AUTORADIOGRAPHIC ANALYSES OF SULFUR-35-SULFATE UPTAKE IN REGENERATE LIMBS OF LARVAL AMBYSTOMA

PRISCILLA MATTSON

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University of New Hampshire, Ph.D., 1971
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AUTORADIOGRAPHIC ANALYSES OF $^{35}$S-SULFATE UPTAKE IN REGENERATING LIMBS OF LARVAL AMBYSTOMA

by

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A.B., Bates College, 1954
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A THESIS

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From 1967-1970, I received financial support from a NASA Predoctoral Traineeship. I was recommended for this award by Dr. Paul A. Wright, who was Chairman of the Department of Zoology at that time.
ABSTRACT

AUTORADIOGRAPHIC ANALYSES OF $^{35}$S-SULFATE UPTAKE IN REGENERATING LIMBS OF LARVAL AMBYSTOMA by PRISCILLA MATTSON

I. Limited dedifferentiation of chondrocytes.

Attempts were made, using combined autoradiographic and histochemical techniques, to determine if chondrocytes continued to synthesize chondroitin sulfate during and after morphological dedifferentiation of these cells in the regenerating limbs of larval Ambystoma. Forelimbs were amputated through the mid-diaphysis of the humerus or through the distal epiphysis and stained autoradiograms were prepared from serial sections of limbs representing daily intervals of regeneration (days 1-15 post-amputation). Each animal was injected twenty-four hours before fixation with 2.5 uc of Na$_2$S$_{35}$O$_4$. Autoradiograms of control, unamputated limbs showed that epiphyseal chondrocytes of the humerus exhibited moderate sulfate incorporation, while isotope uptake was slight in the diaphyseal region.

Epiphyseal-amputated limbs proved to be more amenable for this study. The matrix surrounding epiphyseal chondrocytes underwent very slow dissolution in response to regressive influences and these cells therefore remained spatially identifiable. The chondrocytes continued to show isotope uptake during
early and middle stages of regeneration as their extracellular matrix disappeared and the dedifferentiated cells underwent proliferation. By day 11 post-amputation, redifferentiation was apparent as evidenced by matrix reappearance and an intense incorporation of $^{35}$S-sulfate by cells and matrix.

These results further confirm the idea of tissue-specific regeneration of cartilage.

II. Behavior of muscle and muscle-associated cells.

Analyses were made of stained autoradiograms originally prepared to investigate the extent of cartilage dedifferentiation in regenerating limbs of larval *Ambystoma*. Inspection of early regeneration stages revealed that the incorporation of $^{35}$S-sulfate was not limited to chondrocytes or blastema cells derived from chondrocytes. Fibroblast-like cells which originated from muscle or muscle-associated cells lateral to the limb skeleton also exhibited moderate isotope uptake.

Overall isotope incorporation patterns and related metachromatic responses which occurred during blastemal and early redifferentiation stages of limb regeneration were analyzed. In several respects, results were seen to parallel the results of similar autoradiographic and histochemical studies of differentiating chick limb buds. In both instances, undifferentiated cells of future myogenic and chondrogenic areas initially appeared uniform with regard to the production of sulfated molecules, but localization of isotope uptake later occurred in cell condensations in the central part of the limb bud or blastema. Subsequently, these prochondral cells commenced the production of extracellular matrix.
These data suggest that the regulation of chondroitin sulfate synthesis may be added to previous analogies concerning morphogenesis or cytodifferentiation in developing and regenerating limbs.
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INTRODUCTION

The morphological dedifferentiation of specialized cells is an established prerequisite for amphibian limb regeneration. After wound-healing has occurred at the amputation site, cells apparently derived from the mesodermal tissues of the limb stump lose their distinguishing phenotypic characteristics, accumulate under a specialized layer of epithelium, the apical cap, and then proliferate to form the regeneration bud or blastema. Redifferentiation of blastema cells occurs as the limb is reconstituted.

Early workers in the field of regeneration research noted that blastema cells, since they lacked differentiated characteristics and were well-adapted to proliferation, were similar to embryonic cells (Butler, 1933; Thornton, 1938). More recently, these accounts have been confirmed by ultrastructure studies performed by Hay (1958, 1959, 1965). Her observations of blastema cells from regenerating Ambystoma limbs indicated that the organelles of these cells had an unspecialized arrangement. The endoplasmic reticulum appeared to be discontinuous, numerous free ribosomes were scattered throughout the cytoplasm, and a small vesicular Golgi apparatus was present in a juxtanuclear position. The dedifferentiated cells, in addition to losing all morphological indications of specialization, acquired "embryonic" features associated with cell proliferation such as cytoplasmic basophilia, DNA replication, and an increased nucleo-cytoplasmic ratio.
The conception of blastema cells as embryonic in nature has led many investigators to theorize that the genome of these cells has reverted to an unspecialized condition. Metaplasia (redifferentiation of blastemal components into specialized cell types that are unlike the cells of their origin) seems a likely possibility if this "genomic dedifferentiation" actually occurs.

In attempting to establish the presence or absence of a complete reversal of gene expression during amphibian limb regeneration, it is logical to investigate the dedifferentiation and subsequent redifferentiation of either cartilage cells or muscle cells from limb stump tissues. These cells are microscopically identifiable since they synthesize specialized end products: cartilage cells secrete an extracellular matrix and muscle cells contain contractile intercellular material. Eggert (1967) grafted scapular cartilage to adult newt limbs in which regenerative capacity had been suppressed by irradiation. Amputation through the grafted tissue resulted in blastemas which redifferentiated only into cartilaginous masses. Patrick and Briggs (1964), Steen (1968), and Foret (1970) transplanted cartilage which was labeled with tritiated thymidine and/or by triploidy into humerectomized *Ambystoma* limbs and subsequently amputated these limbs through grafted tissue. Labeled cells were visible during blastema stages. In redifferentiated tissues, the presence of label was detectable only in redifferentiated cartilage. Other attempts were made to trace cells which were derived from labeled, transplanted muscle throughout regeneration stages (Steen, 1968,
Poret, 1970), but these experiments did not provide a definitive answer to whether metaplasia of these cells does or does not occur. The transplants by necessity consisted of mixed muscle and connective tissue components. The appearance of label in redifferentiated cartilage therefore might be attributed to the transformation of connective tissue fibroblasts to cartilaginous tissue and this conversion is not considered an example of true metaplasia.

