COLLAGENASE ACTIVITY IN REGENERATING AND DENERVATION-INDUCED REGRESSING FORELIMBS OF LARVAL AMBYSTOMA MEXICANUM

Dissertation for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

BARBARA P. JOHNSON-MULLER

1976

This is to certify that the

thesis entitled

COLLAGENASE ACTIVITY IN REGENERATING AND DENERVATION-INDUCED REGRESSING FORELIMBS OF LARVAL AMBYSTOMA MEXICANUM

presented by

Barbara P. Johnson-Muller

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Zoology

Stephen C Brown

Major professor

Date Nov. 4, 1976

ABSTRACT

COLLAGENASE ACTIVITY IN REGENERATING AND
DENERVATION-INDUCED REGRESSING FORELIMBS
OF LARVAL AMBYSTOMA MEXICANUM

by

Barbara P. Johnson-Muller

Both regenerating and denervation-induced regressing larval urodele limbs undergo a period of histolysis following amputation and wound closure. During this period phagocytic cells remove cellular and extracellular debris, extracellular matricies disappear, and dedifferentiated cells are released from limb stump tissues. In regenerating limbs dedifferentiated cells form a proliferating blastema, and histolysis of the stump subsides when the blastema begins to differentiate. In amputated, nerveless limbs no blastema forms, and histolysis of the stump continues, resulting in complete resorption of the limb. The factors controlling, and the mechanisms underlying histolysis of regenerating and resorbing larval limbs are not fully understood. However, there is histological evidence that collagen degradation is involved in both processes, and a collagenase has been detected in regenerating adult urodele limbs during the period of limb stump histolysis.

The experiments in this investigation were designed 1) to investigate collagenase activity in regenerating and denervation-induced regressing larval Ambystoma mexicanum forelimbs to assess the phases of regeneration and regression in which collagenase activity is significantly

above unamputated limb tissue levels, and 2) to partially characterize larval A. mexicanum collagenase.

Collagenase activity in larval limb tissues was assayed using the radioactive reconstituted collagen fibril assay at 25°C and 36°C at pH 7.6. This assay was run in conjunction with 1) dialysis of degradation products formed at 36°C to demonstrate that collagen was degraded to small peptides, and 2) disc-gel electrophoresis of degradation products formed at 25°C to demonstrate collagenase characteristic cleavage of collagen to TC^A and TC^B subunits. Crude collagenase extract was obtained by direct extraction of lyophilized tissue with a neutral buffer.

In this investigation the pattern of collagenase activity coincides with the pattern of limb stump histolysis in both regenerating and denervation-induced regressing larval limbs. This suggests that collagen degradation by collagenase is one of the mechanisms underlying histolysis of amputated larval limbs whether or not they subsequently regenerate.

The pattern of collagenase activity in regenerating and denervated regressing limbs differs in this study. In both cases, collagenase activity rises rapidly following amputation to maximum levels. However, collagenase activity in regenerating limbs then returns to essentially normal limb tissue levels, while collagenase activity in denervated regressing limbs remains high. Since nerves stimulate proliferation of the blastema, these results indicate that the blastema may be inhibiting collagenase activity in regenerating limbs, perhaps by producing a collagenase inhibitor.

Collagenase activity was not directly related to the protein content of crude enzyme extracts in this study, suggesting that specific

changes in collagenase activity occur during regeneration and regression of larval urodele limbs.

Larval A. mexicanum collagenase in this investigation shares attributes with other vertebrate collagenases. It is active at slightly alkaline pH, is inhibited by EDTA and mammalian serum, and is not inhibited by cysteine or soybean trypsin inhibitor. It also degrades collagen at 36°C to dialyzable peptides, and at 25°C to characteristic TC^{A}_{75} and TC^{B} subunits, as well as to several discrete slightly smaller TC^{A} subunits. In addition, crude collagenase extract from regenerating limbs, but not from regressing limbs, contains a neutral protease which converts β to α subunits, indicating that connective tissue may be degraded differently in regenerating than in denervated regressing larval limbs.

COLLAGENASE ACTIVITY IN REGENERATING AND DENERVATION-INDUCED REGRESSING FORELIMBS OF LARVAL AMBYSTOMA MEXICANUM

By

Barbara P. Johnson-Muller

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1976

TO DAVID

ACKNOWLEDGMENTS

The author wishes to express thanks to Dr. Stephen Bromley for his special encouragement during the final stages of preparation of this thesis.

Graditude is also extended to the members of my committee, Dr. Stephen Bromley, Dr. Thomas Connelly, Dr. Evelyn Rivera, and Dr. Charles Tweedle, for their editorial assistance in preparation of the thesis.

Special thanks are extended to Dr. Jerome Gross for helpful suggestions concerning the experimentation in this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE Page

FIGURE Page

INTRODUCTION

Regenerating urodele limbs undergo a period of histolysis following amputation and wound closure which is essential to the regenerative process (Carlson, 1974 for review). During this period phagocytic cells remove cellular and extracellular debris, extracellular matricies disappear, and dedifferentiated cells are released from limb stump tissues. These dedifferentiated cells in turn form a proliferating blastema and develop into a new limb. Hence, histolysis of limb stump tissues allows the emergence of the population of cells which become the regeneration blastema and ultimately the new appendage. The factors leading to and controlling histolysis in regenerating limbs, and the mechanisms involved in the process, however, are not fully understood.

Regulation of histolysis, when analyzed at the anatomical or histological level, differs in larval and adult urodeles. In larval limbs, histolysis can be induced by damaging the mesodermal tissues of the limb, and can be prolonged or intensified, or both, by additional experimental manipulations of the limb. Larval limbs which have been amputated and then X-rayed (Butler, 1933; Butler, 1935), UV irradiated (Blum, et al, 1957), treated with colchicine (Thornton, 1943), or denervated (Butler and Schotte, 1941; Schotte and Butler, 1941) undergo extensive histolysis and regression, fail to regenerate, and often are completely resorbed. Similarly, unamputated larval limbs which have been

denervated and subjected to mesodermal tissue damage by breaking a skeletal element undergo extensive regression (Thornton and Kraemer, 1951). Histolysis stops in regenerating larval limbs when a blastema forms (Butler and Puckett, 1940), and may be inhibited by the blastema. Hence, denervated, amputated limbs, which ordinarily undergo extensive histolysis and regression (Butler and Schotte, 1941), will not regress if the limb has developed a blastema before it is denervated (Schotte and Butler, 1944). Likewise, if an undifferentiated blastema is transplanted to the stump of a freshly amputated and denervated limb, the limb will not regress (Schotte, et al., 1941).

In adult urodeles, on the other hand, the mesodermal tissues of the limb are more stable, and histolysis of limb tissues is difficult to maintain. As in larval urodeles, limb amputation in adults induces a period of histolysis which subsides coincident with blastema formation (Norman and Schmidt, 1967). However, experimental manipulations which cause extensive histolysis in larval limbs, cause histolysis to stop in adult limbs. Amputated adult limbs which have been X-rayed (Schmidt, 1968), or denervated (Rose, 1948; Singer and Craven, 1948) do not undergo extensive histolysis and regression, but instead form scar tissue at the tip of the stump.

Despite these differences in its regulation, the process of histolysis itself appears at the histological level to be very similar in larval and adult urodeles. In both larval and adult animals histolysis involves a loss of the formed structures of the limb. In regenerating limbs breakdown of the extracellular matricies of skeleton, dermis, perichondrium, and perineurium has been observed during the regressive phase. Following amputation, the cartilage skeleton of A. maculatum

larvae undergoes erosion involving an extensive area in the distal region of the appendicular skeleton, and the matrix in that region gradually disappears (Butler, 1933; Butler and Schotte, 1941; Hay, 1958). Similarly, injured bone in adult N. viridescens undergoes extensive erosion following amputation (Schmidt, 1968). The dermis of adult newts loses its adepidermal reticulum of fibers during the first phase of regeneration. Collagen fibrils fragment and disappear altogether (Norman and Schmidt, 1967). Perichondrium in amputated limbs of A. maculatum larvae becomes free from the distal end of cartilage, which is simultaneously undergoing dissolution, and disappears around the region of dedifferentiated cartilage (Butler, 1933). In both larval A. maculatum (Thornton, 1938), and adult newts (Norman and Schmidt, 1967), a well organized perineurium is lacking around nerve bundles in the region just proximal to the plane of amputation, and in some cases the perineurium is completely lost, and individual nerves separate from each other.

Similarly, in experimentally induced regressing larval limbs, continued breakdown of the extracellular matricies of the skeleton and perichondrium has been observed. During the extensive histolysis which accompanies resorption of denervated amputated limbs, extreme vacuolation of skeletal elements continues proximally, with distal portions of skeleton collapsing, until the entire skeleton gradually disappears (Butler and Schotte, 1941). During this process, cartilage was described as "melting away in all directions", and it was suggested that "some type of chemical activity" was primarily responsible for cartilage regression. At the same time in the same experimental limbs, perforations appear in perichondrial envelopes, and the envelopes subsequently fold, breakdown, and finally disappear.

Since, as in other vertebrates, collagen is the major structural protein of the extracellular matrices of the limb, and urodele collagen is a typical vertebrate collagen (Mailman, et al., 1974), collagen degradation is presumably one of the mechanisms underlying histolysis of regenerating and regressing limbs. Because native collagen is resistant to general tissue proteases, degradation of collagen fibers in tissues is a complex process which is initiated by the tissue collagenases. To better understand the breakdown of limb collagen, a brief discussion of the properties of collagen and tissue collagenases is appropriate. The information on collagen and tissue collagenases which follows, unless otherwise stated, was obtained from several review articles (Gross, et al., 1963; Seifter and Harper, 1971; Gallop, et al., 1972; Lazarus, 1973; Trelstad, 1973; Harris and Krane, 1974).

The collagen molecule, tropocollagen (TC), is composed of three polypeptide chains, called α chains, each with a molecular weight of approximately 100,000. The α chains can be covalently cross-linked by telopeptides to form dimers (β components), or trimers (γ components). The overall conformation of TC is a helix with short non-helical regions at both ends. Individual α chains are coiled in a left-handed polyglycine or poly-L-proline II structure, and the three α chains are wound around each other to form a coiled-coil structure, designated the major helix. The helical structure of TC is determined by the repeating gly-x-y sequence of the α chains, and by the stabilizing influence of proline and hydroxyproline which together make up 20% of the amino acids of TC. Alpha chains represent at least five different gene products, α 1(I), α 1(II), α 1(III), α 1(IV), and α 2, and so far, four different TC molecules from different combinations of these chains have

been identified. These TC molecules include $[\alpha 1(I)]_2\alpha 2$ found primarily in skin, tendon, bone and ligaments (Miller, et al., 1967; Miller, et al., 1971; Trelstad, 1974), $[\alpha 1(II)]_3$ found in cartilage (Miller and Matukas, 1969; Trelstad, et al., 1970; Miller, et al., 1971; Strawich and Nimni, 1971; Linsenmayer, 1974), $[\alpha 1(III)]_3$ found in aorta and fetal human skin (Miller, et al., 1971; Chung and Miller, 1974; Trelstad, 1974), and $[\alpha 1(IV)]_3$ found in basement membranes (Kefalides, 1971; Trelstad, 1974). Once in the extracellular space, tropocollagen subunits aggregate with one another to form fibrils, and the fibrils further organize into an orthogonally oriented lattice work, or are randomly interlaced with each other depending on the tissue and species. While in fibrils, the TC molecules develop cross-links within single molecules and among adjacent molecules, which stabilize the fibrils, and, depending on the degree of cross-linking, cause them to become insoluble.

Studies on the physical properties of collagen in solution indicate that the resistance of collagen to proteolytic degradation is due to its helical conformation in the native state. Tropocollagen molecules, which are long rigid helical rods (15 X 3000 Å) in the native state, can be denatured over a relatively narrow temperature range to the random coil conformation of gelatin. Native collagen in solution at neutral pH denatures at very close to 37°C; native collagen fibrils at neutral pH do not denature until 55°-60°C. Gelatin, or denatured collagen, is readily degraded by most tissue proteases, while native collagen, at the neutral pH values characteristic of connective tissues, is largely resistant to attack by most proteases (Gross and Lapiere, 1962; Werb and Reynolds, 1974; Steven, et al., 1975). Some regions of the native TC molecule at neutral pH, however, are more susceptible to general proteolytic attack

than others. In high concentrations, trypsin slowly and in small increments degrades as much as 25% of the TC molecule from the carboxy-terminal end. Proteolytic enzymes such as pepsin and chymotrypsin can degrade the short non-helical and telopeptide regions of native collagen, causing the molecule to be more soluble in aqueous solution, but not affecting its helical native form (Drake, et al., 1966). At acid pH values, cathepsin B1, a lysosomal proteinase, can degrade collagen fibrils and insoluble collagen extensively by multiple cleavages of the helical region (Burleigh, et al., 1974).

