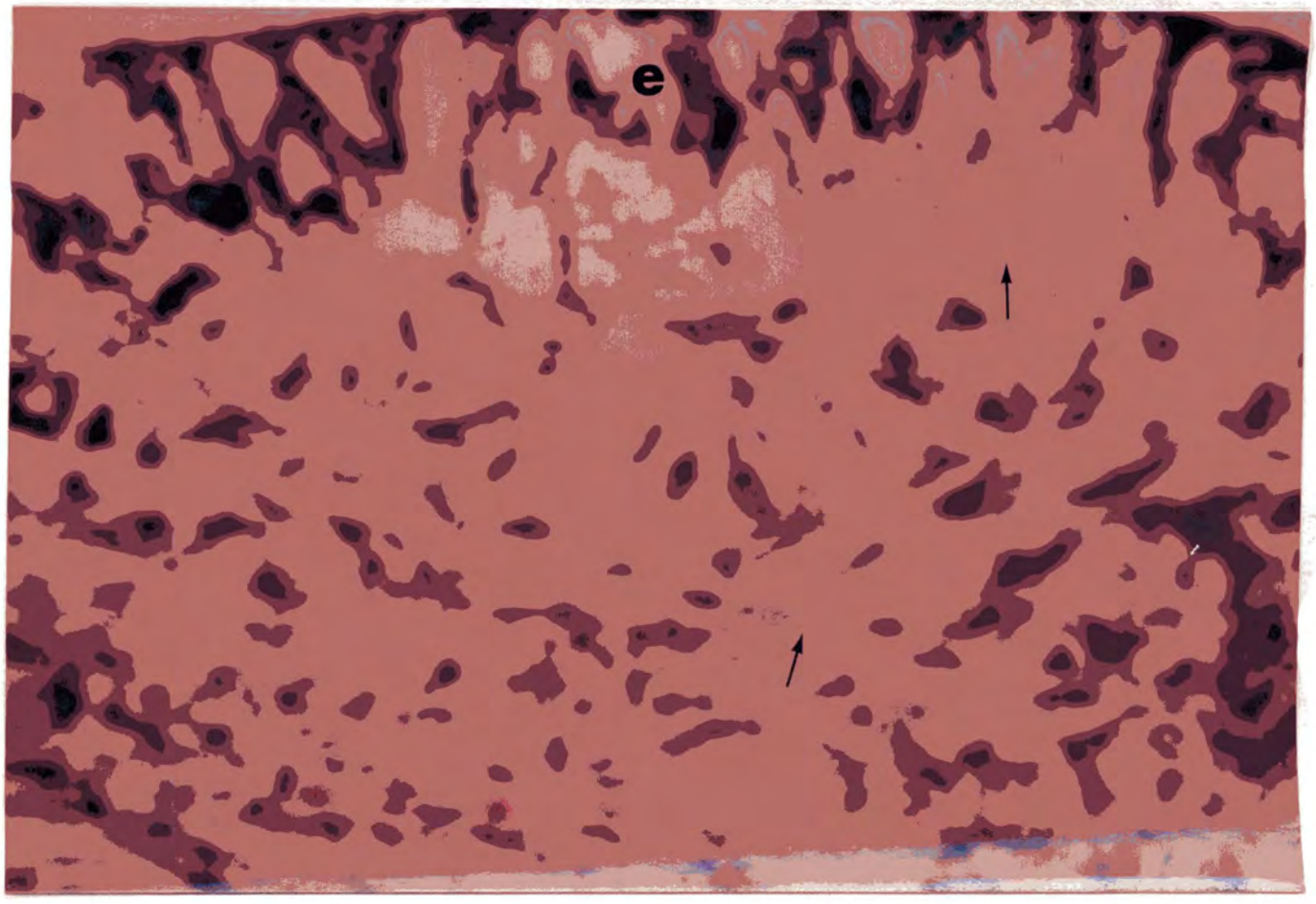

m
g
e

Figure 3 A section through a 3-day anterior regenerate bud. Epithelium (e), mesenchymal cells, and extracellular material of the blastema (arrows) react with alcian blue (pH 2.5) demonstrating the presence of GAG. Notice the free association of mesenchymal cells with the epithelium prior to the deposition of a basal lamina (alcian blue (pH 2.5), 2170X).

Figure 4 A longitudinal section through the anterior region of a 3-day regenerate. Alcian blue (pH 1.0) and P.A.S. staining demonstrates deposition of sulfated GAG associated with epithelial basal lamina and occuring as strands running among the cells of the blastema (arrows) (alcian blue (pH 1.0) - P.A.S., 870X).