In the initial paper presented in this thesis, further attempts were made to investigate chondrocyte dedifferentiation during larval *Ambystoma* limb regeneration. Evidence was sought which would indicate a continuous production of sulfated end products by chondrocytes as they dedifferentiated, became blastema cells, and underwent redifferentiation. Chondroitin sulfate and closely-related sulfated molecules are major constituents of the extracellular matrix of cartilage. The continuous appearance of these sulfated molecules in regenerating limb cells that were originally derived from cartilage would seem to offer further confirmation that chondrocytes remain true to type during regeneration. Consequently, at daily intervals in *Ambystoma* limb regeneration following injection of Na$_2^{35}$SO$_4$ into the body cavity of these animals, autoradiograms of the limb were prepared which recorded the uptake of radioactive sulfate by chondrocytes and cells derived from these chondrocytes.

The conception of blastema cells as embryonic in nature, in addition to stimulating theories and investigations concerned with the developmental potentiality of these cells, also has led many workers to point out analogies between limb bud formation during embryogenesis and reconstitution of the amphibian
limb by regenerative processes. During these two developmental events, several aspects of morphogenesis and cytodifferentiation exhibit similarities.

Recently, certain molecular aspects of chick limb bud development have been investigated. Searls (1965) employed autoradiographic and histochemical techniques to study the cytodifferentiation of cartilage during early stages of chick limb formation. He utilized $^{35}$S-sulfate uptake as an index of sulfated molecule production by limb bud cells and found that this label was uniformly distributed over both future chondrogenic and future myogenic areas of limbs during early developmental stages. Medoff (1967) found that the specific activity of enzymes involved in the synthesis of sulfated molecules increases during growth of the chick limb bud. These results provided experimental confirmation for theories which suggested that changing sulfate incorporation patterns were indicative of localized enzyme repression and enhancement.

In the second investigation included in this thesis, overall $^{35}$S-sulfate incorporation patterns observed during blastemal and redifferentiating stages of amphibian limb regeneration were compared with the uptake of this isotope which occurred during chick limb bud formation. It could then be determined if earlier parallels drawn between regenerating and developing limbs could be extended to include this aspect of cytodifferentiation. Anticipation of these similarities was strengthened by observations that dedifferentiating muscle and muscle-associated cells of the limb stump, as well as de-differentiating cartilage cells, displayed radioactive sulfate
uptake. Therefore, a uniformly labeled early blastema resulted, comparable to the uniformly labeled early chick limb bud. Later stages of limb regeneration were then analyzed to determine if further similarities existed.

Experimental results furnished by both papers in this thesis have permitted speculation concerning genome activity and expression in the cells of the regenerating limb and although no direct evidence is furnished to support these theories, they are presented as logical explanations for the cell behavior observed.
REFERENCES


I. Limited Dedifferentiation of Chondrocytes During Limb Regeneration in *Ambystoma* Larvae

**ABSTRACT**

Attempts were made, using combined autoradiographic and histochemical techniques, to determine if chondrocytes continued to synthesize chondroitin sulfate during and after morphological dedifferentiation of these cells in the regenerating limbs of larval *Ambystoma*. Forelimbs were amputated through the mid-diaphysis of the humerus or through the distal epiphysis and stained autoradiograms were prepared from serial sections of limbs representing daily intervals of regeneration (days 1-15 post-amputation). Each animal was injected twenty-four hours before fixation with 2.5 uc of Na\textsubscript{2}SO\textsubscript{4}. Autoradiograms of control, unamputated limbs showed that epiphyseal chondrocytes of the humerus exhibited moderate sulfate incorporation, while isotope uptake was slight in the diaphyseal region.

Epiphyseal-amputated limbs proved to be more amenable for this study. The matrix surrounding epiphyseal chondrocytes underwent very slow dissolution in response to regressive influences and these cells therefore remained spatially identifiable. The chondrocytes continued to show isotope uptake during early and middle stages of regeneration as their extracellular matrix disappeared and the dedifferentiated cells underwent proliferation. By day 11 post-amputation, redifferentiation was apparent as evidenced by matrix reappearance and an intense incorporation of $^{35}$S-sulfate by cells and matrix.
These results further confirm the idea of tissue-specific regeneration of cartilage and permit speculation regarding the extent of genomic activity during the transient stage of cell dedifferentiation.
INTRODUCTION

Studies of regenerating *Ambystoma* limbs have indicated that cartilage cells do not undergo metaplasia during formation of the regeneration blastema and subsequent reconstitution of the limb (Patrick and Briggs, 1964; Eggert, 1966; Steen, 1968; Foret, 1970). The chondrocytes dedifferentiate, but apparently redifferentiate into the same cell type. In this instance, then, the differentiated state is morphologically reversible and the cellular phenotype eventually will be re-expressed without modification. In the present investigation, attempts were made to determine if these results are reflective of nuclear stability alone, or if the cytoplasm also retains chondrocyte characteristics during the transient stage of cell dedifferentiation.

The mature chondrocyte phenotype is characterized by the presence of a morphological marker consisting of a distinct extracellular matrix. Chondrocytes also possess a relatively cell-specific biochemical marker, i.e. the step-wise, enzyme-catalyzed synthesis of sulfated mucopolysaccharides which are linked to a protein backbone (Delbrück, 1970). In the extracellular phase, these protein-polysaccharide molecules are considered to be interspersed with collagen fibrils and possibly may even be linked to these protein strands (Campo, 1970). If chondrocytes retain the ability to produce sulfated molecules while their morphological dedifferentiation is underway or has been completed, then the cytoplasmic machinery involved in the production of these molecules must remain functional during these stages.
The incorporation of labeled sulfate by cells is detectable by autoradiography and is considered to be indicative of the synthesis of chondroitin sulfate or closely-related compounds. A metachromatic response to toluidine blue indicates that the protein-polysaccharide complex has been extruded from these cells to participate in the formation of an extracellular matrix. These procedures, employed in the present investigation, also have been used in studies involving cytodifferentiation of chick limb bud cartilage (Searls, 1965, 1967; Searls and Janners, 1969). Saito (1967) utilized similar techniques in an investigation of regenerating tissues of the limb of the adult newt, *Triturus pyrogaster*.