In general, however, at the neutral pH values of extracellular fluids, initiation of native collagen degradation requires a true collagenase. Collagenases have been defined as "enzymes capable of degrading native collagen fibrils under physiological conditions of temperature and pH, or are enzymes which cleave native collagen molecules in solution through the characteristic helical part of the molecule" (Lazarus, 1973). The mechanism of action of vertebrate collagenase on the native collagen substrate is unique. It cleaves the TC molecule across all three α chains at one specific bond in each chain (Gross, et al., 1974), producing a fragment 75% of the original length (TC^{A}_{75}), including the A (NH_2) terminus, and a 25% fragment (TC^{B}), including the B (COOH) terminus (Kang, et al., 1966). The TC^{A} fragments produced include both α^{A} and β^{A} fragments. In addition to TC^{A}_{75}, some purified collagenases (from rat and newt) produce TC^{A}_{67} and TC^{A}_{62} fragments, although it is not known if the additional fragments are actually produced by the collagenase or by another contaminating protease.

Collagenases are neutral metal proteinases (Hartley, 1960). As such, they are most active at neutral to slightly alkaline pH, require

Ca^{++} for activity, and are inhibited by EDTA. In addition, some tissue collagenases are inhibited by reagents containing free sulfhydryl groups, such as cysteine, however, none are inhibited by serine proteinase inhibitors such as soybean trypsin inhibitor.

Crude enzyme extracts of collagenase which contain other proteases characteristically degrade native collagen fibrils to small dialyzable peptides at $37^{o}C$, and to undialyzable TC^{A} and TC^{B} fragments at $25^{o}C$.

Vertebrate collagenases, like collagen, are ubiquitous, and have been detected in a number of collagen containing tissues, and in cells involved in collagen breakdown. Among the tissues and cells collagenases have been found in are human skin in vivo and in vitro (Eisen, et al., 1968; Eisen, et al., 1971), papillary dermis of skin (Reddick, et al., 1974), culture medium of fibroblast-like cells (Hook, et al., 1973; Reddick, et al., 1974; Werb and Burleigh, 1974; Werb and Reynolds, 1974), basement lamella (Nagai and Hori, 1972), culture medium of granulation tissue and wound epithelium of cutaneous wounds (Grillo and Gross, 1967; Donoff, et al., 1971) culture medium of epithelial cells of metamorphosing tadpole tail fin (Eisen and Gross, 1965), inflamed rheumatoid synovium in vivo (Bauer, et al., 1971), bone in culture (Walker, et al., 1964; Vaes, 1972), culture medium of activated macrophages (Senior, et al., 1972; Wahl, et al., 1974; Bauer, et al., 1975; Wahl, et al., 1975; Werb and Gordon , 1975), Kupffer cells of liver (Fujiwara, et al., 1973), culture medium of giant cells (Salthouse and Matlaga, 1972), polymorphonuclear leukocyte granules (Lazarus, et al., 1968; Robertson, et al., 1972), cultured remodeling tissues of metamorphosing tadpoles (Gross and Lapiere, 1962), and the culture medium of resorbing postpartum rat uterus (Jeffrey and Gross, 1970; Jeffrey, et al., 1971).

Collagenase degradation of collagen *in vivo* and *in vitro* is primarily regulated by the amount of enzyme present, the form the enzyme is in, and the presence of inhibitors. Except in polymorphonuclear leukocytes, where it is stored in granules, collagenase is synthesized and secreted without storage. This synthesis and secretion has been shown to be inducible in some collagenase producing cell types. Phagocytosis of and continued intravacuolar storage of indigestible particles stimulate macrophages (Werb and Gordon, 1975) and synovial fibroblast-like cells (Werb and Reynolds, 1974) to produce collagenase in tissue culture. Similarly, products secreted by mitogen or antigen stimulated lymphocytes (lymphokines) induce macrophages to produce collagenase *in vitro* (Wahl, et al., 1975). Some collagenases are secreted and stored in tissues as an inactive zymogen or procollagenase, which can be converted to the enzymatically active form by a tissue protease activator (Harper, et al., 1971; Harper and Gross, 1972; Vaes, 1972; Lazarus and Goggins, 1974; Bauer, et al., 1975). Inhibitors of collagenase which form stable, inactive complexes with the enzyme, are present in extracellular fluids. Active collagenase has been shown to be inhibited by the mammalian serum anti-proteinase, α_2-macroglobulin (Bauer, et al., 1971; Eisen, et al., 1971; Abe and Nagai, 1973), by a low molecular weight protein in rheumatoid synovial fluid (Harris, et al., 1969; Abe and Nagai, 1973), and by a factor, about the same size as the collagenase itself, in the culture medium of fibroblast-like cells (Bauer, et al., 1975).

The role of collagenase in connective tissue degradation is thought to be to facilitate primary collagenolysis. It has been suggested that connective tissue collagen fibrils are degraded in a two stage process.

In the first stage, collagenase is secreted into the extracellular matrix, and cleaves collagen molecules in the fibrils into characteristic TC^A and TC^B fragments. These fragments then disperse in the surrounding extracellular fluids, and denature into gelatin because of their lower denaturation temperature (32^oC). In the second phase of degradation, the denatured fragments are broken down by other extracellular neutral tissue proteases into small peptides (Sakai and Gross, 1967; Lazarus, et al., 1968; McCroskery, et al., 1973). Or, alternatively, primary collagenolysis results in small fibril fragments being dissociated from collagen fibrils, and phagocytosed by macrophages. In the second stage of degradation, in this case, collagen is degraded within phagolysosomes at acid pH by proteases such as cathepsin B1 (Burleigh, et al., 1974).

Native collagen in solution or as fibrils must be used as the substrate in assays for collagenase. Breakdown of collagen molecules in solution can be followed by measuring the loss of specific viscosity of the solution. Breakdown of collagen fibrils can be followed by measuring the release of radioactive degradation products into solution from radioactive collagen gels, and by demonstrating that the soluble components are smaller molecules than the original TC molecules and not just solubilized TC molecules. Evidence that the collagen molecule has been characteristically cleaved into TC^A and TC^B fragments can be obtained from SLS crystallites, or from disc-gel electrophoresis of the reaction products.

As was discussed earlier, there is considerable histological evidence of breakdown of the collagenous structures of the limb during the early histolytic phase of regeneration, and throughout the continuing histolysis which characterizes experimentally induced regression. The

biochemical evidence of collagen degradation during histolysis, however, is contradictory. Studies of collagen breakdown during regeneration, where collagenase activity and collagen metabolism were measured, do not agree with each other. Cultured regenerates from adult newts were assayed for their ability to degrade native collagen fibrils, and were found to have maximum collagenolytic activity 15 days post amputation which gradually diminished by 30-35 days post amputation (Grillo, et al., 1968). In addition, highest collagenase activity was found in the stump tissue immediately adjacent to the blastema, with the blastema next most active. These results coincide temporally and spatially with limb histolysis during regeneration, and support the idea that collagenase is initiating degradation of limb collagen during histolysis. On the other hand, a study of collagen metabolism during regeneration of boneless, adult newt limbs, indicates that the amount and synthesis of stump collagen does not change significantly during regeneration (Johnson and Schmidt, 1974). In addition, the same study shows relatively high levels of newly synthesized collagen in the developing blastema with no concomitant formation of insoluble collagen fibrils,which together indicate degradation. These results were interpreted to mean that collagen degradation in early regeneration is occurring primarily in the developing blastema, and not in the existing extracellular matrix of the limb stump. Another study of collagen metabolism during regeneration of adult newt limbs, where just regenerates and not stump tissue were considered, shows that collagen turnover is low during formation of the blastema, and that the period of maximum collagen turnover occurs 5-7 weeks post amputation during digit formation (Mailman and Dresden, 1976).

Since little collagen turnover is occurring in early blastemata in this study, these results support the conclusion of the collagenase study (Grillo, et al., 1968), in which it was demonstrated that collagenase present during the early stages of regeneration is acting primarily on stump collagen. However, high collagen turnover in digit regenerates in this study, suggests the presence of a tissue collagenase during the period 5-7 weeks post amputation which was not detected in the previous collagenase assay (Grillo, et al., 1968).

Part of the reason for the contadictory results regarding when and where collagen is being degraded in regenerating limbs may reside in the experimental procedures employed. In the collagenase study, for instance, cultured blastemata were used to obtain active enzyme (Grillo, et al., 1968). Consequently, it is not clear whether collagenase activity detected by the assay actually reflects the endogenous levels of active enzyme in the regenerating limbs, or de novo synthesis of collagenase by the tissue explants in response to culture. Similarly, the use of boneless limbs to study collagen metabolism in the limb stump during regeneration (Johnson and Schmidt, 1974) may yield misleading results, since the bones are a majoe collagen containing structure of the limb and are known to be eroded during histolysis (Schmidt, 1968).

It is not clear from the biochemical studies mentioned above if limb stump collagen is being degraded during histolysis in regenerating adult newt limbs. Since part of this uncertainty is due to experimental procedure, and since histolysis can be experimentally manipulated in amputated larval urodele limbs, the experiments in this investigation were designed to study collagenase activity in regenerating and experimentally-induced regressing larval Ambystoma mexicanum limbs using a

direct extraction technique to obtain active enzyme. Since most cell types capable of producing collagenase do not store it (Harris and Krane, 1974), direct extraction of limb tissues should reflect endogenous levels of collagenase activity. The time course of collagenase activity in normal regenerating and denervation-induced regressing larval limbs was determined in order to assess the phases of regeneration and regression in which collagenase activity was significantly above normal limb tissue levels. The pattern of collagenase activity in regenerating limbs which were contralateral to the experimentally-induced regressing limbs was also determined as a control on the pattern of activity in the regressing limbs. In addition, to verify that a collagenase was being worked with in this system, larval A. mexicanum collagenase was partially characterized in this investigation.

MATERIALS AND METHODS

Source and Care of Animals

Ambystoma mexicanum larvae (axolotls), used as the source of regenerating and regressing limbs in this study, were spawned and raised in the laboratory at 20±2°C. Newly hatched larvae were raised in large tanks on live brine shrimp. When the average length of the individuals in a spawning reached 3 cm., all the larvae in that spawning were transferred to individual plastic containers to prevent them from cannibalizing each others limbs, and they were switched to a diet of sliced beef liver. Larvae were used in experiments when they reached a length of 5-6 centimeters.

Adult newts, Notophthalmus viridescens, used in this study were ordered from William Lee of Oak Ridge, Tennessee.

Young guinea pigs, from which ^{14}C-collagen was prepared, were obtained through The Center for Laboratory Animal Research at Michigan State University.

Preparation of Regenerating and Regressing Limbs

Three types of experimental limbs were assayed for collagenase activity in this study: 1) normal regenerating limbs, 2) denervated, amputated regressing limbs, which will simply be called regressing limbs, and 3) contralateral regenerating limbs. The basic protocol for

producing these limbs is shown in Figure 1. To produce all three types of limbs, larvae were immobilized in an aqueous solution of 1:500 ethyl-m-aminobenzoate methanesulfonate (EAM) (Eastman), and were amputated through both forelimbs midway between the wrist and elbow. Protruding skeletal elements that resulted from amputation were trimmed flush with the amputation surface to facilitate faster and more uniform regeneration. To produce normal regenerates, larvae were returned to individual containers at this point, and allowed to regenerate for the desired length of time. To produce regressing limbs and contralateral regenerates, larvae were denervated at the time of amputation by severing the third, fourth, and fifth spinal nerves in the shoulder region of the right limb. These larvae were then returned to individual containers, and allowed to regress on their right side, and regenerate on their contralateral left side. Denervated limbs were maintained in a nerveless condition by re-denervating them every six days. All experimental animals were maintained at 22±1°C while their limbs were regenerating, or regressing.