e

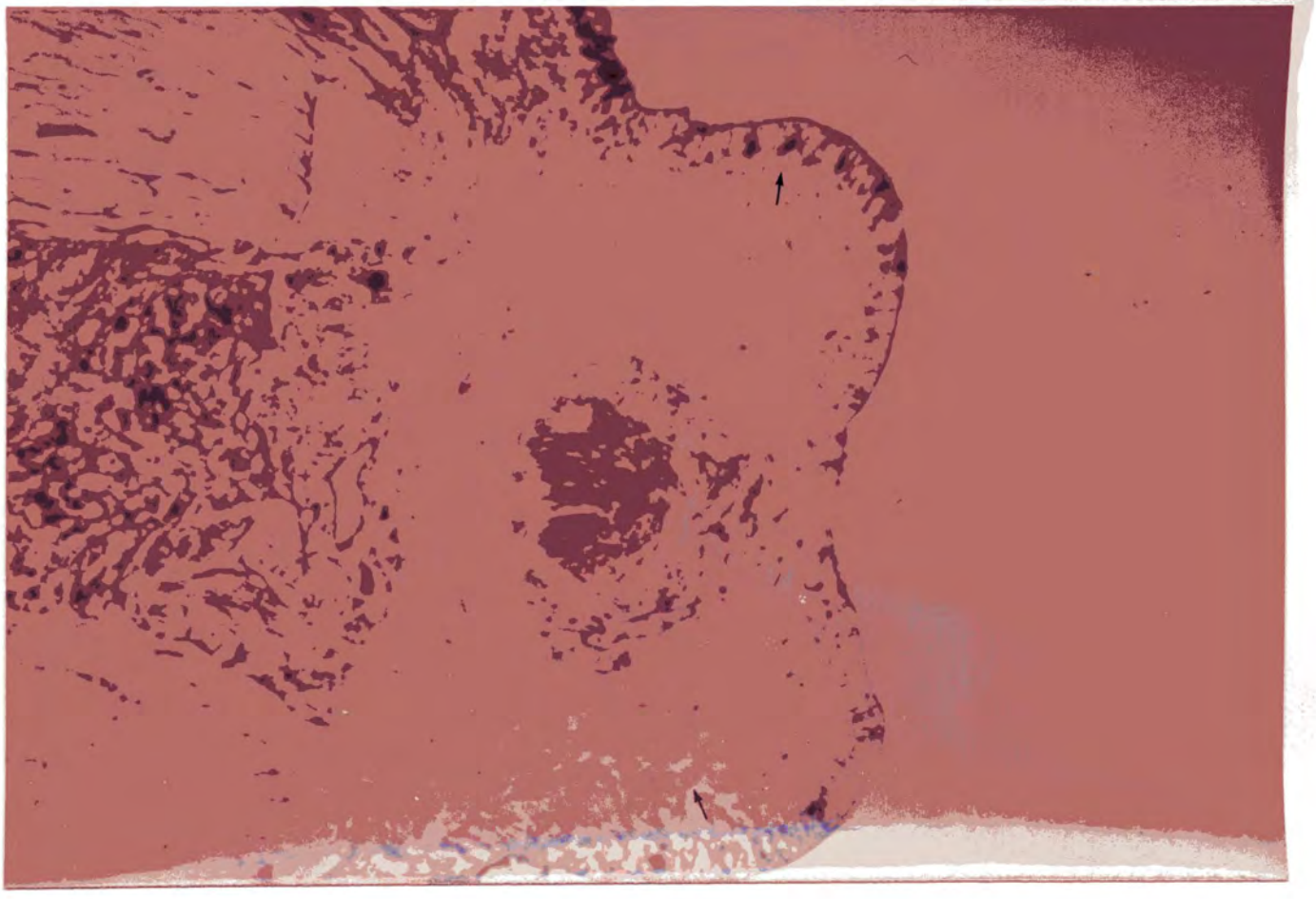
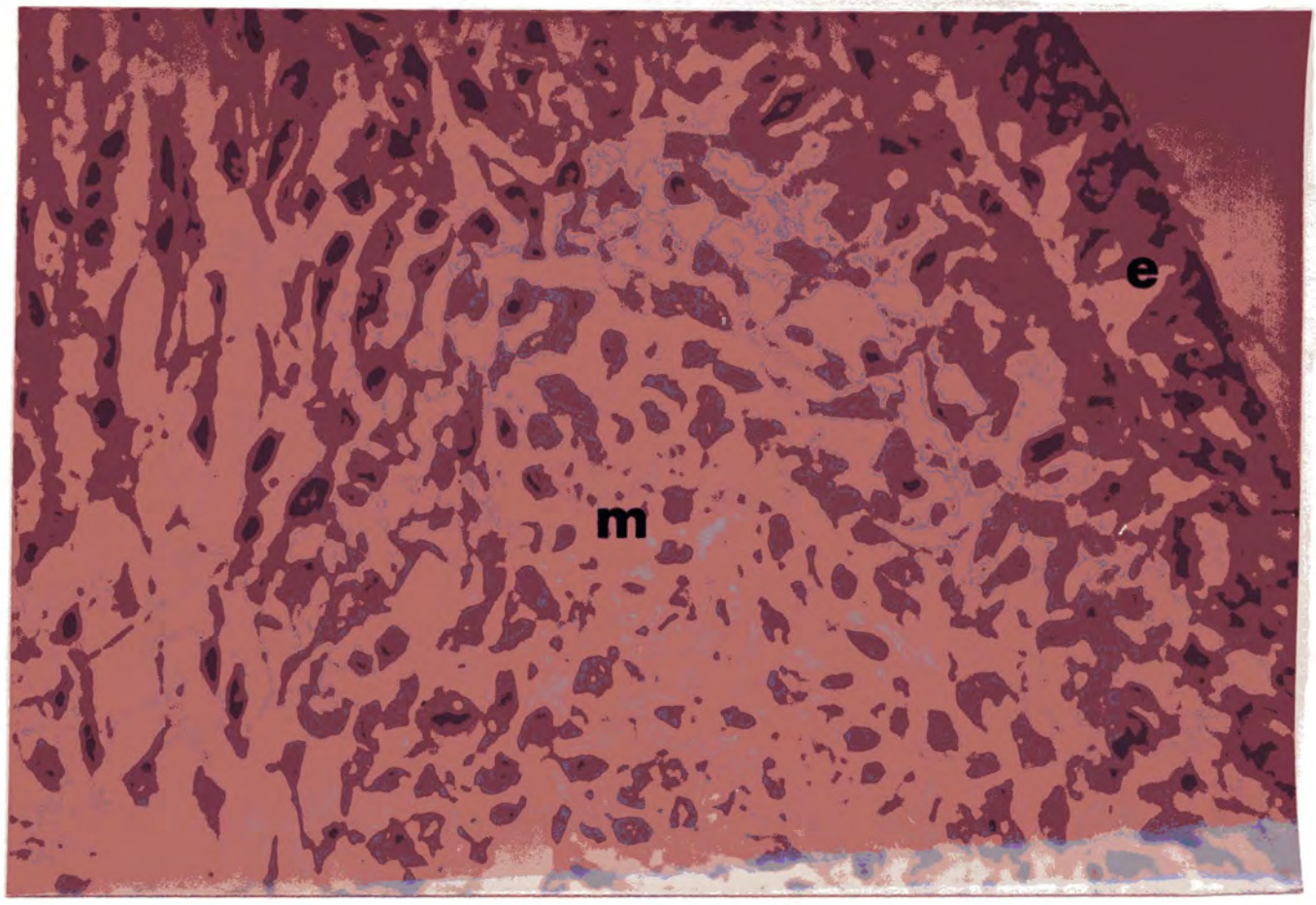


Figure 5 A section through a 3-day anterior regenerate bud. This control section was incubated in buffer without hyaluronidase. GAG associated with epithelium (e), blastemal cells, and extracellular material (m) is clearly evident. (alcian blue (pH 2.5), 2170X).