In the present work, factors which might affect patterns of cartilage dedifferentiation were considered. Thornton (1953) noted that in injured, denervated, larval *Ambystoma* limbs, the difference in cartilage densities of the epiphyseal and diaphyseal regions resulted in dissimilar dedifferentiation responses. He found that epiphyseal hyaline cartilage persisted until late stages of limb regression, while vacuolated cartilage of the diaphyses proved much less resistant to demolition effects. Similar differences in susceptibility to regression have been observed by Trampusch and Harrebomée (1965), Steen (1968), and Foret (1970) in studies of regenerating amphibian limbs possessing transplanted skeletal units.

The relative quantities of sulfated mucopolysaccharides likely to be produced at different regions of the limb skeleton also were considered. $^{35}$S-sulfate uptake by mouse and rat
costal cartilage has been investigated using autoradiographic and biochemical analyses (Herbai, 1970a, 1970b). The main growth zone in ribs, the osteochondrial junction, exhibited intense sulfate incorporation. Medial "resting" cartilage segments which do not grow longitudinally showed low isotope uptake. Similar regional differences in $^{35}$S-sulfate uptake are to be expected in the skeletal components of Ambystoma limbs, since growth zones are known to exist at the extremities of vertebrate long bones.

Consequently, in the following procedures, it was anticipated that daily autoradiographic recordings of regenerating Ambystoma forelimbs which had been amputated through the level of the humerus epiphysis could trace chondrocytes which (1) were originally metabolically active in the production of sulfated molecules and (2) would remain spatially identifiable during blastema formation due to the slow dissolution of their extracellular matrix. Therefore, experimental variation of the level of amputation was performed in order to obtain evidence for the extent of chondrocyte dedifferentiation during regeneration.
MATERIALS AND METHODS

Ambystoma maculatum and A. opacum larvae were raised from eggs obtained locally or from Mr. Glen Gentry, Donelson, Tennessee, respectively. The larvae were maintained in individual containers of dechlorinated tap water and fed Enchytraeus three times weekly.

Larvae 31-34 mm. in length were employed in two experimental series. In the first series, forelimbs were amputated through the mid-diaphysis of the humerus, while in the second series, the amputation plane passed through the distal epiphysis. (Prior to the operations, animals were anesthetized in MS 222 at a concentration of 1:10,000). In both series, limbs representing daily intervals of regeneration (days 1-15 post amputation) were fixed in Bouin's fixative and briefly decalcified in Jenkin's Fluid before routine histological processing. Operations were performed on 6 larvae per day, 3 animals being utilized daily for each of the two amputation levels. Each animal was injected intraperitoneally 24 hours before fixation with 2.5 uc of Na$_2^{35}$SO$_4$ (specific activity $484$ mc/mM; New England Nuclear Corporation). In order to determine sulfate incorporation in the unamputated limb, 6 additional animals were fixed 24 hours following injection and processed as above. Experimental and control limb serial sections 10 microns in thickness were mounted on glass slides and prepared for autoradiography.

The slides were coated with melted Kodak NTB-2 nuclear track emulsion diluted 1:1 with distilled water, dried for 1
hour at 28°C, and stored at 5°C in light-tight boxes containing dessicant for 10-14 days. Autoradiograms were developed for 2 minutes in Kodak Dektol developer (diluted 1:1) at 15°C and fixed in Kodak F5 fixing bath for 5 minutes at the same temperature. The emulsion-coated sections were stained for metachromasia by a modification of the Quick Toluidine Blue method (Humason, 1970).

In analyzing autoradiograms for the degree of labeling intensity, an ocular grid was employed to make rough counts of silver grains found above pertinent regions of limb sections. For any given autoradiogram, the number of grains counted above segments of the proximal epiphysis arbitrarily was considered to represent "moderate" sulfate incorporation. In areas of the same autoradiogram which exhibited from 0-50% of this activity, the label was classified as "negligible" to "light", while in areas having values of 150% or higher of the proximal epiphysis counts, uptake was recorded as "heavy" to "intense".
RESULTS

Stained autoradiograms of control, unamputated limbs indicated a strong metachromatic response in both epiphyseal and diaphyseal regions of limb skeletal units. However, few silver grains appeared above diaphyseal chondrocytes or matrix. The light isotope uptake here suggested that matrix production by these cells was minimal (Figures 1 and 2). In contrast to the incorporation pattern of diaphyseal cartilage, the chondrocytes and matrix of the metaphyseal and epiphyseal regions displayed moderate isotope uptake, indicating an active involvement in the production of sulfated molecules (Figures 3 and 4).

Autoradiograms representing daily intervals in the regeneration phases of the two experimental series were examined and $^{35}$S-sulfate incorporation patterns during the preblastema, blastema, and early redifferentiation stages of regeneration were determined. Since the variation in amputation levels resulted in distinctly different morphological changes and isotope incorporation patterns during regeneration, the two series will be described separately.

$^{35}$S-Sulfate Incorporation In Limbs Amputated Through The Diaphysis

Characteristic morphological and cytological changes which follow amputation of the larval Ambystoma limb at this level were observed. During the first several days following wound healing, vacuolation of the cartilaginous matrix of the humerus occurred, the humerus was attacked by giant cells, and muscle sarcosynthesis proceeded in a distal-proximal direction. By the 7th day of regeneration there was little evidence of matrix
remaining within the humeral diaphysis, although the peri-
chondrium remained largely intact. Chondrocytes released from
their matrix demonstrated negligible uptake of $^{35}$S and were
intermingled with blood cells which had entered through peri-
chondrial perforations. Other diaphyseal chondrocytes display-
ing negligible label were accumulating at the severed end of
the humerus (Figures 5 and 6). This low degree of labeling
was not unexpected since the chondrocytes visible were origi-
ally diaphyseal components and were not actively engaged in
matrix production at the time of amputation. A moderate label
was obvious over fibroblast-like cells lateral to the humerus.
The labeling of these cells was observed in many of the auto-
radiograms prepared in both of the experimental series and will
be described and discussed in the second paper in this thesis.