Regenerating and regressing limbs were collected for homogenization using sterile technique. To prevent bacterial and fungal contamination of limb tissues, all glassware and operating tools were autoclaved before use, and all solutions were sterilized with millipore filters (0.45u pore size). In addition, larvae were surface sterilized with antibiotics (Na-penicillin G, Sigma, 0.2 mg/ml.; streptomycin sulfate, Sigma, 0.5 mg/ml.; fungizone, GIBCO, 0.02 mg/ml.) four hours before their limb tissues were removed. To collect limb tissues, larvae were immobilized in EAM, and were examined under a dissecting microscope to determine the morphological stage of regenerates, and the regressive progress of

FIGURE 1. The procedure used to obtain regenerating and regressing larval axolotl limbs to be assayed for collagenase activity. A. Diagram of protocol for obtaining normal regenerates (a). B. Diagram of protocol for obtaining regressing limbs (b) and contralateral regenerates (c). Dashed lines represent amputations when on limbs, and denervations when in the shoulder region.

denervated limbs. After examination, regenerating limbs were amputated at the elbow, and regressing limbs were amputated either at the elbow, or 3 mm from the end of the stump depending on how much regression had occurred. All limb tissues distal to these amputations, which includes stump tissues as well as regenerates and regressing tissues, were included in the crude enzyme extracts. Limb segments from the mid forelimb to the elbow of normal unamputated limbs were collected as controls on the amount of collagenase activity in normal limb tissues. All limb tissues were transferred to sterile plastic petri dishes as soon as they were removed, were frozen at -20°C, and were immediately lyophilized.

Regenerates were obtained from adult newts in the same manner as mentioned above.

Preparation of Crude Enzyme Extracts

Crude enzyme extract was prepared from regenerating, regressing, and control limb tissues by the direct extraction method of Nagai and Hori (1972). These extracts differed from those of Nagai and Hori, in that they were prepared from lyophilized tissue instead of freeze-thawed wet tissue. Using sterile glassware and buffer, a homogenate was prepared of ten lyophilized regenerates in 0.5 ml. of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl, 5 mM $CaCl_2 \cdot 2H_2O$, and antibiotics (Na-penicillin G, streptomycin sulfate, and fungizone at the concentrations specified in the preceding section) in a 1 ml. capacity glass tissue homogenizer at 0°C. Such homogenates were incubated at 37°C for 66 hrs. in sterile test tubes to extract collagenase. After incubation the homogenates were

centrifuged, and the supernatants collected for use as crude enzyme extracts in the collagenase assay.

The protein content of each crude enzyme extract was determined using the Lowry method (Lowry, et al., 1951) at one-tenth the suggested volumes.

Preparation of ^{14}C-Collagen

Radioactive guinea pig skin collagen for the radioactive reconstituted collagen fibril assay was prepared from young growing animals (250-300 gm.) which were injected intraperitoneally with 100 uc of glycine-^{14}C-UL six hours before their skins were collected (Gross and Lapiere, 1962). Collagen was extracted from the dermis with 0.5 M acetic acid (Gross and Kirk, 1958), was purified by repeated salt precipitation (Jackson and Fessler, 1955), and was lyophilized. Four grams of purified radioactive guinea pig skin collagen with activity of 600 DPM/mg. were obtained from 5 coriums. The identity and purity of the guinea pig collagen was checked using disc-gel electrophoresis. Guinea pig collagen was compared with acid soluble calf skin collagen (Sigma) (Figure 2) using the gel electrophoresis method of Nagai, et al. (1964). Both collagen preparations contained characteristic α, β, and γ bands which co-migrated, few contaminant protein bands, and an unidentified faster migrating species, x.

Radioactive Reconstituted Collagen Fibril Assay for Collagenase

Collagenase activity in urodele limb tissues was measured using the radioactive reconstituted collagen fibril assay (Nagai, et al., 1966). All glassware and solutions used in the assay were sterile. To prepare

^{14}C-collagen fibrils, 2 mg./ml. of lyophilized radioactive guinea pig skin collagen were dissolved in 0.5 M acetic acid by stirring gently overnight at 4°C. The resulting collagen solution was dialyzed with stirring against 0.15 M phosphate buffer, pH 7.6, for 24 hrs. at 4°C, and then against an 0.4 M NaCl solution at 4°C for 24 hours. Aliquots of the final collagen solution, 0.25 ml. (300 DPM), were pipetted into 3-ml. test tubes, and were incubated at 36°C for 12 hrs. to gel. To the gel, which consisted of native, reconstituted collagen fibrils, were added: 1) 0.25 ml. of 0.1 M Tris buffer, pH 7.5, containing 1 mM $CaCl_2 \cdot 2H_2O$, and antibiotics (final reaction mixture concentration: Na-penicillin G, Sigma, 0.2 mg/ml.; streptomycin sulfate, Sigma, 0.5 mg/ml.; fungizone, GIBCO, 0.02 ml/ml.), and 2) 0.1 ml. of the enzyme extract to be assayed. Reaction mixtures were incubated at 36°C for 5-66 hrs. depending on the experiment. At the end of the incubation period reaction mixtures were centrifuged at 25,000 x g for 15 min. to separate undegraded collagen fibrils from peptide breakdown products released into the reaction buffer. The supernatant was decanted from the pellet, and both fractions were saved to be analyzed. The collagen pellet was solubilized in 0.15 ml. of glacial acetic acid (99.7%), was added to 15 ml. of dioxane scintillation cocktail, and was counted in a liquid scintillation spectrometer. The CPM thus obtained were converted to DPM to standardize all results. Total collagenolytic activity, the total number of counts released as breakdown products, was determined by subtracting the DPM of experimental reaction mixtures containing crude enzyme extracts from the DPM of a control reaction mixture containing only enzyme buffer. This method of determining how much degradation product was formed, by

measuring the amount of substrate left unreacted, was used because of the low radioactivity of the substrate collagen (600 DPM/mg.). It differed from the method of Nagai, et al. (1966), who measured the radioactivity of the degradation products in the supernatant. The specific activity of each crude enzyme extract was determined also.

Calculation of Limits of Detection of Radioactivity

Because of the low radioactivity of the collagen substrate in the collagenase assay (300 DPM per reaction mixture), the precision of the counting setup (least detectable amount of radioactivity) was calculated for all experiments (Brewer, et al., 1974). Using the formula:

$$\text{Precision} = \frac{K(2R_b/t_b)^{1/2}}{\text{c.f.}}$$

where R_b = background counting rate, t_b = counting time, c.f. = overall efficiency of the counting system, and K = proportionality constant for statistical significance, all counts (CPM) in all experiments were found to be higher than the least detectable amount of radioactivity of the system at $p = 0.01$.

Assay for Dialyzable Degradation Products of ^{14}C-Collagen

The supernatants of reaction mixtures run at 36°C were pooled and checked for dialyzable counts to verify that the collagen fibril substrate of the assay was being degraded and not just solubilized during the incubation period. Half of each pooled supernatant was dialyzed against distilled H_2O at 4°C for 72 hours. Then 0.5 ml. aliquots of dialyzed and undialyzed supernatant were added separately to 15 ml. portions of dioxane scintillation cocktail, and were counted. The

dialyzable counts in the supernatant were determined by subtracting the counts remaining in 0.5 ml. of dialyzed supernatant from the counts in 0.5 ml. of undialyzed supernatant.

A control was run on the above assay procedure to rule out the possibility that intact solubilized collagen was precipitating on or sticking to the dialysis tubing during dialysis, and thereby removing itself from solution, and mimicing the results that would be expected if dialyzable peptides were in the supernatants. Undegraded ^{14}C-collagen, 3 mg/ml., was dissolved in 0.5 M acetic acid, and half of the resulting solution dialyzed against distilled H_2O for 72 hrs. at 4°C. Aliquots, 0.25 ml., of the resulting dialyzed and undialyzed solutions were added to 15 ml. of dioxane scintillation cocktail, and were counted. After adjusting the DPM of the dialyzed collagen solution for the increase in volume it had undergone during dialysis, the counts in dialyzed and undialyzed solutions were found to be not significantly different from each other at p=0.05 (Table 4), indicating that intact collagen in solution is not being removed from solution during dialysis.

Assay for Characteristic TC^A Degradation Products

Collagenase characteristic TC^A degradation products were detected using disc-gel electrophoresis (Nagai, et al., 1964). Reaction mixtures were prepared in exactly the same manner as for the radioactive reconstituted collagen fibril assay, and were incubated at 25°C, so TC^A degradation products would not be degraded to small peptides, for 12-72 hours. At the end of the incubation period, 18 ul of glacial acetic acid (99.7%) were added to each reaction mixture to make it 0.5 M acetic acid, and the mixtures were placed at 4°C overnight to dissolve all collagen.

An aliquot, 0.1 ml., of each reaction mixture was run on a separate polyacrylamide gel, and the resultant gels were stained, destained, and photographed for observation.

Histology

Since regressing limbs were only re-denervated every 6 days, a study was conducted to make sure that nerves were not growing back into limbs between denervations. Denervated limbs were checked histologically at the light microscope level for the presence of nerves. The limbs of 25 larval axolotls were amputated and denervated, and five limbs were collected on each day 2-6 days post operation. Upon collection, limbs were fixed in Bouin's fixative for 24 hrs., incubated in Lenois' solution for 3 days to remove picric acid, dehydrated, embedded in paraplast, and sectioned at 7 microns. Sectioned tissues were stained for nerves using Samuel's silver stain (Samuel, 1953). Control innervated limbs were fixed and stained in the same way. Slides were examined with a light microscope and photographed. It was found that by two days post amputation and denervation no small nerve fibers remained in limb muscle, and by 6 days no new nerve fibers had grown back into the limb muscle (Figure 3), demonstrating that regressing limbs remained nerveless between denervations.

Morphological Staging of Regenerates and Regressing Limbs

As a rough guide of how fast larval axolotl limbs were regenerating or regressing, experimental limbs were staged while being collected to be assayed for collagenase activity. Limbs were classified as being one of the following stages: 1) no change- no change in stump length, no

FIGURE 2. Disc-gel electrophoretic patterns of radioactive guinea pig skin collagen and a collagen standard showing characteristic α, β, and γ bands, and an unknown band, x. (a) the collagen standard, Sigma acid soluble calf skin collagen; (b) radioactive guinea pig skin collagen; (c) equal concentrations of the collagen standard plus radioactive guinea pig skin collagen. Each gel contains the same amount of protein.

FIGURE 3. Light micrographs of larval axolotl limb muscle stained for nerves with Samuel's silver stain. A. Normal innervated limb muscle. Arrows point to several nerve fibers. B. Muscle from a limb which has been denervated and amputated for 6 days. No nerve fibers present. (500X)

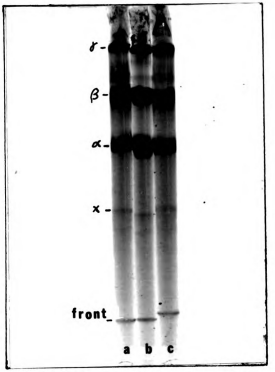


FIGURE 2

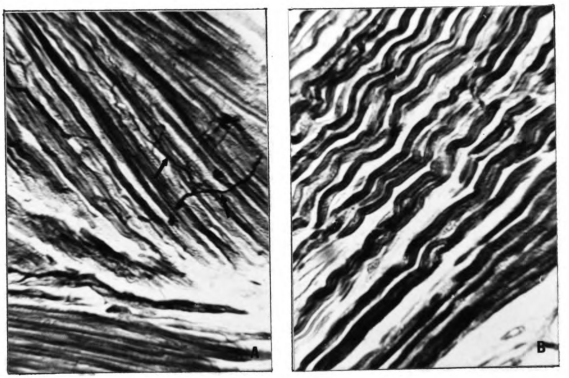


FIGURE 3

noticable accumulation of tissue beyond the level of amputation, 2) regressing limb- shortening of stump, no noticable accumulation of tissue beyond the level of amputation, 3) bulb blastema- accumulation of tissue beyond the level of amputation, rounded outgrowth, 4) cone blastema- cone shaped outgrowth, larger than bulb, 5) paddle blastema- dorsal-ventral flattening of distal outgrowth, same size or larger than cone blastema, 6) 2-digit regenerate- formation of a notch in the distal edge of the paddle, same size or larger than paddle, and 7) 3-and 4-digit regenerate-formation successively of a second and third notch posterior to the first notch in the distal edge of the paddle, larger than the preceding stages, beginning of digit outgrowth.

Statistics

The patterns of collagenase activity during the period 2-16 days post amputation in normal regenerating, regressing, and contralateral regenerating limbs were analyzed using a split plot analysis of variance. This analysis was performed to demonstrate if generally the trend of collagenase activity over time is the same for the three experimental groups, and to compare specifically 1) collagenolytic activity in two experimental groups on a given day, and 2) collagenolytic activity within an experimental group on any day with the control levels of collagenolytic activity in that group. Collagenolytic activity on the same day in different groups were compared using a one-sided t-test of a comparison; collagenolytic activities in the same group were compared with a control using a one-sided Dunnett's t-test for comparisons with a control.

Other statistical comparisons of collagenolytic activity done in this study used the Student's t-test.