Figure 6 A section through a 3-day anterior regenerate bud. This hyaluronidase-treated section shows a significant reduction in reaction with alcian blue (pH 2.5). Removal of hyaluronidase-sensitive material reveals distinguishable chondrocytes (c) with visible matrix deposition as well as enzyme-resistant material associated with the epithelial basal lamina (arrow). (alcian blue (pH 2.5), 2170X).


e
m

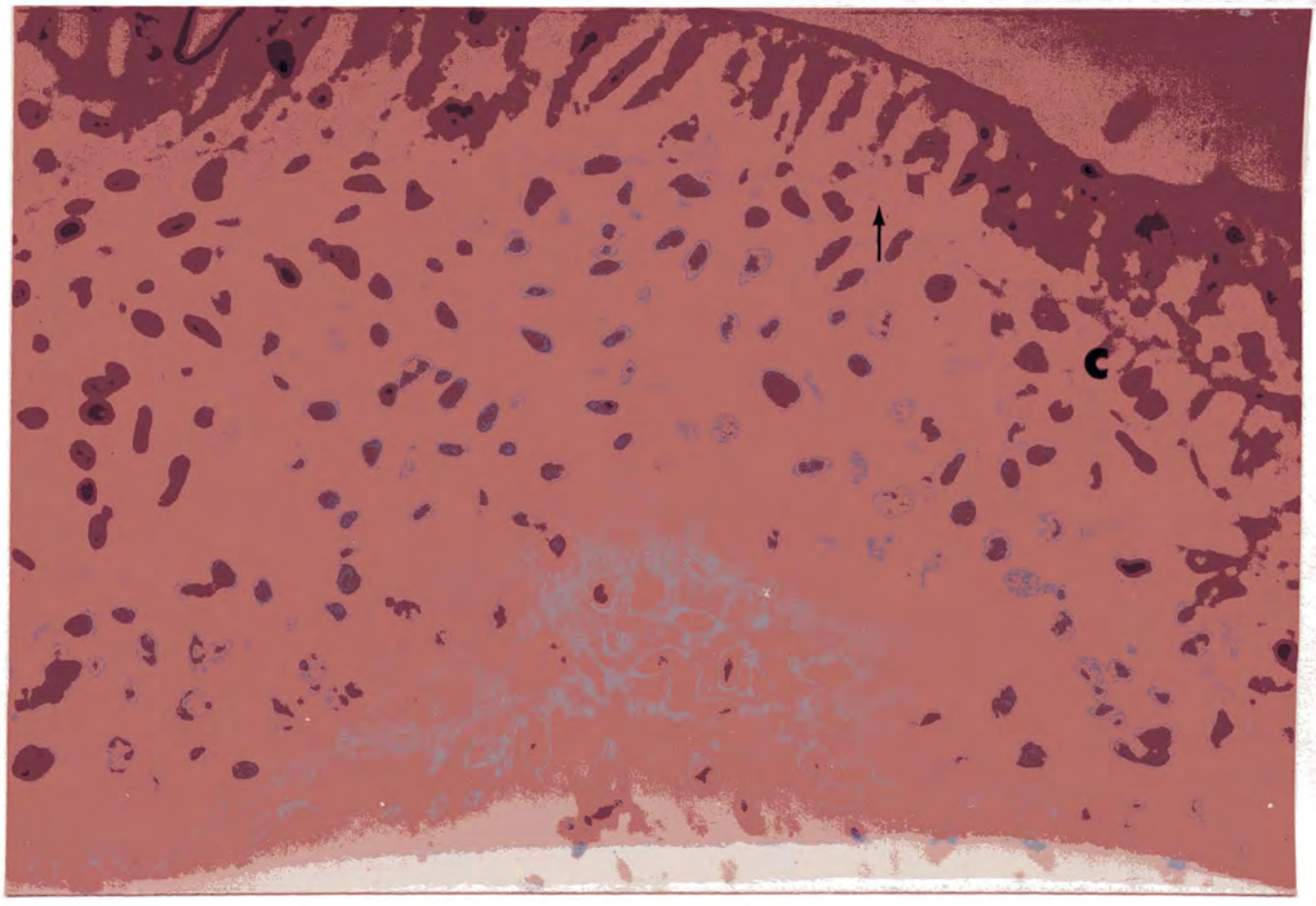
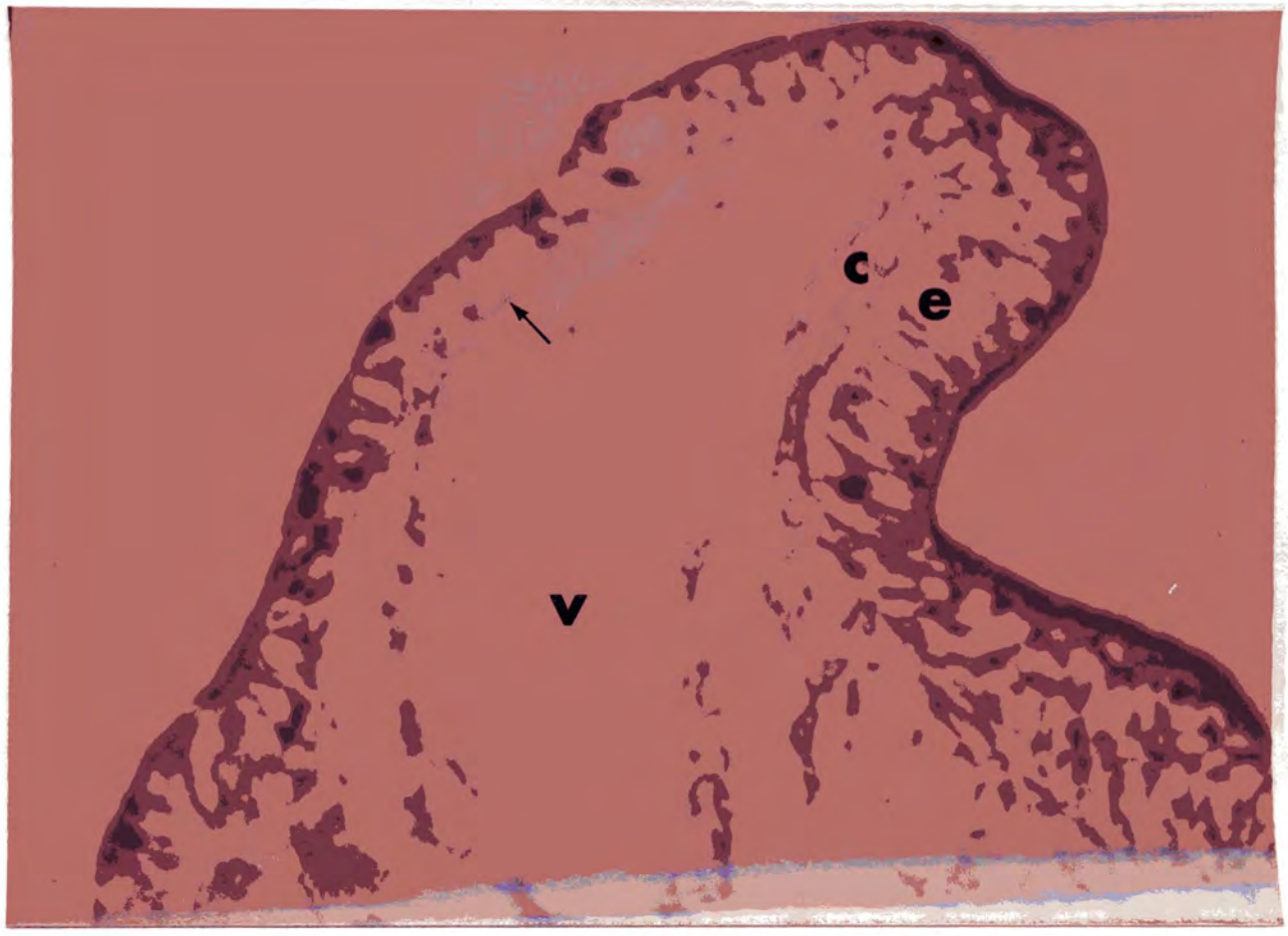