Sections of limbs which had been regenerating for 8
days indicated that an extensive demolition of the distal
humerus by unlabeled giant cells was underway. A few chondro-
cytes were still apparent within the borders of the remaining
perichondrium.

Limbs regenerating for 11 days were considered to be
in the redifferentiation phase. The formation of a cartilaginous
"callus" had occurred directly lateral to the humerus, at the
limit of dissolution of the former matrix. The extracellular
matrix of cells comprising the "callus" was strongly meta-
chromatic and both cells and matrix exhibited intense isotope
uptake (Figure 7). In the interior of the humerus at this level,
chondrocytes and matrix also were labeled intensely, while more
distally, moderately labeled fibroblast-like cells apparently
had converged towards the center of the limb by passing through openings in the still-fragmenting perichondrium.

**35S Sulfate Incorporation In Limbs Amputated Through The Distal Epiphysis Of The Humerus**

By the end of the second day of regeneration, after limb amputation through the distal epiphysis, some vacuolation of the remaining hyaline cartilage had occurred. While the matrix in this region of the humerus remained intact, it produced a much lighter metachromatomatic response than the diaphyseal matrix. Epiphyseal chondrocytes were labeled moderately. There was little musculature near the amputation plane, and since muscle sarcolysis proceeds in a distal-proximal direction, little or no muscle demolition was visible (Figures 8 and 9).

Typical sections of limbs in this series which had regenerated for 4 days indicated that the remaining epiphyseal chondrocytes were still surrounded by matrix. Perichondrial disintegration had extended more proximally and the apical cap had increased considerably in thickness.

By the sixth day of regeneration, the epiphyseal chondrocytes remained in essentially the same configuration as previously. These chondrocytes and their intercellular matrix continued to exhibit moderate isotope labeling. Moderately labeled fibroblast-like cells now were visible lateral to the ex-chondrocytes and seemed to be migrating distally to participate in blastema formation.

From the above description, it is obvious that the behavior of skeletal components during days 1-6 post-amputation of limbs in this experimental series differed considerably from
responses customarily observed in the early stages of limb regeneration. Sections through limbs amputated through the epiphysis and regenerating for 7 days therefore appeared distinctly different from limbs amputated through the diaphysis and regenerating for the same period of time. The humerus matrix had undergone very slow dissolution and a distinct elongated clump of labeled ex-epiphyseal cells and their progeny extended distally into the blastema (Figure 10). These cells were now separated by only slight traces of intercellular matrix and were moderately labeled. A number of these cells were observed in various stages of mitosis which indicated that the cells were proliferating.

Sections of limbs in this series that had been regenerating for 8 days indicated that the distal epiphyseal cells still remained identifiable. The blastema had elongated considerably. The majority of the former epiphyseal components and their progeny were labeled with approximately the same moderate degree of intensity as the fibroblast-like cells which were located laterally and distally to them. Cells at the distal end of the intact perichondrium, near the original amputation plane, exhibited initial indications of increased $^{35}S$ uptake and the accelerated production of matrix associated with redifferentiation (Figures 11 and 12).

Limbs in the eleventh day of regeneration indicated that restoration of the epiphysis was well underway. Isotope uptake by cells and matrix at the distal end of the perichondrium had become intense, but the cartilaginous "callus" was much less distinct in this series than in limbs amputated through the
diaphysis. Ex-epiphyseal cells and their progeny were
surrounded by lightly staining matrix and both cells and re­
forming matrix were heavily labeled (Figure 13).
DISCUSSION

During the blastema-forming phase of amphibian limb regeneration, differentiated cells undergo a visible morphological reversion to a mesenchymatous condition. Numerous investigators have postulated that metaplasia may occur during this process. Such an occurrence would of course imply that production of specialized cell molecules ceases and blastema nuclei return to an embryonic state.

Dasgupta (1970) transplanted nuclei from Xenopus blastema cells into enucleated eggs of the same species and achieved considerable success in attaining development to post-neurula embryos. Serial transplant techniques resulted in considerable improvement in the degree of development achieved. These experiments provided a means of assessing the developmental potentiality of nuclei from dedifferentiated blastema cells and furnished evidence that the restriction of gene expression which occurs during cell differentiation (as manifested in the synthesis of specialized cell end-products) is not an irreversible effect. However, Dasgupta's findings do not reveal anything about the actual degree of dedifferentiation of these cells at the time when their nuclei were transplanted. In fact, certain nuclear transplantation experiments which involved donor nuclei from several types of fully differentiated somatic cells were equally or more successful in promoting the development of enucleated egg cells (Laskey and Gurdon, 1970).

The present investigation has attempted to consider the extent of dedifferentiation of blastema cells as measured by specialized syntheses. Although a number of stump tissues may
make cellular contributions to the blastema, this paper was limited to a consideration of chondrocyte dedifferentiation.

Speculation that dedifferentiated chondrocytes undergo metaplasia has remained unsupported by experimental evidence. Scapular cartilage grafted to x-rayed, amputated newt limbs resulted in the formation of blastemas which were derived exclusively from the grafted tissues (Eggert, 1966). Patrick and Briggs (1964), Steen (1968), and Foret (1970) employed transplants of tritiated thymidine-labeled and/or triploid cartilage into humerectomized Ambystoma limbs which were subsequently amputated. They found evidence that chondrocytes eventually redifferentiated into the same cell type during regeneration. DeBoth (1970) has shown that the range of capability of presumptive chondrocytes may be modified by environmental factors and suggested that differential gene action may be involved, but the modifications with which he was concerned resulted only in the production of skeletal elements other than those normally expected; no conversion of one cell type to another was observed.

Several previous studies on developing or regenerating limbs have attempted to detect sequential changes in the metabolic activity of chondrocytes by utilizing the same techniques employed in this investigation, i.e. $^{35}$S-sulfate incorporation patterns and metachromatic responses to toluidine blue staining. The production of chondroitin sulfate and/or other sulfated mucopolysaccharides by these cells and the extrusion of this material to become a component of the extracellular matrix consequently could be detected and conclusions drawn.
regarding the metabolic activity or degree of differentiation of the chondrocytes. These techniques have been employed to detect critical stages in the differentiation of cartilage during chick limb development (Searls, 1965), to establish that cell migration is not involved in these processes (Searls, 1967), and to determine the stage in formation of the chick limb when cartilage cells have become "stabilized" (Searls and Janners, 1969). Saito (1967) in a study of the regenerating tissues of *Triturus pyrrogaster* forelimbs, identified the stage at which incorporation of $^{35}$S-sulfate into blastema cells first became obvious. His autoradiograms showed localized isotope incorporation, first by precartilage and later by well-defined chondrocytes. These investigations concerned the adult newt and amputations were performed through the distal third of the humerus. Since no cartilage is found at this level in the bony skeleton of the adult amphibian, chondrocyte dedifferentiation was not observed.