Experiments

The Pattern of Collagenase Activity in Regenerating and Regressing Limbs

Series 1: Collagenase Activity in Normal Regenerating Limbs

Normal regenerates were collected daily over the period 2-16 days post amputation, and together with segments from unamputated limbs were assayed for collagenase activity using the reconstituted radioactive collagen fibril assay at 36°C. To produce each crude enzyme extract for the assay, 10 right limbs from 10 larvae were pooled at each time post amputation. The entire experiment was run three times, and the mean (n=3) values of collagenase activity at each time post amputation were determined (Table 1). A total of 480 larvae were used in this series.

Series 2: Collagenase Activity in Regressing and Contralateral Regenerating Limbs

Denervated regressing limbs and the contralateral regenerates on the same animals were collected on the same time schedule, and assayed for collagenase activity in the same way as the normal regenerates in Series 1. Ten denervated regressing right limbs and 10 regenerating contralateral left limbs from 10 larvae were pooled separately at each time period to produce the crude enzyme extracts for the collagenase assay. As in Series 1, these experiments were run three times, and the mean (n=3) values of collagenase activity at each time post amputation were determined (Table 1). A total of 450 larvae were used in this series.

Characterization of Larval Axolotl Collagenase

Series 3: Kinetics of Larval Axolotl Collagenase

The kinetics of collagenase activity from normal 7-day regenerates was investigated using the reconstituted radioactive collagen fibril assay at 36°C. Three crude enzyme extracts were produced by pooling 80 regenerates from 40 larvae for each extract, and each extract was assayed at three concentrations, 0.05 ml., 0.10 ml., and 0.20 ml. Reaction mixtures at each concentration were incubated for 5, 7, 10, 20, 30, 40, 50, 60, and 66 hours. Mean values (n=3) of collagenase activity at each time of incubation and concentration were determined, and plotted as progress curves of reaction velocities. From these curves the effect of enzyme concentration on reaction velocities at different times was determined. A total of 120 larvae were used in this series.

Series 4: Formation of Dialyzable Degradation Products at 36°C

The supernatants of reaction mixtures of crude enzyme extracts which had been assayed for collagenase activity using the reconstituted radioactive collagen fibril assay at 36°C were used to assay for dialyzable degradation products according to the procedure outlined earlier in this section. Supernatants of reaction mixtures containing enzyme extract from normal 8-day regenerates, and from 8-16 day regressing limbs were obtained from Series 1 and Series 2 respectively.

Series 5: Formation of Collagenase Characteristic TC^A Degradation Products at 25°C

Normal 7-day regenerates and 7-day regressing limbs were assayed for the ability to produce collagenase characteristic TC^A degradation

products using the reconstituted collagen fibril assay and disc-gel electrophoresis. Crude enzyme extracts of regenerating and regressing limbs were both produced by pooling 40 limbs from 20 larvae. Reaction mixtures containing crude enzyme extract from regenerating and regressing limbs were incubated at 25°C for 0, 12, 24, 36, 48, 60, and 72 hours. The following controls were also run: 1) reaction mixture minus enzyme incubated for 0 and 72 hrs., 2) reaction mixture containing crude enzyme extract from 7-day regenerates minus collagen incubated for 0 and 72 hrs., 3) reaction mixture containing crude enzyme extract from 7-day regressing limbs minus collagen incubated for 0 and 72 hrs., and 4) complete reaction mixtures incubated for 72 hrs. to which 0.01% and 0.06% trypsin (Sigma, 2X crystallized, from Bovine pancreas) solutions had been added, giving final reaction mixture concentrations of 0.002% and 0.01% trypsin. All experiments were run twice. Forty larvae were used in this experiment.

Series 6: Effect of Known Collagenase Inhibitors

Crude enzyme extract from normal 8-day regenerates was assayed for collagenase activity in the presence of known vertebrate collagenase inhibitors using the reconstituted radioactive collagen fibril assay at 36°C. Reaction mixtures contained the following final concentrations of inhibitors: 2 mM Na_4-EDTA, 5 mM cysteine, and 10% fetal calf serum (GIBCO). Three crude enzyme extracts were produced by pooling 20 regenerates from 10 larvae for each extract. This experiment was run three times, and the mean collagen degradation (n=3) with each treatment determined. Thirty larvae were used in this series.

Series 7: Non-Specific Collagen Degradation by Trypsin

Using the reconstituted radioactive collagen fibril assay at 36°C, the following solutions were assayed for collagenase activity: 1) 0.01% trypsin, 2) 0.06% trypsin, 3) 0.06% trypsin + 0.01% soybean trypsin inhibitor, 4) crude enzyme extract from normal 7-day regenerates + 0.01% soybean trypsin inhibitor. Three crude enzyme extracts from normal 7-day regenerates, the same extracts used in Series 3, were used in this series, and each extract was incubated for 30, 50, and 66 hours. Each trypsin containing reaction mixture was run three times. The mean (n=3) collagen degradation with each treatment was calculated.

Collagenase Activity of Adult Newt Regenerates

Series 8: Lyophilized Regenerates

Normal 16-day adult newt regenerates, the stage were maximum collagenolytic activity was obtained in cultures, were lyophilized, directly extracted, and assayed for collagenase activity using the reconstituted radioactive collagen fibril assay at 36°C. Reaction mixtures were incubated for 20, 48, and 72 hours. To produce crude enzyme extracts for the assay, 18 regenerating limb segments were homogenized in 5 ml. of enzyme buffer. The experiment was run twice with two different enzyme homogenates, and mean values (n=2) of enzyme activity were determined. A total of 18 newts were used in this series.

RESULTS

Morphological Development

Normal and contralateral larval axolotl regenerates appear morphologically to be developing at essentially the same rate during the period 2-16 days post amputation (Figure 4). In both cases a bulb blastema appears at 5 days post amputation, a cone at 8 days, a paddle at 9 days, and digits at 12 days post amputation. Denervated amputated limbs, on the other hand, during the same time period do not change morphologically until 8 days post amputation (Figure 4). At that time, the limbs begin to shorten between the amputation surface and the elbow, and continue to shorten for the next 8 days, until, by 16 days post amputation, they have regressed to the elbow.

The Pattern of Collagenase Activity in Regenerating and Regressing Limbs

Like the rate of morphogenesis, the general pattern of collagenase activity in normal and contralateral larval axolotl regenerates is essentially the same (Figures 5 and 6, Table 1). Collagenase activity in both types of regenerating limb rises rapidly after amputation to maximum levels at 6-7 days post amputation, and thereafter returns rapidly to normal tissue levels. Total and specific collagenase activity in normal regenerates are significantly above control tissue levels ($p=0.05$) 3-9 days and 3-8 days post amputation respectively. Similarly, in

FIGURE 4. **The morphological development of regenerating and regressing larval axolotl limbs at different times post amputation.**

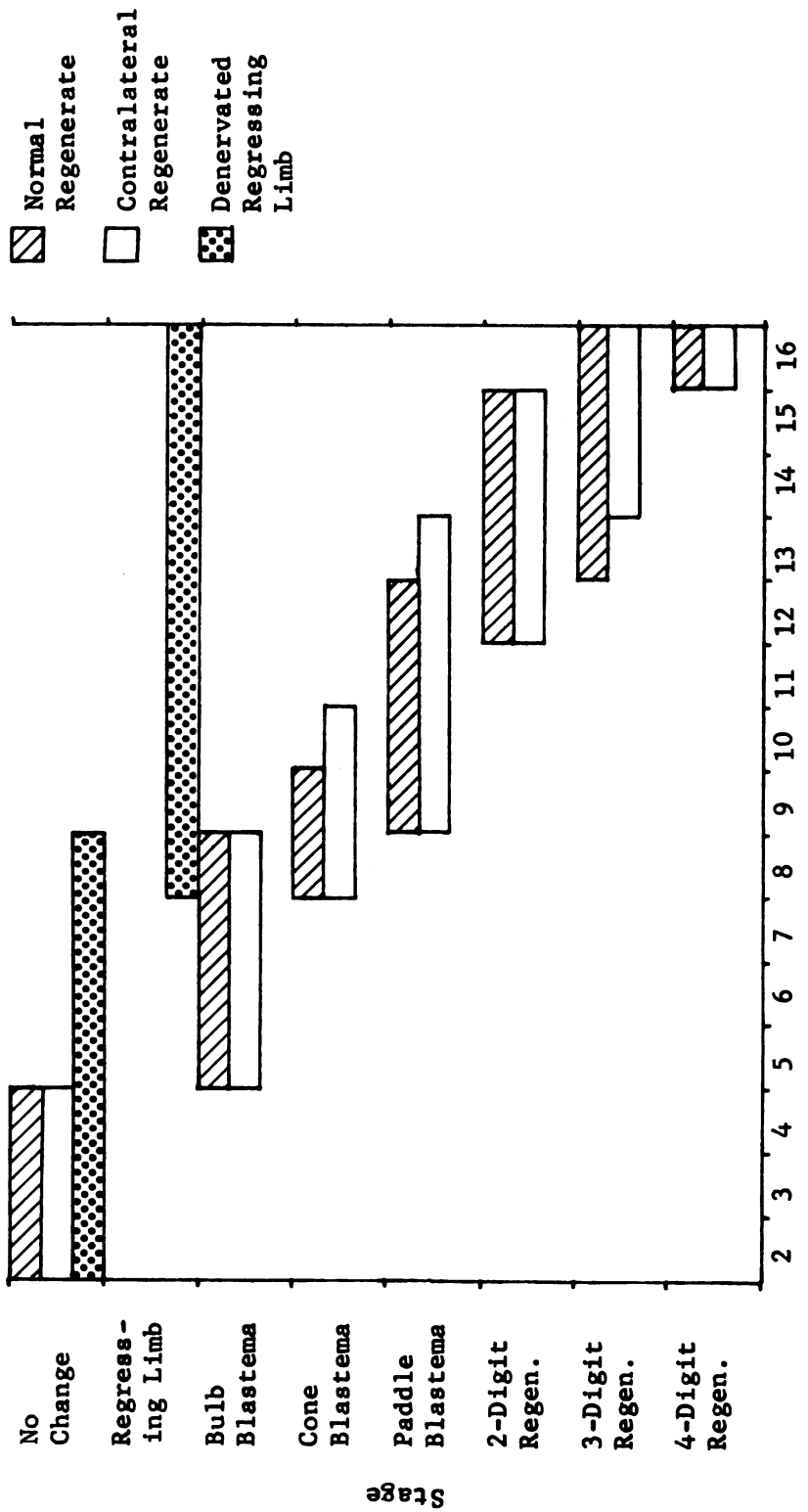

Normal Regenerate
Contralateral Regenerate
Denervated Regressing Limb
No Change
Regress-ing Limb
Bulb Blastema
Cone Blastema
Paddle Blastema
2-Digit Regen.
3-Digit Regen.
4-Digit Regen.
Stage
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Days Post Amputation

FIGURE 4

contralateral regenerates, total and specific collagenase activity are significantly above control tissue levels ($p=0.05$) 3-10 days and 3-9 days post amputation respectively.

The general pattern of collagenase activity in regressing limbs differs from that of regenerating limbs (Figures 5 and 6, Table 1). Like regenerating limbs, collagenase activity in regressing limbs rises rapidly after amputation to maximum levels at 8 days post amputation, however, unlike regenerating limbs, collagenase activity then remains high instead of returning to control tissue levels. In regressing limbs total and specific collagenase activity are higher ($p=0.05$) than control tissue levels 2-16 days and 3-16 days post amputation respectively.

The trends in collagenase activity over time in normal regenerating, regressing, and contralateral regenerating limbs were compared to determine if and when the three groups of limb tissues differed from each other in enzyme activity. The F statistics obtained from a split plot analysis of variance for a groups times days interaction, strongly indicates that collagenase activity over time is not the same for the three groups of limbs, either for total activity ($p=0.001$), or for specific activity ($p=0.01$). A more specific analysis to determine on which days post amputation collagenase activity is different in the three experimental groups, however, failed to show significant differences below $p=0.1$. An analysis of collagenase activity in regenerating and regressing limbs at 11-16 days post amputation, the time of greatest difference in enzyme activity among these experimental groups, showed significant differences only at somewhat greater than $p=0.1$. It is felt, however, that since the F statistic strongly indicates a difference in these experimental groups, and since the greatest difference in enzyme

FIGURE 5. Total collagenase activity of regenerating and regressing larval axolotl forelimbs at different times post amputation using the radioactive reconstituted collagen fibril assay at 36°C. Values at 0 time represent enzyme activity in unamputated limb segments. Standard error of difference between two points on different lines= 179 DPM; standard error of difference between two points on the same line= 22 DPM.