c

Figure 7 A longitudinal section of a 4-day developing radiole stained with alcian blue (pH 1.0) - P.A.S. This figure demonstrates sulfated GAG associated with epithelial basal lamina (arrow) and developing chondrocytes (c). The blood vessel (v) fills the developing radiole, compressing mesenchymal cells between it and the epithelium. Notice the thickened area of epithelium (e) under which the chondrocytes first form. (alcian blue (pH 1.0) - P.A.S., 870X).

Figure 8 A cross section of 6-day developing radioles stained with alcian blue (pH 1.0) - P.A.S. Areas of thickened epithelium (e) are clearly visible in areas of developing chondrocytes (c). Deposition of sulfated GAG associated with chondrocytes and epithelial basal lamina is visible (arrows). The blood vessel (v) fills the interior of the developing radioles. (alcian blue (pH 1.0) - P.A.S., 2170X).


c
e
v

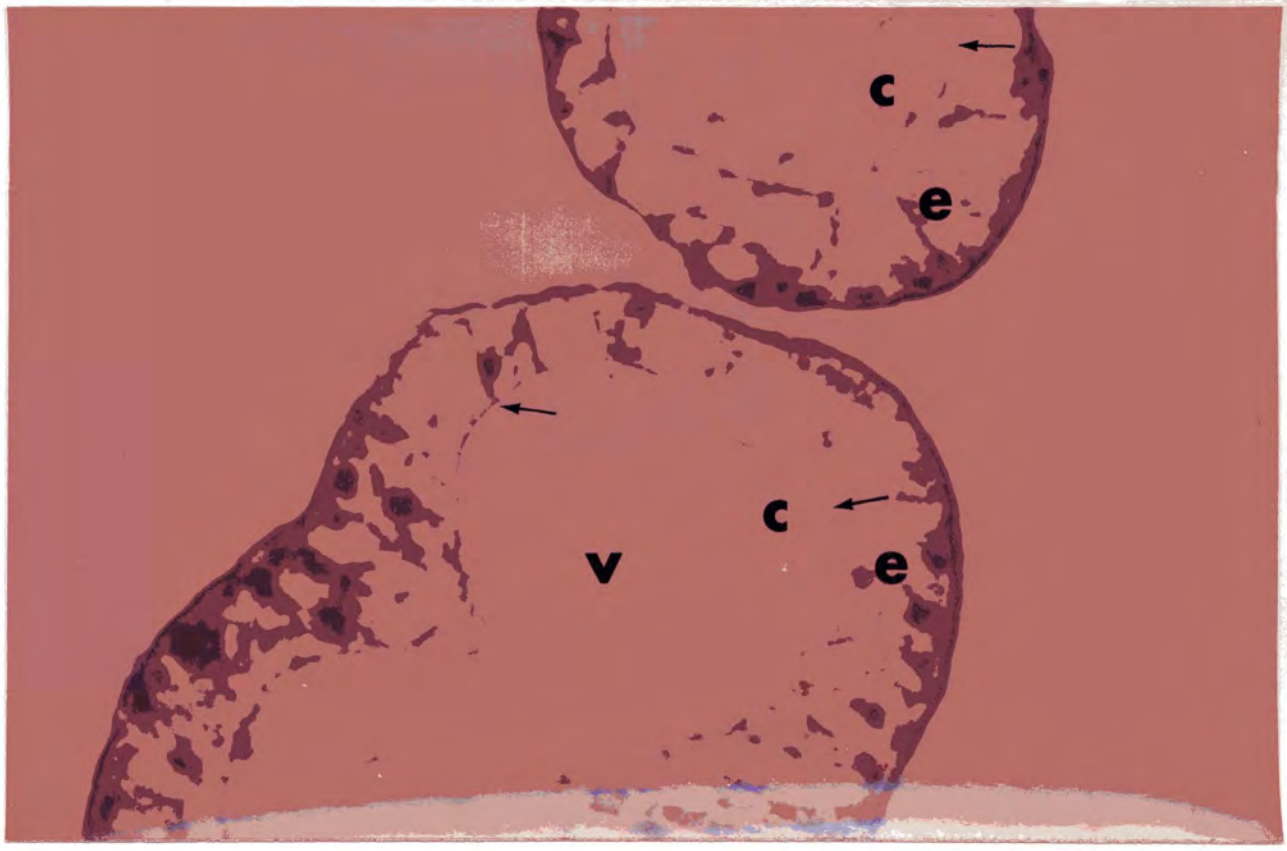
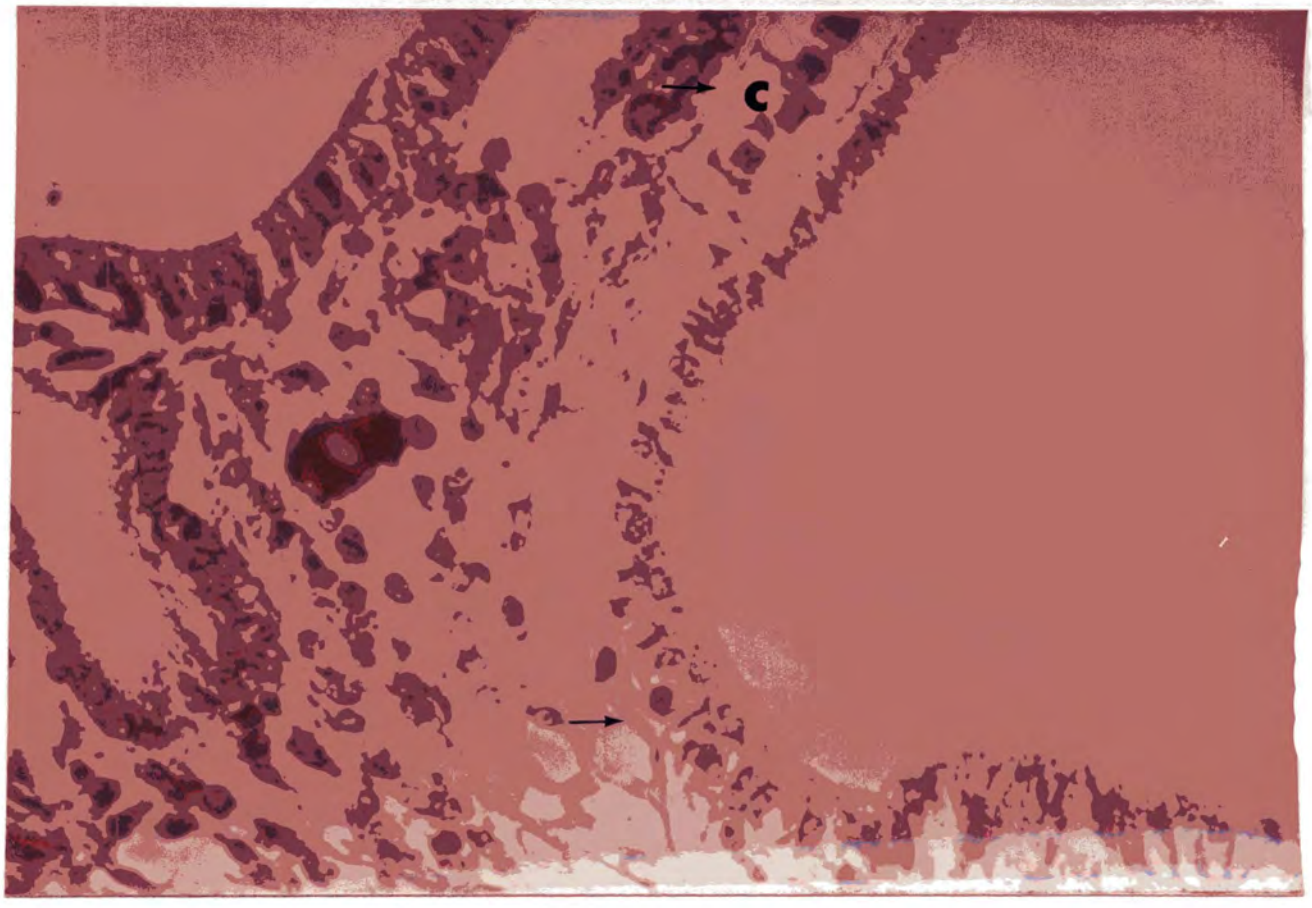