Results from the present investigation indicated that synthetic activity typical of differentiated epiphyseal chondrocytes continued to occur as the matrix surrounding these cells gradually disappeared and the dedifferentiated chondrocytes underwent proliferation during blastemal stages. As redifferentiation of these cells took place, they exhibited intensified isotope uptake and histochemical indications of renewed matrix production. The cells remained spatially identifiable in autoradiograms prepared at daily intervals in the regenerative processes.
Possible interpretations of the continuous isotope uptake observed throughout the regenerative stages must include consideration of the gene-controlled metabolic pathways which produce sulfated, cell-specific molecules linked to a non-collagenous protein backbone, as well as the cellular components which participate in the formation and extrusion of this end-product.

Delbrück (1970) reviewed the enzyme-catalyzed reaction sequence which has been established for the synthesis of various glycosaminoglycans-protein complexes. Activation of monomeric metabolites by ATP and UTP precedes polymerization processes. In the case of chondroitin sulfate, alternating molecules of N-acetyl galactosamine and glucuronic acid (GAG) are formed into a polysaccharide chain. Sulfate activating enzymes catalyze the formation of adenosine-3'-phosphate-5'-sulfate (PAPS) and a sulfate transferase specifically transfers sulfate from PAPS to the polymer. The sulfated GAG are linked to a protein backbone by a serine-xylose-galactose-galactose bridge.

In an attempt to establish more precisely the role of cellular organelles concerned with the synthesis and transfer of these extracellular materials, Goel (1970) performed electron microscope studies on chick limbs undergoing chondrogenesis. His analyses support earlier conclusion that non-collagenous protein is synthesized in the endoplasmic reticulum and then transported to the Golgi apparatus, where it is combined with mucopolysaccharides. Citing other authors who have found evidence of the sulfation of acid mucopolysaccharides in the Golgi apparatus, he concluded that the chondrogenic "granules" which
he observed here represented sulfated protein-polysaccharide complexes.

The continuous incorporation of \(^{35}S\)-sulfate by epiphysseal chondrocytes and their progeny throughout regenerative stages indicated that cytoplasmic components necessary for the production and accumulation of chondroitin sulfate must remain operative as the epiphysseal chondrocytes dedifferentiate morphologically and become proliferating constituents of the blastema. Since the sulfation involved in chondroitin sulfate synthesis is the final step in the production of these molecules not only sulfate-activating and transferase enzymes must be present, but also those enzymes which regulate the step-wise construction of the polysaccharide moiety and formation of the protein backbone. Organelles responsible for this enzyme synthesis and accumulation of sulfated molecules apparently also remain functional. These results indicate that the cytoplasmic machinery involved in chondroitin sulfate synthesis and accumulation can be considered stable throughout the stages observed. The concept of tissue specific regeneration of cartilage cells therefore is supported, at least under the experimental conditions described here.

It is possible that messenger RNA's associated with the manufacture of specialized products may continue to be synthesized during the proliferative phase of regeneration. Cessation of gene activity coupled with the existence of stable mRNA's for production of certain enzymes in the cells under consideration would be an alternative explanation for the continuous appearance of the differentiated cell end product.
Investigations of a more direct nature would be necessary to establish conclusively the state of the genome as chondrocytes undergo morphological dedifferentiation during regeneration.
REFERENCES


EXPLANATION OF FIGURES

Note: Limb sections were mounted and photographed at an angle of 45° from the vertical. For orientation purposes, the symbols "p" and "d" are used on many photographs to indicate the proximal and distal portions of the limb, respectively.

Figure 1. The approximate junction of the distal metaphysis of the humerus (MET) and the diaphysis (DIA) in a control, unamputated limb. C-5-1. X 400

Figure 2. Higher magnification of the area outlined in Figure 1. Moderate labeling is visible in cells and matrix of the metaphysis. The diaphyseal region is lightly labeled. C-5-1. X 625
Figure 3. The distal epiphysis (EPI) in a control, un-amputated limb. C-5-1. X 400

Figure 4. Higher magnification of the area outlined in Figure 3. Moderate labeling is visible in cells and matrix. C-5-1. X 600
Figure 5. Distal end of a limb which has regenerated for 7 days following amputation through the humeral diaphysis. Chondrocytes (CH), intermingled with blood cells, are being released from the severed end of the humerus (H) to form the blastema. S-7-1-#2. X 400

Figure 6. Higher magnification of the area outlined in Figure 5. The chondrocytes exhibit only slight uptake of $^{35}$S-sulfate. S-7-1-#2. X 650
Figure 7. Central part of a limb which has regenerated for 11 days following amputation through the humeral diaphysis. An intensely labeled cartilaginous "callus" has formed, external to the perichondrium (PC) at the limit of dissolution of the diaphyseal matrix. Cells and matrix of the reconstituting humerus also exhibit intense isotope uptake (arrow). S-11-2. X 340
Figure 8. Distal end of a limb which has regenerated for 2 days following amputation through the humeral epiphysis. The matrix of the epiphyseal region has become vacuolated and is stained much less intensely than in control limbs. E-2-2-#1. X 460

Figure 9. Higher magnification of the area outlined in Figure 8. Chondrocytes have retained a moderate label. E-2-2-#1. X 690
Figure 10. Blastema of a limb which has regenerated for 7 days following amputation through the distal epiphysis of the humerus. Epiphyseal-originating cells and their progeny (EPI) remain spatially identifiable due to the slow dissolution of the original epiphyseal matrix. Arrows indicate artifactual separation of these cells from the other components of the blastema. A portion of the perichondrium and matrix of the intact distal humeral metaphysis is visible. RE-7-3-#1. X 320.
Figure 11. Blastema of a limb regenerating for 8 days following amputation through the distal humeral epiphysis. Epiphyseal cells and their progeny (EPI) remain identifiable. Fibroblast-like cells (FB) are accumulating distally as the blastema elongates. RE-8-1-#1. X 400