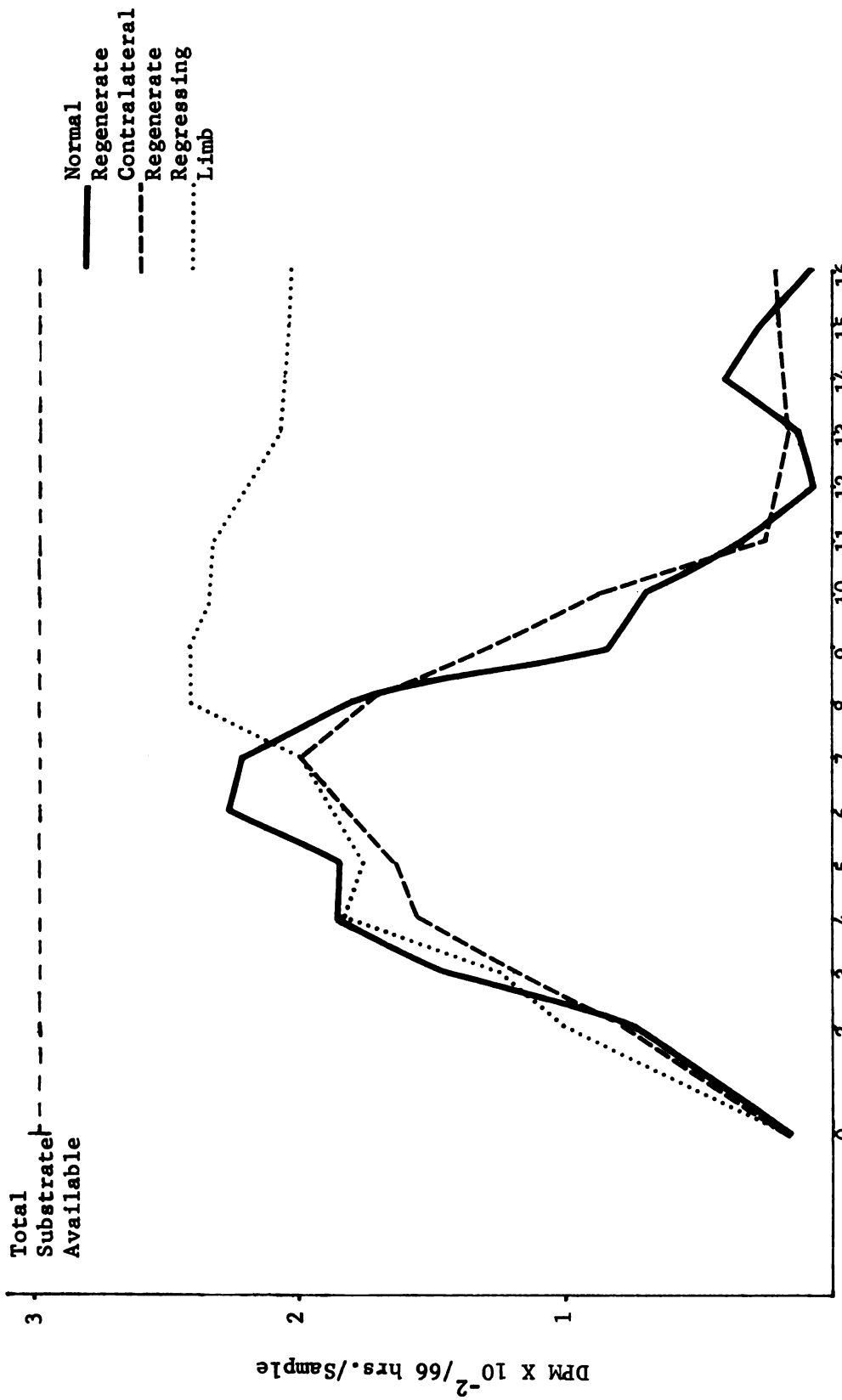

Normal
Regenerate
Contralateral
Regenerate
Regressing
Limb
Total
Substrate
Available
DPM X 10^{-2}/66 hrs./Sample
Days Post Amputation
0
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
1
2
3

FIGURE 5

FIGURE 6. Specific collagenase activity of regenerating and regressing larval axolotl forelimbs at different times post amputation using the radioactive reconstituted collagen fibril assay at 36°C. Values at 0 time represent enzyme activity in unamputated limb segments. Standard error of difference between two points on different lines= 2431 DPM; standard error of difference between two points on the same line= 304 DPM.

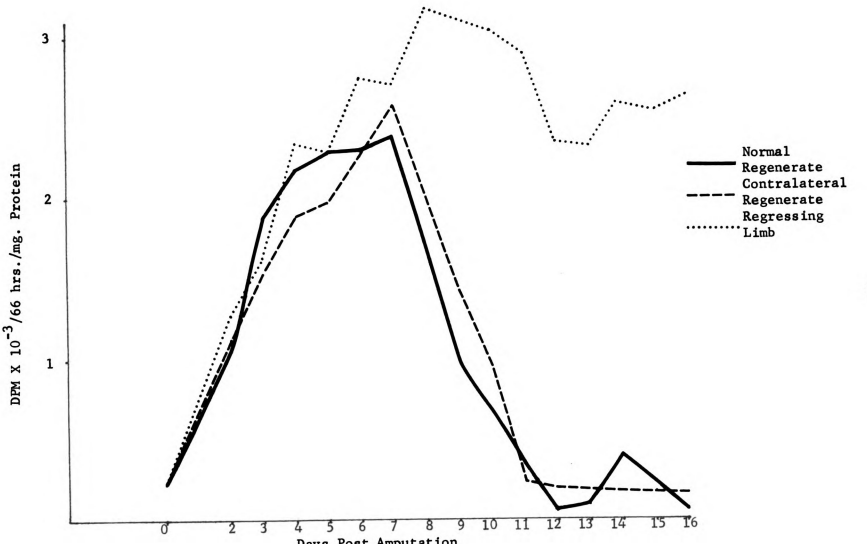

3
2
1
DPM X 10^{-3}/66 hrs./mg. Protein
0
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Days Post Amputation
Normal Regenerate
Contralateral Regenerate
Regressing Limb

FIGURE 6

TABLE 1

COLLAGENOLYTIC ACTIVITY AT DIFFERENT TIMES POST AMPUTATION IN NORMAL REGENERATING, DENERVATED REGRESSING, AND CONTRALATERAL REGENERATING LIMBS OF LARVAL AXOLOTLS USING THE ^{14}C-COLLAGEN FIBRIL ASSAY AT 36°C

Days Post Amp.	Total Activity- DPM Substrate Degraded/66 hrs.				Protein Content of Crude Enzyme Extract- mg/ml			Specific Activity- DPM Substrate Degraded/66 hrs./mg. Protein			
	Run#1	Run#2	Run#3	Mean	Run#1	Run#2	Run#3	Run#1	Run#2	Run#3	Mean
0	20	18	8	15	0.64	0.62	0.74	304	290	108	234
Normal Regenerate											
2	45	88	90	74	0.96	0.59	0.73	469	1492	1233	1065
3	127	124	189	147	1.04	0.64	0.77	1240	1938	2455	1878
4	177	199	182	186	0.92	0.88	0.78	1924	2261	2333	2173
5	205	139	212	185	0.98	0.79	0.71	2092	1759	2986	2279
6	222	238	221	227	1.04	1.17	0.81	2135	2034	2728	2299
7	227	226	210	221	0.82	1.14	0.87	2768	1982	2414	2388
8	195	176	171	181	1.30	1.03	0.90	1500	1709	1900	1703
9	57	79	120	85	1.18	0.80	0.82	483	988	1463	978
10	0	112	99	70	1.49	1.19	0.90	0	941	1100	680
11	18	51	26	32	1.49	0.86	0.87	121	593	299	338
12	0	16	0	5	1.30	1.13	0.85	0	142	0	47
13	38	0	0	13	1.42	1.08	0.86	268	0	0	89
14	51	59	12	41	1.54	0.76	1.00	331	776	120	409
15	31	48	0	26	1.76	0.87	1.09	176	552	0	243
16	0	25	0	8	1.80	1.36	1.03	0	184	0	61
Regressing Limb											
2	76	120	108	101	0.86	0.83	0.72	884	1446	1500	1277
3	76	175	125	125	0.79	0.80	0.72	962	2188	1736	1629
4	143	209	200	184	0.89	0.79	0.72	1607	2646	2778	2344
5	172	168	189	176	0.80	0.74	0.79	2150	2270	2392	2271
6	166	181	212	186	0.60	0.69	0.74	2767	2623	2865	2752
7	212	168	218	199	0.67	0.83	0.74	3164	2024	2946	2711
8	250	252	221	241	0.82	0.71	0.76	3049	3549	2908	3169
9	261	229	236	242	1.01	0.68	0.70	2584	3368	3371	3108
10	250	208	244	234	0.78	0.70	0.82	3205	2971	2976	3051
11	258	202	238	233	0.81	0.82	0.78	3185	2463	3051	2900
12	222	209	230	220	0.92	0.98	0.92	2413	2133	2500	2349
13	194	215	212	207	0.76	1.02	0.92	2553	2108	2304	2322
14	244	130	245	206	0.88	0.58	0.90	2773	2241	2722	2579
15	240	173	200	204	0.86	0.76	0.78	2791	2276	2564	2544
16	211	181	221	204	1.04	0.59	0.80	2029	3068	2762	2620

TABLE 1 (cont'd.)

Days Post Amp.	Total Activity- DPM Substrate Degraded/66 hrs,				Protein Content of Crude Enzyme Extract- mg/ml			Specific Activity- DPM Substrate Degraded/66 hrs./mg. protein			
	Run#1	Run#2	Run#3	Mean	Run#1	Run#2	Run#3	Run#1	Run#2	Run#3	Mean
Contralateral Regenerate											
2	35	99	97	77	0.65	0.74	0.68	538	1338	1426	1101
3	56	180	125	120	0.87	0.81	0.72	644	2222	1736	1534
4	120	167	180	156	1.01	0.68	0.90	1188	2456	2008	1881
5	137	155	199	164	0.80	0.86	0.83	1712	1802	2398	1971
6	166	182	206	185	0.78	0.87	0.80	2128	2092	2575	2265
7	201	188	209	199	0.69	0.86	0.80	2913	2186	2612	2570
8	190	173	161	175	0.82	0.89	0.94	2317	1944	1713	1991
9	135	148	100	128	0.97	0.92	0.77	1392	1609	1299	1433
10	92	107	64	88	0.94	0.89	0.90	979	1202	711	964
11	14	25	34	24	0.98	1.08	1.02	143	231	333	236
12	19	23	17	20	1.01	0.96	0.92	173	240	185	199
13	20	17	11	16	0.92	0.87	0.86	217	195	128	180
14	13	32	10	18	1.04	1.16	0.97	125	276	103	168
15	11	29	21	20	1.00	1.28	1.00	110	227	210	182
16	7	34	20	20	1.15	1.31	1.22	61	260	164	162

activity between experimental groups occurs 11-16 days post amputation, that collagenase activity in regenerating and regressing limbs is significantly different during this time period, and that the small sample size (n=3) for each data point is preventing significant differences from being detected. Further, it is felt that since only relatively small differences in enzyme activity occur between normal and contralateral regenerates during the entire time period studied, and between regenerating and regressing limbs 2-8 days post amputation, that 1) the trend in collagenase activity in normal and contralateral regenerates is the same over the time period studied, and 2) the trend in collagenase activity in regressing limbs is the same as that of regenerating limbs 2-8 days post amputation, but differs from regenerating limbs 9-16 days post amputation.

Characterization of Larval Axolotl Collagenase

Kinetics-

The reaction velocity progress curves of collagenase from 7-day axolotl regenerates (Figure 7, Table 2) are of the general form produced by most enzyme reactions, in which velocity falls with time.

In most enzyme reactions initial velocities are proportional to the enzyme concentration, and hence a straight line is produced if initial velocity is plotted against enzyme concentration. When apparent velocities at different times from Figure 7 were plotted against the collagenase concentration, the resulting curves become straighter as t approaches 0, indicating that velocity is proportional to enzyme concentration in this system (Figure 8, Table 3).

FIGURE 7. Reaction velocity progress curve of collagenase from 7-day larval axolotl regenerates at three concentrations using the radioactive reconstituted collagen fibril assay at 36°C. (●), 0.05 ml. of crude enzyme extract; (x), 0.10 ml. of crude enzyme extract; (o), 0.20 ml. of crude enzyme extract.