c
e
c
v
e

Figure 9 A longitudinal section of a 5-day developing radiole stained with Masson's trichrome stain. Faint blue staining demonstrating collagen (arrows) is visible associated with development chondrocytes (c), under the epithelium, and scattered throughout the blastema. (Masson's trichrome, 2170X).

Figure 10 A cross section through the base of one lobe of the developing brachial crown stained with Masson's trichrome stain. The thick fibrous lamina lying between the epithelium and chondrocytic tissue reacts with the anline blue component of the stain indicating the presence of collagen (arrows). Faint blue staining associated with the chrondrocytic tissue (c) is also visible. (Masson's trichrome, 870X).


C

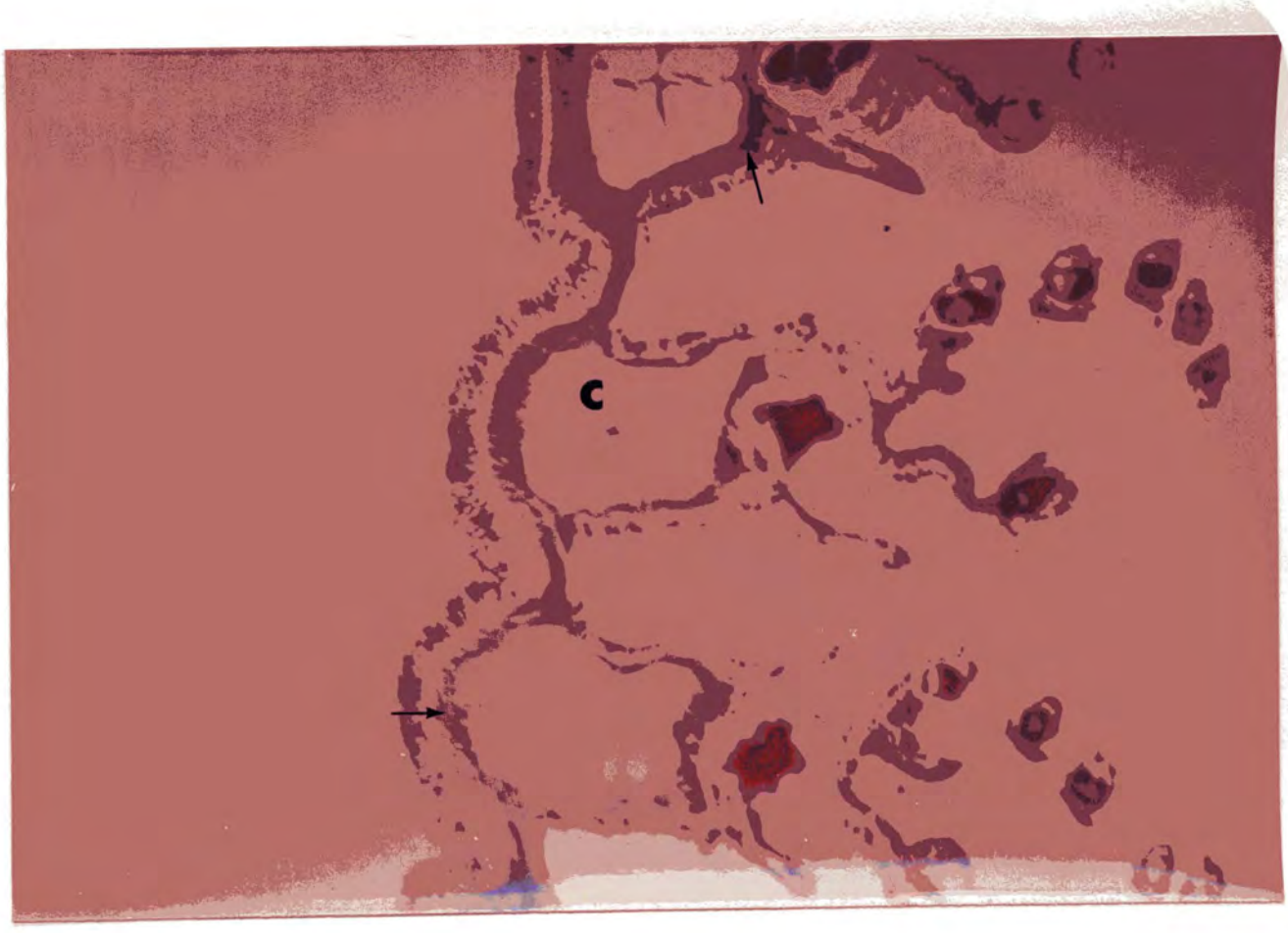
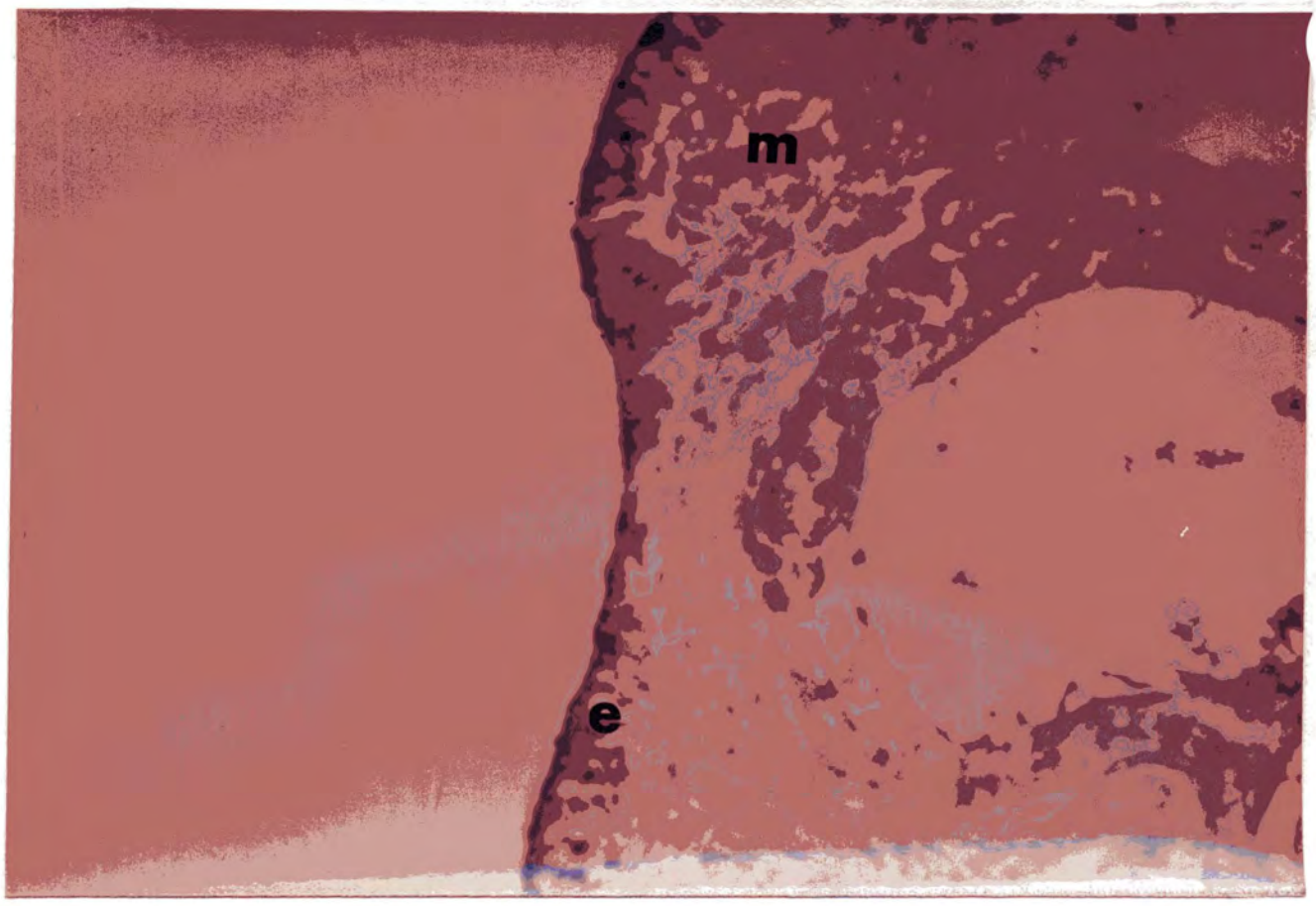

C

Figure 11 A longitudinal section through early (2 day) regenerate buds. Rinehart-Abul-Haj dialyzed iron preparation reacts with acidic mucins and is rendered visible by Prussian blue stain. Intense staining of the epithelium (e) and the external mucous coat as well as mesenchymal cells (m) and extracellular materials is visible. (Dialyzed iron - Prussian blue, 870X).