Figure 12. Higher magnification of the area outlined in Figure 11. Distally, epiphyseal-originating cells and their progeny remain moderately labeled. Proximally, some matrix production has resumed near the level of the original amputation and cells and matrix in this region are somewhat more heavily labeled (arrows). RE-8-1-#1. X 560
Figure 13. Central part of a limb which has been regenerating for 11 days following amputation through the humeral epiphysis. Redifferentiation of the original epiphyseal cells and their progeny is occurring and these cells exhibit heavy to intense isotope incorporation (arrows). RE-113A. X 450
II. Behavior Of Muscle And Muscle-Associated Cells

ABSTRACT

Analyses were made of stained autoradiograms originally prepared to investigate the extent of cartilage dedifferentiation in regenerating limbs of larval Ambystoma. Inspection of early regeneration stages revealed that the incorporation of $^{35}$S-sulfate was not limited to chondrocytes or blastema cells derived from chondrocytes. Fibroblast-like cells which originated from muscle or muscle-associated cells lateral to the limb skeleton also exhibited moderate isotope uptake.

Overall isotope incorporation patterns and related metachromatic responses which occurred during blastemal and early redifferentiation stages of limb regeneration were analyzed. In several respects, results were seen to parallel the results of similar autoradiographic and histochemical studies of differentiating chick limb buds. In both instances, undifferentiated cells of future myogenic and chondrogenic areas initially appeared uniform with regard to the production of sulfated molecules, but localization of isotope uptake later occurred in cell condensations in the central part of the limb bud or blastema. Subsequently, these prochondral cells commenced the production of extracellular matrix.

These data suggest that the regulation of chondroitin sulfate synthesis may be added to previous analogies concerning morphogenesis or cytodifferentiation in developing and regenerating limbs.
INTRODUCTION

The regeneration of an amphibian limb exhibits many similarities to limb formation during chick embryogenesis. The early blastema of a regenerating limb is composed of dedifferentiated, proliferating cells which have been derived from the mesodermal tissues of the limb stump. Wound epithelium covering the blastema terminates distally in an apical cap several cells in thickness. Similarly, in early states of chick limb development, undifferentiated mesodermal cells constitute the growing limb bud and the bud is surrounded by ectoderm which distally forms a thickened ridge. In both developing and regenerating limbs, cell condensation subsequently occurs in the central part of the mesenchymal cell mass, possibly related to the low oxygen tension in this region (Schmidt and Weidman, 1964). These prochondral cells then secrete extracellular matrix and the outlines of the cartilaginous skeletal elements gradually appear. Myogenesis does not occur until well after the skeleton has taken shape.

In the initial paper in this thesis, histochemical and autoradiographic techniques were employed in a study of chondrocyte dedifferentiation during larval Ambystoma limb regeneration. The incorporation of $^{35}$S-sulfate was recorded at daily intervals during regeneration as an index of chondroitin sulfate production. Identifiable epiphyseal chondrocytes gradually ceased matrix production, proliferated while in this dedifferentiated state, and eventually regained their phenotypic identity, yet continued to produce sulfated molecules throughout these stages.
In the present work, further analyses were made of the stained autoradiograms originally prepared to detect cartilage dedifferentiation patterns. Inspection of early regenerative stages revealed that $^{35}$S-sulfate uptake was not limited to chondrocytes or blastema cells derived from chondrocytes. Isotope incorporation was also displayed by fibroblast-like cells which originated from muscle or muscle-associated cells of the limb stump and apparently migrated distally. Regardless of their source, the dedifferentiated cells of the early blastema displayed a uniform uptake of $^{35}$S-sulfate. This effect, as well as subsequent localized labeling patterns and related metachromatic responses observed during redifferentiation stages of the regenerating limb proved to be similar to results obtained by Searls (1965a) in his autoradiographic and histochemical studies of differentiating chick limb buds.

The experiments reported here were performed in order to determine whether the regulation of chondroitin sulfate synthesis may be added to previous analogies concerning morphogenesis or cytodifferentiation in developing andregenerating limbs. Several authors (Searls, 1965a; Medoff, 1967; Zwilling, 1968) have suggested that localized enzyme amplification and repression may serve as a regulatory mechanism for the synthesis of chondroitin sulfate during chick limb development and it is reasonable to speculate that similar controls for the production of this macromolecule may be operative during limb regeneration.
MATERIALS AND METHODS

Autoradiographic evidence of $^{35}$S-sulfate incorporation by cells of regenerating larval Ambystoma limbs is employed in the present work. These records consisted of toluidine blue-stained autoradiograms prepared from serial sections of 6 control, unamputated limbs and limbs representing daily intervals in the regeneration of two experimental series. In the first series, limb amputation had been performed through the humeral diaphysis, while in the second series, the amputation plane passed through the distal epiphysis. Three animals were utilized daily for each of the two amputation levels. Each control and experimental animal had received 2.5 uc of Na$_2$$^{35}$SO$_4$ by intrapleuroperitoneal injection twenty-four hours prior to the fixation of the limbs for histological processing. The autoradiographic records originally were intended to exhibit the extent of chondrocyte dedifferentiation during regeneration and details of their preparation and analysis can be found by reference to the initial paper in this thesis.
RESULTS

Stained autoradiograms of control, unamputated limb sections indicated a negligible or light uptake of $^{35}$S-sulfate by musculature lateral to the limb skeleton, loose connective tissue, epidermis, and the humeral diaphysis and perichondrium. Cells and matrix of the humeral epiphyses and metaphyses were moderately labeled.