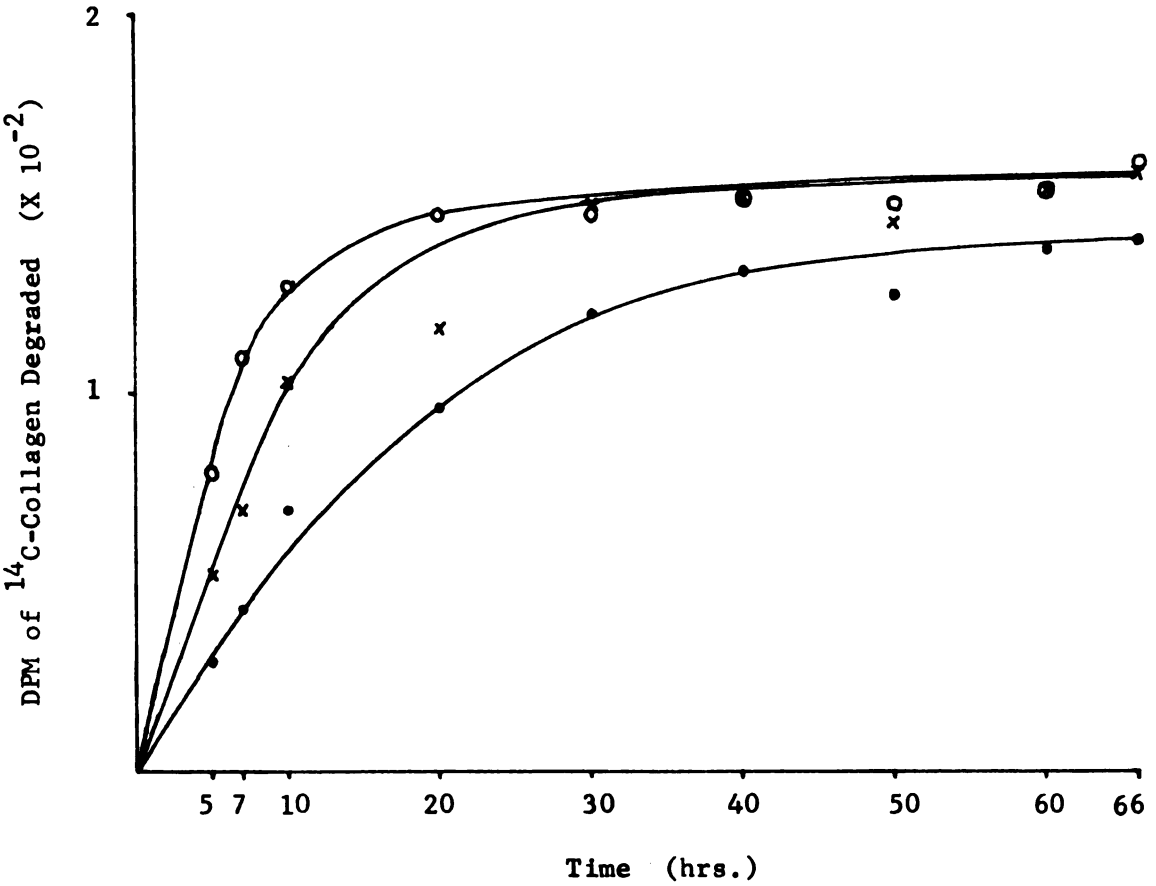

2
1
DPM of ^{14}C-Collagen Degraded (X 10^{-2})
5 7 10 20 30 40 50 60 66
Time (hrs.)

FIGURE 7

FIGURE 8. Effect of collagenase concentration on reaction velocity at different times. Values obtained from graphs in Figure 7. t_0, 0 time incubation of reaction mixture; t_1, 1 hour incubation of reaction mixture; t_5, 5 hour incubation of reaction mixture; t_{10}, 10 hour incubation of reaction mixture; t_{20}, 20 hour incubation of reaction mixture.

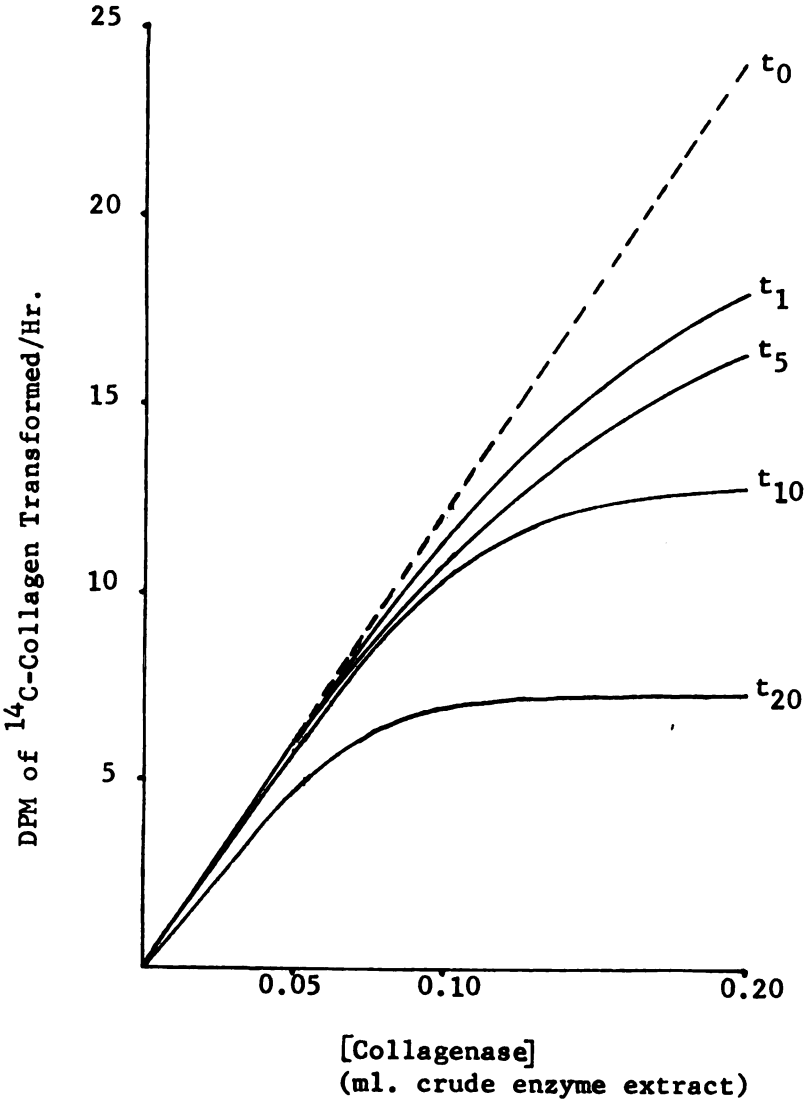

25
20
15
10
5
DPM of ^{14}C-Collagen Transformed/Hr.
t_0
t_1
t_5
t_{10}
t_{20}
0.05
0.10
0.20
[Collagenase]
(ml. crude enzyme extract)

FIGURE 8

TABLE 2

REACTION VELOCITY PROGRESS CURVES OF COLLAGENASE FROM 7-DAY AXOLOTL REGENERATES AT THREE CONCENTRATIONS

Mean DPM of ^{14}C-Collagen Degraded ± $\sigma_{\bar{n}}$

Hrs.	0.05 ml. of Enzyme	0.10 ml. of Enzyme	0.20 ml. of Enzyme
5	29±2	52±2	79±5
7	43±5	69±6	109±8
10	69±12	103±11	128±12
20	96±6	117±5	147±15
30	121±11	150±17	147±17
40	132±8	151±15	152±20
50	126±15	145±15	150±16
60	138±15	153±27	154±23
66	141±12	158±25	160±24

TABLE 3

EFFECT OF COLLAGENASE CONCENTRATION ON REACTION VELOCITY AT DIFFERENT TIMES

Apparent DPM of ^{14}C-Collagen Degraded/Hr.

Hrs.	0.05 ml. of Enzyme	0.10 ml. of Enzyme	0.20 ml. of Enzyme
0	6.0	12.0	24.0
1	6.0	11.5	18.0
5	6.0	10.8	16.4
10	5.8	10.3	12.8
20	4.8	7.0	7.3

Formation of Dialyzable Degradation Products at 36°C-

Crude enzyme extracts from 8-day regenerating and 8-16 day regressing limbs are both able to breakdown collagen to dialyzable peptides at 36°C (Table 4). In reaction mixtures of both extracts, 81% of the counts released into the supernatant were dialyzable.

Formation of Characteristic TC^A Degradation Products at 25°C-

Crude enzyme extracts from both regenerating and regressing larval axolotl limbs degraded collagen to collagenase characteristic TC^A fragments at 25°C. The disc-gel electrophoresis patterns of collagen incubated with crude enzyme extract from 7-day regenerates for 0-72 hrs. (Figure 9a-g) show α^A and β^A degradation products in the gels by 12 hrs. of incubation. The β^A band, however, disappears by 36 hrs. of incubation and does not appear again, even though the β band almost completely disappears by 72 hrs. of incubation. The α^A component, on the other hand, is present throughout the incubation period, as are several discrete degradation products of α^A which are the major species accumulating. These results indicate that at least three reactions are occurring in this system: 1) collagenase is degrading TC molecules to TC^A_{75} degradation product, which results in formation of α^A_{75} and β^A_{75}, 2) a neutral proteinase is catalyzing hydrolysis of the cross-linked portions of collagen molecules, resulting in conversion of β and β degradation products to α and α degradation products, and 3) either collagenase or proteases are degrading TC^A_{75} molecules to several slightly smaller molecules.

The disc-gel electrophoresis patterns of collagen incubated with crude enzyme extract from 7-day regressing limbs for 0-72 hrs.

TABLE 4

FORMATION OF DIALYZABLE DEGRADATION PRODUCTS AT 36°C BY CRUDE ENZYME EXTRACT FROM NORMAL REGENERATES AND REGRESSING LIMBS

	Mean DPM	σ_n	% of Counts in A
Control-			
A. Undialyzed ^{14}C-Collagen Solution	457	2	100
B. Dialyzed ^{14}C-Collagen Solution	452	6	100
Normal Regenerates-			
A. Counts in Supernatant	340	5	100
B. Counts in Dialyzed Supernatant	66	2	19
C. Dialyzable Counts (A-B)	274	-	81
Regressing Limbs-			
A. Counts in Supernatant	304	8	100
B. Counts in Dialyzed Supernatant	58	2	19
C. Dialyzable Counts (A-B)	246	-	81

FIGURE 9. Disc-gel electrophoresis patterns of collagen degradation products released by collagenase from 7-day larval axolotl regenerates. Enzyme extract was incubated with guinea pig collagen at 25°C for (a) 0 hrs., (b) 12 hrs., (c) 24 hrs., (d) 36 hrs., (e) 48 hrs., (f) 60 hrs., and (g) 72 hrs. Reaction mixture minus enzyme was incubated at 25°C for (h) 0 hrs., and (i) 72 hrs. Fr, front.

FIGURE 10. Disc-gel electrophoresis patterns of collagen degradation products released by collagenase from 7-day denervated regressing larval axolotl limbs. Enzyme extract was incubated with guinea pig collagen at 25°C for (a) 0 hrs., (b) 12 hrs., (c) 24 hrs., (d) 36 hrs., (e) 48 hrs., (f) 60 hrs., and (g) 72 hrs. Fr, front.

FIGURE 11. Disc-gel electrophoresis patterns of reaction mixtures containing crude enzyme extracts from larval axolotl limbs incubated without collagen at 25°C. Control guinea pig collagen incubated for 72 hrs. (a); crude enzyme extract from 7-day regenerates incubated for (b) 0 and (c) 72 hrs.; crude enzyme extract from 7-day regressing limbs incubated for (d) 0 and (e) 72 hrs. Fr, front.

FIGURE 12. Disc-gel electrophoresis patterns of collagen incubated with trypsin at 25°C. Guinea pig collagen minus enzyme incubated for (a) 0 and (b) 72 hrs.; Guinea pig collagen incubated for 72 hrs. with (c) 0.01% trypsin, and (d) 0.06% trypsin. Fr, front.