m
e

LIST OF REFERENCES

Banerjee, S.D., R.H. Cohn, and M.R. Bernfield. "Basal Lamina of Embryonic Salivary Epithelia. (Production by the Epithelium and Role in Maintaining Lobular Morphology.)" J. Cell Biol. 73:445-463, 1977.

Chamberlain, J.G. and M.M. Nelson. "Congenital Abnormalities in the Rat Resulting from Single Injections of 6-Aminonicotinamide During Pregnancy." J. Expt. Zool. 153:285-299, 1963.

Chvapil, M., E. Ehrlichova and J. Hurych. "Two Forms of the Effect of α,α'-Dipyridyl and 1,10-Phenanthroline on Synthesized Hydroxyproline-Deficient Collagen." Experientia 22:584-585, 1966.

Cohen, A.M. and E.D. Hay. "Secretion of Collagen by Embryonic Neuroepithelium at the Time of Spinal Cord-Somite Interaction." Dev. Biol. 26:578-605, 1971.

Coulombre, A.J. and J.L. Coulombre. "Corneal Development. III. The Role of the Thyroid in Dehydration and the Development of Transparency." Exp. Eye Res. 3: 105-114, 1964.

Crow, H.J., Jr. "The Effects of β-Aminopropionitrile on the Cushion Tissue Mesenchyme of the Rat Bulbus Cordis." M.S. Thesis. Medical University of South Carolina, Charleston, South Carolina, 1979.