In sections of diaphyseal-amputated limbs which had been regenerating for 5 days, the lightly labeled matrix of the humerus had become highly vacuolated distally in response to regressive influences. Extensive muscle sarcolysis extending proximally to the mid-diaphyseal level, was evident. Moderately labeled fibroblast-like cells were accumulating lateral to the perichondrium and distal to the intact musculature (Figures 1 and 2). In sections of epiphyseal-amputated limbs which had been regenerating for the same period of time, the shaft of the humerus appeared unaffected by regressive influences. The matrix of the humeral epiphysis had undergone slow dissolution and chondrocytes originating from this region remained spatially identifiable. Muscle sarcolysis had not extended as far proximally as in diaphyseal-amputated limbs since less musculature was present at the original amputation site. However, some moderately labeled fibroblast-like cells were found distal to the intact musculature and lateral to the moderately labeled epiphyseal chondrocytes and lightly labeled diaphysis of the humerus.

By the 7th day of regeneration, muscle sarcolysis had
proceeded more proximally in both series and the moderately labeled fibroblast-like cells were correspondingly more numerous. These cells were especially conspicuous in the diaphyseal-amputated series. Here, they were located laterally to the still intact perichondrium of the humerus which surrounded only a few chondrocytes exhibiting negligible label (Figures 3 and 4). In the epiphyseal-amputated series, some fibroblast-like cells apparently had migrated apically to participate in formation of the blastema and could be seen both laterally and distally to the dedifferentiated, proliferating chondrocytes. Both types of cells appeared to be moderately labeled.

In autoradiograms of diaphyseal-amputated limbs which had been regenerating for the interval between 8 and 11 days, the fibroblast-like cells apparently had migrated distally and also seemed to have moved to the central part of the limb through perforations in the fragmenting perichondrium. By the time demolition of the perichondrium was completed, a substantial blastema had formed in this matter. The intermingling of the lightly labeled chondrocytes of the diaphysis with the more numerous, moderately labeled fibroblast-like cells resulted in a blastema composed of cells which gave the impression of uniform $^{35}$S-sulfate incorporation. In sections of 11 day regenerates of this series, intensive label was observed at the most distal part of the intact perichondrium, indicating the formation of a cartilaginous "callus" here. A metachromatic response also was visible in this region.
Records of the 8-11 day interval of regeneration in epiphyseal-amputated limbs showed marked blastema elongation, largely as a result of proliferation of the fibroblast-like cells distal to cells originating from the epiphysis. Both types of cells remained labeled. Signs of redifferentiation (noticeably heavier $^{35}$S-sulfate uptake and renewed metachromasia) gradually became evident in the proximal part of the epiphysial-originating chondrocytes, although a much less noticeable cartilaginous "callus" formed near the distal end of the perichondrium than was seen in limbs of the diaphyseal series.

In autoradiograms of limbs which had been regenerating for days 12-14 in both experimental series, cell condensation gradually appeared in the central part of the blastema following a proximo-distal gradient. Subsequently, labeling became heavy to intense in these cells. The lateral parts of the blastema now were labeled only lightly. Matrix secretion, as evidenced by metachromatic staining of extracellular material produced by the condensed cells, appeared approximately 1 day after a differential labeling pattern had been established and followed the same proximo-distal gradient (Figures 5 and 6).
DISCUSSION

One of the established results of "demolition effects" which occur during early phases of amphibian limb regeneration is the production of fibroblast-like cells at the sarcoelyzing ends of injured stump musculature.

The precise origin of the fibroblast-like cells has remained an unsettled question. Chalkley (1954, 1959) counted mitoses in dedifferentiating stump tissues of regenerating adult newt limbs. From these results, he concluded that dedifferentiating muscle accounts for a minimum of 8% of blastema cells. Other workers have employed light and electron microscope studies to investigate this problem. Results of Thornton's (1938) light microscope studies of regenerating larval Ambystoma limbs indicated a myogenic origin for the fibroblast-like cells. Using light and electron microscope studies, Hay (1959) arrived at the same conclusion and stated that the muscle-derived cells probably constituted at least 25% of the blastema. Recently, Lentz (1969) has interpreted his light and electron microscope observations of regenerating Triturus limbs as providing further substantiation for a complete dedifferentiation of muscle cells to mesenchymal blastema cells. Schmidt (1968) presented a conflicting viewpoint. In his opinion, the connective tissues of the adult newt are the sole source of supply for the regeneration blastema. He regards the dedifferentiated cells appearing adjacent to fragmenting muscle fibers as fibroblasts which originated from myseal sheath connective tissue and he proposed that these "activated fibroblasts" proliferate and mi-
grate apically to participate in blastema formation.

In the present work, autoradiographic evidence indicated that the fibroblast-like cells displayed a moderate uptake of $^{35}$S-sulfate at the time when they were first identifiable and continued to incorporate this isotope as they apparently contributed to the blastema. In epiphyseal-amputated limbs, the blastema formed somewhat differently from limbs of the diaphyseal-amputated series and epiphyseal chondrocytes which participated in blastema formation were identifiable as they de-differentiated and proliferated. However, the net effect in both series was an early blastema in which the dedifferentiated cells derived from the mesodermal tissues of the stump appeared to exhibit a uniform uptake of $^{35}$S-sulfate.

Searls (1965a) studied uptake of $^{35}$S-sulfate in differentiating chick limb buds. His autoradiographic evidence demonstrated that mesenchymal limb bud cells were uniform with respect to the uptake of this isotope until the middle of stage 22. Similarities between the early blastema of a regeneration of Ambystoma limb and the chick limb bud during early stages of its formation are therefore not limited to morphological resemblances. In both instances, undifferentiated cells of future myogenic and chondrogenic areas appeared uniform with regard to the production of sulfated molecules.

Searls (1965b) and Medoff (1967) attempted to identify the sulfated material produced during early stages of chick limb bud development. Their results have indicated that the balance of this material is identical to the sulfated mucopolysaccharide which is regarded as the end product of differ-
entiated cartilage metabolism. Searls (1965b) biochemical analyses of radioactive material from stage 22 limbs suggested the presence of chondroitin sulfate A and C and a small amount of B at this stage. Biochemical analyses were also employed by Medoff (1967) in a study of mucopolysaccharide synthesis by cells of chick limb buds at various stages in embryonic development. She assayed not only for the presence of sulfated material, but also for several enzymes involved in its biosynthesis. Her results indicated that chondroitin sulfate and three enzymes participating in the synthesis of this product are present in the presumptive limb tissue as early as stage 15.