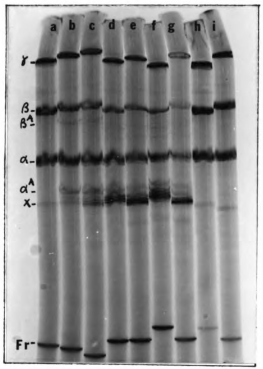


FIGURE 9

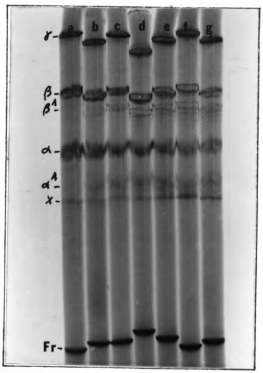


FIGURE 10

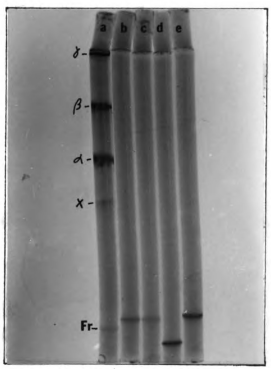


FIGURE 11

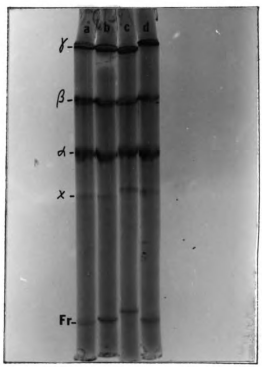
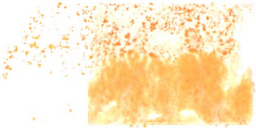


FIGURE 12



(Figure 10) are essentially the same as those of Figure 9 except: 1) β^A and its degradation products accumulate, indicating the absence of a neutral proteinase capable of converting β to α subunits in this system, and 2) there appears to be less degradation of the collagen after 72 hrs., even though crude enzyme extract from 7-day regressing limbs contained more protein (1.06 mg./ml.) than crude enzyme extract from 7-day regenerates (0.78 mg./ml.) and the same size aliquot of each was used in reaction mixtures.

Disc-gel electrophoresis patterns of control reaction mixtures containing 1) collagen minus enzyme incubated for 0 and 72 hrs. (Figure 9h and i), 2) crude enzyme extracts from 7-day regenerating and 7-day regressing limbs minus collagen incubated for 0 and 72 hrs. (Figure 11), and 3) collagen plus 0.01% and 0.06% trypsin incubated for 72 hrs. (Figure 12) demonstrate respectively that 1) collagen in the assay is not being degraded by a collagenase endogenous to the collagen substrate itself (Pardo and Tamayo, 1975), 2) TC^A degradation products are not a component of, or a degradation product of the crude enzyme extracts themselves, and 3) TC^A degradation products are not the result of non-specific trypsin degradation of collagen.

Effect of Known Collagenase Inhibitors-

Both total and specific collagenase activity in reaction mixtures containing crude enzyme extract from normal 8-day regenerates and EDTA or fetal calf serum are significantly lower ($p=0.001$) than the equivalent reaction mixtures containing no inhibitors. EDTA inhibits 90%, and fetal calf serum 65% of the collagenase activity. Cysteine, on the other hand, has no effect on collagenase activity ($p=0.05$).

FIGURE 13. Effect of known collagenase inhibitors on total collagenase activity of 8-day larval axolotl regenerates using the radioactive reconstituted collagen fibril assay at 36°C. (A) no inhibitors; (B) plus 2 mM Na_4-EDTA; (C) plus 5 mM cysteine; (D) plus 10% fetal calf serum.

FIGURE 14. Effect of known collagenase inhibitors on specific collagenase activity of 8-day larval axolotl regenerates using the radioactive reconstituted collagen fibril assay at 36°C. (A) no inhibitors; (B) plus 2 mM Na_4-EDTA; (C) plus 5 mM cysteine; (D) plus 10% fetal calf serum.

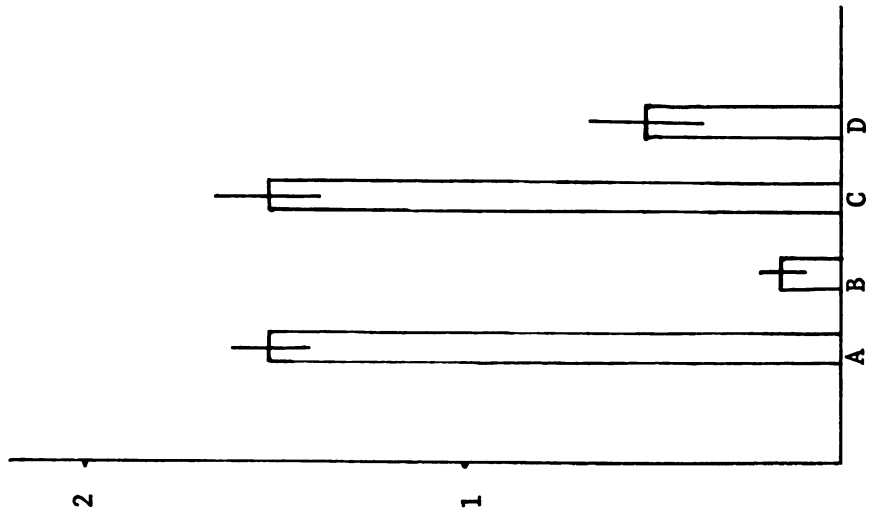


FIGURE 14

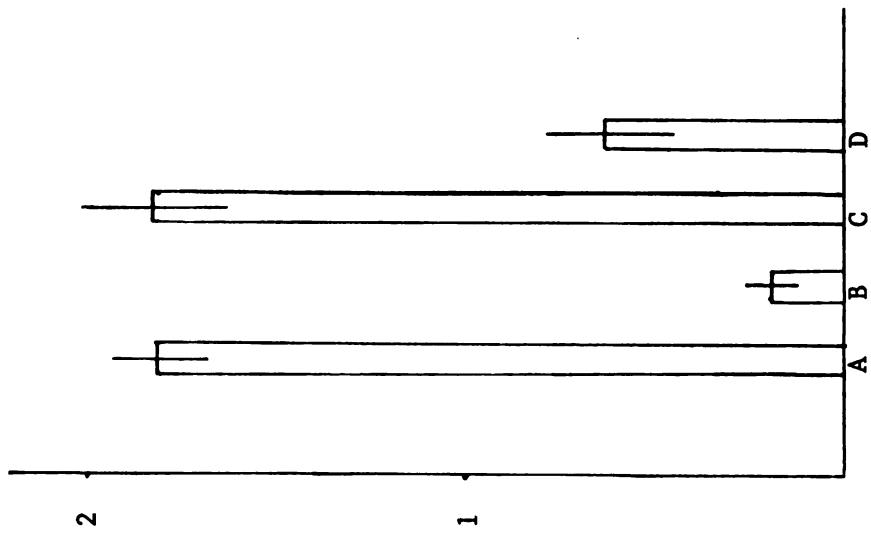


FIGURE 13

TABLE 5

EFFECT OF KNOWN COLLAGENASE INHIBITORS ON COLLAGENOLYTIC ACTIVITY OF 8-DAY REGENERATES OF LARVAL AXOLOTLS

	Total Activity- DPM of Substrate Degraded/66 hrs.					Protein Content of Crude Enzyme Extract- mg/ml			Specific Activity- DPM Substrate Degraded/ 66 hrs./mg. protein				
Treatment	Run#1	Run#2	Run#3	Mean	σ_n	Run#1	Run#2	Run#3	Run#1	Run#2	Run#3	Mean	σ_n
A. No Inhibitors	156	194	194	181	13	1.19	1.26	1.16	1311	1540	1672	1508	105
B. Plus 2 X 10^{-3} M Na_4-EDTA	14	34	10	19	7	1.19	1.26	1.16	118	270	86	158	57
C. Plus 5 X 10^{-3} M Cysteine	148	212	188	183	19	1.19	1.26	1.16	1244	1683	1621	1516	137
D. Plus 10% Fetal Calf Serum	26	80	82	63	18	1.19	1.26	1.16	218	635	707	520	152

Non-Specific Collagen Degradation by Trypsin-

Trypsin causes counts to be released into the supernatant in the reconstituted radioactive collagen fibril assay at 36°C (Table 6). An 0.01% trypsin solution added to the reaction mixture and incubated for 66 hrs., causes 20% (57 out of 300 DPM) of available counts to be released from the substrate, and an 0.06% trypsin solution causes 25% (76 out of 300 DPM) of the counts to be released from the substrate. Soybean trypsin inhibitor completely prevents breakdown of collagen fibrils by trypsin. When a solution containing 0.06% trypsin plus 0.01% soybean trypsin inhibitor is added to a reaction mixture, the counts released into the supernatant after 66 hrs. of incubation are not significantly different from 0 ($p=0.05$).

Since soybean trypsin inhibitor completely inhibits trypsin activity in the reconstituted radioactive collagen fibril assay, soybean trypsin inhibitor was added to reaction mixtures containing crude enzyme extract from regenerating larval axolotl limbs to determine if any of the collagenase activity of the crude enzyme extract in the assay is due to non-specific trypsin breakdown of the collagen fibrils. When 0.1 ml. of crude enzyme extract from 7-day regenerates containing 0.01% soybean trypsin inhibitor was added to a reaction mixture and incubated at 36°C for 30, 50, and 66 hrs., the counts released into the supernatants (Table 6) were not significantly different from ($p=0.05$) the counts released into the supernatants of reaction mixtures containing the enzyme alone at the same times (Table 2). This indicates that non-specific trypsin degradation is not contributing to the breakdown of collagen by crude enzyme extracts.

TABLE 6

NON-SPECIFIC DEGRADATION OF COLLAGEN BY TRYPSIN USING THE ^{14}C-COLLAGEN FIBRIL ASSAY AT 36°C

	Mean DPM of ^{14}C-Collagen Degraded ± σ_n
0.01% Trypsin- 66 hrs.	57±4
0.06% Trypsin- 66 hrs.	76±6
0.06% Trypsin + 0.01% Soybean Trypsin Inhibitor- 66 hrs.	10±8
0.10 ml. Collagenase from 7-Day Axolotl Regenerates + 0.01% Soybean Trypsin Inhibitor-	
30 hrs.	137±6
50 hrs.	153±22
66 hrs.	154±28

TABLE 7

COLLAGENOLYTIC ACTIVITY OF LYOPHILIZED NEWT REGENERATES USING THE ^{14}C-COLLAGEN FIBRIL ASSAY AT 36°C

	Mean DPM of ^{14}C-Collagen Degraded ± σ_n
0.10 ml. of Crude Enzyme Extract From 16-Day Newt Regenerates-	
20 hrs.	11±3
45 hrs.	14±2
72 hrs.	16±2

Collagenase Activity in Lyophilized Newt Regenerates

Crude enzyme extract from lyophilized 16-day newt regenerates prepared by the direct extraction method, when assayed for collagenase activity using the reconstituted radioactive collagen fibril assay at $36^{o}C$, showed very low levels of collagenase activity after 72 hrs. of incubation (Table 7). DPM of ^{14}C-collagen degraded were significantly above 0 ($p=0.05$), but not significantly different from ($p=0.05$) unamputated limb tissue levels (Table 1, time 0) of axolotls.

DISCUSSION

The results obtained in this investigation demonstrate that the level of collagenase activity in regenerating and denervation-induced regressing larval Ambystoma mexicanum limbs coincides with histolysis of limb stump tissues. In both normal regenerates and those contralateral to regressing limbs, collagenase activity is above normal non-regenerating limb tissue levels from 3 to 9-10 days post amputation (Figures 5 and 6). During this period regenerates proceed to the early paddle stage of regeneration (Figure 4). This rate of morphological development is the same as that of regenerating Ambystoma maculatum limbs in which histolysis and dedifferentiation of muscle, cartilage, and other stump tissues is occurring simultaneously. Regenerating larval A. maculatum limbs dedifferentiate 3 to 10 days post amputation, with most extensive dedifferentiation occurring 3 to 6-7 days after amputation (Butler, 1933; Hay, 1958). Hence, the pattern of limb stump histolysis coincides with the pattern of collagenase activity in regenerating larval urodele limbs of the two species thus far examined. The correlation found in this study between limb histolysis and collagenase activity using enzyme extracted directly from larval limb tissues, agrees with similar data on the time course of collagenase activity in adult newts where enzyme was obtained from the culture medium of limb tissues in vitro (Grillo, et al., 1968). In adult newts the regressive phase of regeneration continues for 21-28 days after amputation (Hay, 1966).

Collagenase activity in newts reaches maximum levels at 15 days post amputation and returns to normal tissue levels by 30-35 days post amputation (Grillo, et al., 1968). Thus, even though regeneration and its phases occur more slowly in adult than in larval urodeles (Schmidt, 1968), and the methods of obtaining active enzyme in the two studies were different, measured collagenase activity and limb histolysis can still be demonstrated to occur simultaneously.