Ehrmann, R.L. and G.O. Gey. "The Growth of Cells on a Transparent Gel of Reconstituted Rat-Tail Collagen." J. Nat. Cancer Inst. 16:1375-1403, 1956.

Fitzharris, T. "The Ultrastructural Effect of Colchicine on Annelid Regeneration." J. Cell Biol. 47:63a, 1970.

Fitzharris, T.P. "Regeneration in Sabellid Annelids." Amer. Zool. 16:593-616, 1976.

Fitzharris, T. and G. Lesh. "Gut and Nerve-Cord Interaction in Sabellid Regeneration." J. Embryol. exp. Morph. 22:279-293, 1969.

Goetinck, P.F., J.P. Pennypacker and P.D. Royal. "Proteochondroitin Sulfate Synthesis and Chondrogenic Expression." Exp. Cell Res. 87:241-248, 1974.

Gona, A.G. "Effects of Thyroxine, Thyrotropin, Prolactin, and Growth Hormone on the Maturation of the Frog Cerebellum." Exp. Neurol. 38:494-501, 1973.

Grobstein, C. "In vitro Induction of Cartilage in Mouse Somite Mesoderm by Embryonic Spinal Cord." Proc. Soc. Exp. Biol. and Med. 85:477-481, 1954.

Grobstein,C. "Mechanisms of Organogenetic Tissue Interaction." Natl. Cancer Inst. Monogr. 26:279-299, 1967.

Grobstein, C. and J. Cohen. "Collagenase: Effect on the Morphogenesis of Embryonic Salivary Epithelium in vitro." Science 150:626-628, 1965.

Hamburgh, M., L.A. Mendoza, J.F. Burkart and F. Weil. "Thyroid-Dependent Processes in the Deveolping Nervous System." In: Hormones in Development, edited by M. Hamburgh and E.J.W. Barrington. New York: Appleton-Century-Crofts, 1971, pp. 403-415.

Hay, E.D. "Origin and Role of Collagen in the Embryo." Amer. Zool. 13:1085-1107, 1973.

Hay E.D. and S. Meier. "Glycosaminoglycan Synthesis by Embryonic Inductors: Neural Tube, Notochord, and Lens." J. Cell Biol. 12:889-898, 1974.

Hay, E.D. and J.P. Revel. "Autoradiographic Studies of the Origin of the Basement Lamella in Ambystoma." Dev. Biol. 7:152-168, 1963.

Holtfretter, J. "Mesenchyme and Epithelia in Inductive and Morphogenetic Processes." In: Epithelial-Mesenchymal Interactions, edited by R. Fleischmajer and R.E. Billingham. Baltimore: The Williams and Wilkins Co., 1968, pp. 1-30.

Hurych, J. and A. Nordwig. "Inhibition of Collagen Hydroxylysine Formation by Chelating Agents." Biochim. Biophys. Acta 140:168-170, 1967.

Johnston, P.M. and C.L. Comar. "Autoradiographic Studies of the Utilization of S^{35}-Sulfate by the Chick Embryo." J. Biophys. and Biochem. Cytol. 3:231-238, 1957.

Kallman F. and C. Grobstein. "Source of Collagen at Epitheliomesenchymal Interfaces during Inductive Interaction." Dev. Biol. 11: 169-183, 1965.

Kallman, F. and C. Grobstein. "Localization of Glucosamine-Incorporating Materials at Epithelial Surfaces during Salivary Epithelio-mesenchymal Interaction in vitro." Dev. Biol. 14:52-67, 1966.

Kiortsis, V. and M. Moraitou. "Factors of Regeneration in Spirographis spallaznii." In: Regeneration in Animals and Related Problems, edited by V. Kiortsis and H.A.L. Trampusch. Amsterdam: North Holland Publishing Co., 1965, pp. 250-261.

Konigsberg, I.R. "The Relationship of Collagen to the Clonal Development of Embryonic Skeletal Muscle." In: Chemistry and Molecular Biology of the Intracellular Matrix, Vol. 3, edited by E.A. Balazs. New York: Academic Press, 1970, pp. 1779-1810.

Kosher, R.A. and R.L. Church. "Stimulation of in vitro Somite Chondrogenesis by Procollagen and Collagen." Nature 258:327-330, 1975.

Kosher, R.A. and J.W. Lash. "Notochordal Stimulation of in vitro Somite Chondrogenesis Before and After Enzymatic Removal of Perinotochordal Materials." Dev. Biol. 42:362-378, 1975.

Kosher, R.A., J.W. Lash and R.R. Minor. "Environmental Enhancement of in vitro Chondrogenesis. IV. Stimulation of Somite Chondrogenesis by Exogenous Chondromucoprotein." Dev. Biol. 35:210-220, 1973.

Kvist, T.N. and C.V. Finnegan. "The Distribution of Glycosaminoglycans in the Axial Region of the Devleoping Chick Embryo. I. Histochemical Anaylsis." J. Exp. Zool. 175:221-240, 1970.

Lash, J.W. "Somitic Mesenchyme and Its Response to Cartilage Induction." In: Epithelial-Mesenchymal Interactions, edited by R. Fleischmajer and R.E. Billingham. Baltimore: The Williams and Wilkins Co., 1968, pp. 165-172.

Lash, J.W. and N.S. Vasan. "Somite Chondrogenesis in vitro." Dev. Biol 66:151-171, 1978.

Legrand, J. "Comparative Effects of Thyroid Deficiency and Undernutrition on Maturation of Nervous System and Particularly on Myelination in Young Rats." In: Hormones in Development, edited by M. Hamburgh and E.J.W. Barrington. New York: Appleton-Century-Crofts, 1971, pp. 381-390.

Levene, C.I. and J. Gross. "Alterations in State of Molecular Aggregation of Collagen Induced in Chick Embryos by β-Aminopropionitrile (Lathyrus Factor)." J. Exp. Med. 110:771-790, 1959.

Linsenmayer, T.F., B.P. Toole and R.L. Trelstad. "Temporal and Spatial Transitions in Collagen Types during Embryonic Chick Limb Development." Dev. Biol. 35:232-239, 1973.

Lippman, M. "Glycosaminoglycans and Cell Division." In: Epithelial-Mesenchymal Interactions, edited by R. Fleischmajer and R.E. Billingham. Baltimore: The Williams and Wilkins Co., 1968, pp. 208-229.

Manasek, F.J., M. Reid, W. Vinson, J. Seyer and R. Johnson. "Glycosaminoglycan Synthesis by the Early Embryonic Chick Heart." Dev. Biol. 35:332-348, 1973.