The analogous patterns of sulfated molecule synthesis which were exhibited by cells of developing and regenerating limbs at early stages of morphogenesis continued to be manifest when autoradiograms of later regenerative stages were studied. In late blastemas of regenerating Ambystoma limbs an intensification of label appeared in centrally located blastema cells that were undergoing condensation in a proximo-distal gradient and a concomitant decline of isotope uptake occurred peripheral to this core of cells. These results parallel Searls (1965a) autoradiographic records of radioactive sulfate uptake from late stage 22 through stage 24 of chick limb formation. He reported that a localization of $^{35}$S-sulfate label was observed in these stages above condensed, centrally-located cells in the proximal part of the limb bud, while isotope incorporation gradually declined in future myogenic regions. In autoradiograms of regenerating limbs, a metachromatic response to toluidine blue staining first appeared between condensed
blastema cells showing localized $^{35}$S-sulfate uptake, approximately a day after the uptake pattern had become definitive. Similarly, Searls (1965a) noted that metachromasia was initially detected in the central core of limb bud cells at stage 25. In both instances, then, presumptive chondrocytes demonstrated an increased rate of chondroitin sulfate synthesis prior to expression of their cellular phenotype.

It should be noted that $^{35}$S-sulfate uptake patterns exhibited by cells of redifferentiating, regenerating limbs showed some differences from the incorporation patterns of differentiating chick limb buds. In autoradiograms of late blastemal stages of regenerating limbs, the condensation of blastema cells was preceded by the formation of an intensely-labeled, metachromatically-responding cartilaginous "callus" at the distal end of the shaft (in limbs of the diaphyseal-amputated series) or by the intensified isotope uptake and renewed metachromasia of redifferentiating epiphyseal-derived chondrocytes (in limbs of the epiphyseal-amputated series). In both instances, this redifferentiating cartilage eventually fused with cartilage derived from cell condensations as reformation of the limb skeleton took place.

Searls (1965a, 1967) proposed that the change from a uniform incorporation of $^{35}$S-sulfate by all of the mesenchymal cells of the limb bud to a pattern of preferential incorporation during later stages in embryogenesis could occur in either of two ways: (1) By migration of cells capable of synthesizing mucopolysaccharides to the center of the limb starting at stage
22, or (2) by a mechanism which controlled the relative rates of mucopolysaccharide synthesis by localization of the enzymes responsible for this synthesis at the center of the limb. He then proceeded to rule out the first possibility by transplanting labeled tissue from future chondrogenic areas of the limb bud to other limb regions and employing autoradiographic techniques to record the fate of these cells during development (Searls, 1967). No evidence of cell migration was detected.

Medoff (1968) investigated the developmental kinetics of three enzymes (sulfating-activating enzyme, UDPG dehydrogenase, and UDPN Ac-4-epimerase) in chick limb bud cells differentiating in vivo and in vitro. These enzymes are involved in the synthesis of sulfated glycosaminoglycans. Her results established that the specific activity for all three enzymes both in vivo and in vitro was low prior to chondrogenic differentiation, but rose steadily as cartilage became histologically identifiable. She interpreted these findings as confirming Searls (1967) alternative hypothesis for the localization of chondroitin sulfate synthesis during limb development. Her results indicated that gradual restriction of $^{35}$S-sulfate uptake to chondrogenic regions of developing limb buds involved a progressive enhancement of pre-existing enzyme activity in this region and repression of this same enzyme activity in myogenic regions of the limb.

Zwilling (1967) summarized the data of Searls (1965a) and Medoff (1967) and concluded that genome activation of chondroitin sulfate synthesis occurs in all mesoderm limb tissues before active cytodifferentiation of cartilage is initiated,
but chondrogenesis will only take place in those cells where there is an augmentation of this synthetic activity. Whether control of this augmentation occurs at the transcriptional or translational level is unknown (E. Zwilling, 1970 meetings of the Society for Developmental Biology).

It is possible to speculate that a derepression of the synthesis of enzymes involved in the production of chondroitin sulfate may be one of the molecular events taking place as muscle or muscle-associate cells dedifferentiate and apparently become blastemal components during amphibian limb regeneration. At any rate, it seems reasonable to suggest that the regulation of chondroitin sulfate synthesis during de novo cartilage differentiation is analogous in both developing and regenerating limbs. The synthesis of this macromolecule appears to be an initial step in cytodifferentiation of limb mesodermal tissues during regeneration as well as embryonic development, regardless of the ultimate fate of these cells.
REFERENCES


Note: Limb sections were mounted and photographed at an angle of \( 45^\circ \) from the vertical. For orientation purposes, the symbols "p" and "d" are used on many photographs to indicate the proximal and distal portions of the limb, respectively.

Figure 1. Central part of a limb which has regenerated for 5 days following amputation through the humeral diaphysis. The approximate boundary is shown between intact musculature and fibroblast-like cells accumulating distally near sarcolyzing muscle. The matrix of the diaphysis (DIA) has become highly vacuolated. S-5-3-#2. X 400

Figure 2. Higher magnification of the area outlined in Figure 1. Fibroblast-like cells (FB) are moderately labeled. Intact musculature (MUS) exhibits only slight uptake of \( ^{35}S \)-sulfate. S-5-3-#2. X 600
Figure 3. Central part of a limb which has regenerated for 7 days following amputation through the humeral diaphysis. The diaphyseal perichondrium (PC) remains intact, but the matrix in this region has undergone dissolution, liberating the chondrocytes it formerly surrounded. Numerous fibroblast-like cells (FB) have accumulated lateral to the perichondrium. S-7-l-#2. X 280

Figure 4. Higher magnification of the area outlined in Figure 3. The few remaining diaphyseal chondrocytes exhibit negligible labeling. Fibroblast-like cells (FB) are moderately labeled. S-7-l-#2. X 480
Figure 5. Distal part of a limb which has regenerated for 13 days following amputation through the humeral epiphysis. Condensed prochondral cells (CH) are visible in the central part of the blastema, distal to epiphyseal-originating cells and their progeny. Matrix production is occurring in a proximo-distal gradient. Distally, cell condensation is still occurring (arrows). RE-13-1. X 345

Figure 6. Higher magnification of the area outlined in Figure 5. The condensed core of cells exhibits heavy to intense uptake. Peripheral myogenic areas (MYO) are labeled slightly. RE-13-1. X 600