Margolis, R.U., R.K. Margolis, L.B. Chang and C. Preti. "Glycosaminoglycans of Brain during Development." Biochemistry 14:85-88, 1975.

von der Mark, H., K. von der Mark and S. Gay. "Study of Differential Collagen Synthesis during Development of the Chick Embryo by Immunofluorescence. i. Preparation of Collagen Type I and Type II Specific Antibodies and Their Application to Early Stages of the Chick Embryo." Dev. Biol. 48:237-249, 1976.

Markwald, R.R. and W.N. Adams Smith. "Distribution of Mucosubstances in the Developing Rat Heart." J. Histochem. Cytochem. 20:896-907, 1972.

Martin, G.R., J. Gross, K.A. Piez and M.S. Lewis. "On the Intramolecular Cross-Linking of Collagen in Lathyritic Rats." Biochim. Biophys. Acta 53:599-601, 1961.

Masson, P. "Some Histological Methods. Trichrome Staining and their Preliminary Technique." J. Tech. Methods 12:75-90, 1929.

McManus, J.F.A. "Histological and Histochemical Uses of Periodic Acid." Stain Techn. 23:99-108, 1948.

Meier, S. and E.D. Hay. "Control of Corneal Differentiation by Extracellular Materials. Collagen as a Promoter and Stabilizer of Epithelial Stroma Production." Dev. Biol. 38:249-270, 1974a.

Meier S. and E.D. Hay. "Stimulation of Extracellular Matrix Synthesis in the Developing Cornea by Glycosaminoglycans." Proc. Nat. Acad. Sci. U. S. A. 71:2310-2313, 1974b.

Mills, W.P., Jr. "Ultrastructural Changes in the Secretory Organelles of Endocardium and Endocardial Cushion Tissue of Embryonic Rat Heart After Teratogenic Treatment." M.S. Thesis. Medical University of South Carolina, Charleston, South Carolina, 1974.

Mowry, R.W. "The Special Value of Methods that Color both Acidic and Vicinal Hydroxyl Groups in the Histochemical Study of Mucins. With Revised Directions for the Colloidal Iron Stain, the Use of Alcian Blue G8X and their Combinations with the Periodic Acid-Schiff Reaction." Ann. N. Y. Acad. Sci. 106:402-423, 1963.

O'Hare, M.J. "Chondrogenesis in Chick Embryo Somites Grafted with Adjacent and Heterologous Tissues." J. Embryol. exp. Morph. 27:229-234, 1972.

Orkin, R.W. and B.P. Toole. "Hyaluronidase Activity and Hyaluronate Content of the Developing Chick Embryo Heart." Dev. Biol. 66:308-320, 1978.

Overman, D.O. and A.R. Beaudoin. "Early Biochemical Changes in the Embryonic Rat Heart after Teratogen Treatment." Teratology 4:183-190, 1971.

Page, R.C. and E.P. Benditt. "Diseases of Connective Vascular Tissues. IV. The Molecular Basis for Lathyrism." Lab. Invest. 26:22-26, 1972.

Polansky, J.R., B.P. Toole and J. Gross. "Brain Hyaluronidase: Changes in Activity during Chick Development." Science 183:862-864, 1974.

Ponseti, I.V. and R.S. Shepard. "Lesions of the Skeleton and of Other Mesodermal Tissues in Rats Fed Sweet-Pea (Lathyrus Odoratus) Seeds." J. Bone and Joint Surg. 36:1031-1058, 1954.

Prockop, D.J., E. Weinstein and T. Mulveny. "Hydroxylation of Lysine in a Polypeptide Precursor of Collagen." Biochem. Biophys. Res. Comm. 22:124-128, 1966.

Rinehart, J.F. and S.K. Abul-Haj. "An Improved Method for Histologic Demonstration of Acid Mucopolysaccharides in Tissues." Arch. Path. 52:189-194, 1951.

Solursh, M. "Glycosaminoglycan Synthesis in the Chick Gastrula." Dev. Biol. 50:525-530, 1976.

Solursh, M., S.A. Vaerewyck and R.S. Reiter. "Depression by Hyaluronic Acid of Glycosaminoglycan Synthesis by Cultured Chick Embryo Chondrocytes." Dev. Biol. 41:233-244, 1974.

Thornton, C.S. "The Relation of Epidermal Innervation to Limb Regeneration in Amblystoma Larvae." J. Exp. Zool. 127:577-597, 1954.

Toole, B.P. "Hyaluronate Turnover during Chondrogenesis in the Developing Chick Limb and Axial Skeleton." Dev. Biol. 29:325-329, 1972.

Toole, B.P. "Hyaluronate and Hyaluronidase in Morphogenesis and Differentiation." Amer. Zool. 13:1061-1065, 1973.

Toole, B.P. "Morphogenetic Role of Glycosaminoglycans (Acid Mucopolysaccharides) in Brain and Other Tissues." In: Neuronal Recognition, edited by S.A. Barondes. New York: Plenum Press, 1976, pp. 275-329.

Toole, B.P. and J. Gross. "The Extracellular Matrix of the Regenerating Newt Limb: Synthesis and Removal of Hyaluronate prior to Differentiation." Dev. Biol. 25:57-77, 1971.

Toole, B.P. and T.F. Linsenmayer. "Proteoglycan-Collagen Interaction: Possible Developmental Significance." In:Extracellular Matrix Influences on Gene Expression, edited by H.C. Salvkin and R.C. Greulich. New York: Academic Press Inc., 1975, pp. 34-346.

Toole, B.P. and D.A. Lowther. "The Effect of Chondroitin Sulphate-Protein on the Formation of Collagen Fibrils in vitro." Biochem. J. 109:857-866, 1968.

Toole, B.P. and R.L. Trelstad. "Hyaluronate Production and Removal during Corneal Development in the Chick". Dev. Biol. 26:28-35, 1971.

Trelstad, R.L., E.D. Hay and J.P. Revel. "Cell Contact during Early Morphogenesis in the Chick Embryo." Dev. Biol. 16:78-106, 1967.

Trelstad, R.L., K. Hayashi and B.P. Toole. "Epithelial Collagens and Glycosaminoglycans in the Embryonic Cornea." J. Cell Biol. 62:815-830, 1974.

Trelstad, R.L., A.H. Kang, A.M.. Cohen and E.D. Hay. "Collagen Synthesis in vitro by Embryonic Spinal Cord Epithelium." Science 179:295-296, 1973.

Wessells, N.K. and J.H. Cohen. "Effects of Collagenase on Developing Epithelia in vitro: Lung, Ureteric bud, and Pancreas." Dev. Biol. 18:294-309, 1968.