The level of collagenase activity and histolysis of limb stump tissues also coincide in denervated regressing larval A. mexicanum limbs. In regressing limbs in these experiments, collagenase activity is above normal limb tissue levels from 2-3 days to 16 days post amputation inclusive with enzyme activity reaching the highest levels at 7 days post amputation and remaining high thereafter (Figures 5 and 6). Morphologically, these limbs regress from the mid-forelimb to the elbow during this 16 day period (Figure 4). The rate of regression of these limbs is the same as that of nerveless, amputated larval Notophthalmus viridescens (newt) limbs, which histologically dedifferentiate and partially disintegrate during this period. Denervated larval newt limbs, amputated through the mid-forelimb, begin to dedifferentiate 2-3 days post amputation, and continue to dedifferentiate as long as they remain nerveless, with the radius and ulna disappearing by 15-20 days post amputation (Butler and Schotte, 1941; Schotte and Butler, 1941). Again, just as in regenerating limbs, the pattern of collagenase activity in regressing larval urodele limbs coincides with limb stump histolysis in the two species examined. These results agree with findings in other systems, where resorbing tissues, such as involuting postpartum rat uterus (Jeffrey, et al., 1971) and metamorphosing tadpole tail fin (Gross and

Lapiere, 1962; Gross, 1964; Dresden, 1971; Gross and Bruschi, 1971; Nagai and Hori, 1972), are high in collagenolytic activity during periods of rapid connective tissue degradation *in vivo*.

The correlation between collagenase activity and limb stump histolysis in both regenerating and regressing larval *A. mexicanum* limbs in this study, together with abundant histological evidence that collagen is degraded during limb stump histolysis in both regenerating and regressing larval urodele limbs (see Introduction, pps. 2 and 3), suggests that collagen degradation by collagenase is one of the mechanisms underlying histolysis of larval limbs whether or not they subsequently regenerate.

The pattern of collagenase activity, like the pattern of histolysis, differs in regenerating and denervation-induced regressing larval axolotl limbs. In both cases, collagenase activity begins at the same time post amputation, and rises to the same maximum level at the same rate (Figures 5 and 6). However, having reached maximum levels, collagenase activity in regenerating limbs returns to essentially normal limb tissue levels, while collagenase activity in denervated regressing limbs remains high. It would appear then, that nerves are not involved in stimulation of collagenase activity in either regenerating or regressing limbs, since the early pattern of collagenase activity is the same in both, but nerves may play a role in the cessation of collagenase activity in regenerating larval limbs. The patterns of collagenase activity in regenerating and regressing limbs in this study suggest the presence of a collagenase inhibitor in regenerating limbs, similar to the protease inhibitor found in allergic reactions in skin (Hayashi, et al., 1969). In cutaneous allergic reactions in mammals, a neutral protease (Arthus protease) is produced which causes tissue damage. The

appearance of Arthus protease in an allergic reaction is followed closely by the appearance of its inhibitor, a protein, and subsequently, levels of protease activity return to normal tissue levels. The bell shaped curve describing specific Arthus protease activity versus time in allergic reactions resembles that of specific collagenase activity in regenerating larval urodele limbs (Figure 6). Furthermore, when the protease inhibitor is removed by column chromatography from crude Arthus protease preparations, the resulting plot of specific protease activity versus time resembles that of specific collagenase activity in denervation-induced regressing larval urodele limbs (Figure 6). Hence, it is possible that collagenase activity in regenerating limbs is returned to normal tissue levels by a collagenase inhibitor which binds with collagenase and inactivates it, and that collagenase activity in denervated regressing limbs remains high because a collagenase inhibitor is absent. Other mechanisms of limiting collagenase activity in regenerating larval limbs involving its synthesis or activation are also possible. A demonstration of inhibitors would be useful in distinguishing between these possibilities, and would not appear to present technical difficulties.

The role of nerves in inhibiting collagenase activity in regenerating urodele limbs may be either direct or indirect. It is unlikely, however, that nerves act directly on collagenase, since larval limbs can be experimentally induced to undergo extensive regression under conditions where nerves maintain their usual relationship to the amputated limb, such as occurs when limbs are amputated and x-irradiated (Butler, 1933; Butler, 1935) or UV irradiated (Blum, et al., 1957). It is more likely that nerves act indirectly to inhibit collagenase activity. In regenerating urodele limbs, nerves provide a trophic influence (Singer,

1965) which promotes proliferation of blastema cells. The biochemical nature of the neurotrophic substance necessary for limb regeneration is not known, although it has been suggested (Gospodarowicz, 1975; Gospodarowicz, et al., 1975) that it may be fibroblast growth factor, a peptide found in neural tissue which provokes cell division in vitro and in vivo in fibroblasts, chondroblasts, and several other cell types (Gospodarowicz, 1974; Rudland, et al., 1974; Gospodarowicz, 1975). Experiments where fibroblast growth factor was injected into non-regenerating amputated adult frog limbs (Gospodarowicz, et al., 1975) or brain extract was infused into denervated, amputated adult newt limbs (Singer, et al., 1976) have augmented the regenerative response of these experimental limbs, but have not yet produced a complete regenerated limb. The effect of nerves on cell proliferation can be seen both in limbs which are amputated and denervated simultaneously, and in limbs which are denervated after a regenerate has formed. Innervated and denervated amputated urodele limbs both form a wound epithelium, undergo histolysis and dedifferentiation, and synthesize DNA. However, only in innervated limbs do dedifferentiated cells then divide to form a blastema (Tassava, et al., 1974; Mescher and Tassava, 1975). Similarly, denervation of a proliferating regenerate results in a 40%-50% decrease in protein synthesis (Dresden, 1969; Lebowitz and Singer, 1970; Singer and Caston, 1972; Dearlove and Stocum, 1974; Singer, et al., 1976), and RNA and DNA synthesis (Dresden, 1969; Singer and Caston, 1972).

Since nerves are essential for blastema formation in regenerating limbs, and since collagenase activity begins to decrease with the formation of a cone blastema in this study (Figures 4, 5, and 6), it is possible that a property of the blastema inhibits collagenase activity

in regenerating urodele limbs, The blastema may produce a collagenase inhibitor. It contains numerous fibroblasts (Schmidt, 1968), and fibroblast-like cells have been shown to be capable of producing a collagenase inhibitor *in vitro* (Bauer, et al., 1975). Production of a collagenase inhibitor by the blastema which prevents limb stump collagenolysis, would explain why in larval urodele limbs experimental treatments which prevent blastema formation, such as x-irradiation (Butler, 1933; Butler, 1935), UV irradiation (Blum, et al., 1957), treatment with colchicine (Thornton, 1943), or denervation (Butler and Schotte, 1941; Schotte and Butler, 1941), result in extensive histolysis and regression of amputated larval limbs. It would also explain why denervated, amputated larval limbs will not regress if the limb has developed a blastema before it is denervated (Schotte abd Butler,1944), and why a freshly amputated and denervated limb will not regress if an undifferentiated blastema is transplanted to it (Schotte, et al., 1941).

No difference was seen in the pattern of collagenase activity in normal and contralateral regenerates in this study (Figures 5 and 6). It has been shown (Tweedle, 1971) that regenerating limbs which are contralateral to limbs which have suffered nerve damage, either by amputation or denervation, regenerate more slowly than regenerating limbs which are contralateral to uninjured limbs. Regenerates contralateral to either denervated or amputated limbs, however, regenerate at the same rate. In the present study, "normal" regenerates were contralateral to regenerating limbs, and "contralateral" regenerates were opposite denervated limbs (Figure 1). Hence, both types of regenerate were contralateral to limbs which had suffered nerve damage, and showed no contralateral effect on collagenase activity when compared to each other.

These findings agree with those of Tweedle (1971), that regenerating limbs contralateral to nerves damaged either by amputation or denervation regenerate at the same rate.

There was no direct relationship between the amount of protein which was in the crude enzyme extracts from regenerating and regressing larval limbs of various ages, and the level of collagenase activity in the extract (Table 1, Figure 6). This indicates that the increases and decreases in collagenase activity observed at different times reflect specific changes in enzyme activity, and not just changes in a constant portion of a changing total protein content in regenerating or regressing limbs.

The radioactive reconstituted collagen fibril assay for collagenase which was used in this study differed from the standard assay procedures in that 1) lyophilized tissue was used to prepare crude enzyme extracts instead of freeze-thawed tissue (Nagai and Hori, 1972), and 2) unreacted substrate remaining after the reaction was measured instead of degradation product formed (Nagai, et al., 1966). The assay employed does show normal enzyme kinetics (Figures 7 and 8; Dixon and Webb, 1964). The enzyme kinetics for collagenase obtained in this study agree with those obtained for partially purified tadpole collagenase (Kang, et al., 1966) and crude mouse bone collagenase (Vaes, 1972), in which a linear relationship exists between both the amount of substrate degraded and the enzyme concentration, and the time of incubation during the early part of the reaction.

Larval A. mexicanum collagenase appears to share attributes with other vertebrate collagenases. Like other metal proteinases (Hartley, 1960), it is active at slightly alkaline pH, is inhibited by EDTA and

mammalian serum (Figures 13 and 14), but is not inhibited by cysteine (Figures 13 and 14) or soybean trypsin inhibitor (Tables 2 and 6). Also typical of a vertebrate collagenase, axolotl collagenase degrades collagen to dialyzable peptides at 36°C (Table 4). Disc electrophoretic patterns of reaction mixtures run at 25°C show that axolotl collagenase degrades collagen to characteristic TC^{A}_{75}, and several slightly smaller TC^{A} fragments, perhaps TC^{A}_{67} and TC^{A}_{62} (Figures 9 and 10). Axolotl collagenase initially cleaves the TC molecule at the characteristic site 75% from the A end of the molecule, and then either the collagenase or a contaminating protease degrades the TC^{A}_{75} fragment at its B end to several discrete additional fragments. In addition, the collagenase preparations from regenerating limbs, but not from regressing limbs, contained a neutral proteinase which cleaved TC cross-links, converting β to α subunits (Figure 9). Axolotl collagenase behaves like collagenase obtained from the culture medium of postpartum rat uterus (Jeffery and Gross, 1970), and the culture medium of regenerating adult newt limbs (Dresden and Gross, 1970). It differs from the newt enzyme in that the newt enzyme is inhibited by cysteine. Axolotl collagenase from regenerating limbs, but not regressing limbs, differs from both uterine and newt enzyme preparations in its ability to convert β to α subunits. This difference may be due to the fact that the uterine and newt collagenase preparations in the studies mentioned above were both partially purified by ammonium sulfate precipitation, a process which may have removed some neutral proteinases, while the axolotl preparation was a crude enzyme extract. Crude enzyme extract from rat dermis obtained by direct extraction (Nagai and Hori, 1972) also contained collagenase and a neutral proteinase which converts β to α subunits. It is not clear why

regressing larval limbs do not contain a neutral proteinase which converts β to α subunits (Figure 10). Perhaps this reflects a difference in the way connective tissue collagen is degraded in regenerating and regressing limbs. It has been proposed (Harris and Krane, 1974) that collagen degradation in tissues undergoing very rapid collagen removal occurs primarily in phagolysosomes at acid pH, while slower remodeling processes occur primarily in the extracellular matrix at neutral pH. It is possible that in regressing limbs a neutral proteinase capable of converting β to α is not produced because collagen is degraded mainly within phagolysosomes, and hence, the enzyme is not secreted, while in regenerating limbs collagen is degraded both extracellularly and in phagolysosomes, and a neutral proteinase is part of the mechanism of collagen removal. Intracellular degradation of collagen during the most active phases of limb stump breakdown agrees with the observation that acid proteases are found in regenerating amphibian limbs and tails during the period of stump histolysis (Schmidt, 1966).

Living cells were not necessary to obtain active collagenase from larval axolotl limb tissues. Active enzyme was obtained from lyophilized regenerating and regressing limbs by direct extraction with a neutral buffer. This agrees with direct extraction of collagenase from tadpole skin, rat skin, rat dermis, and human synovial membrane (Nagai and Hori, 1972). On the other hand, active collagenase could not be directly extracted from lyophilized 16 day adult newt regenerates (Table 7). This agrees with an earlier study (Grillo, et al., 1968) in which it was found that living cells were necessary for production of collagenase in culture by adult newt blastemata, since freeze-thawing the tissue prior

to culture resulted in no enzyme activity. It is possible that the difference in ability to directly extract collagenase from larval axolotl and adult newt regenerates is due to the level of collagenase inhibitors in their respective limb stump tissues and regenerates. Since the same histological evidence exists that collagen is being degraded in adult newt regenerates during limb histolysis (Schmidt, 1968), presumably collagenase is present in these tissues. Perhaps adult newt stump tissues and regenerates contain higher levels of collagenase inhibitors which, when the tissues are extracted directly, are extracted with the collagenase and inactivate it, masking the presence of active enzyme. Similarly, perhaps larval limb tissues and regenerates have lower concentrations of collagenase inhibitors, and hence, when extracted directly, not all of the active collagenase in the tissue is inactivated. Protein collagenase inhibitors have been found bound to collagenase in enzymatically inactive connective tissues (Harris, et al., 1969; Bauer, et al., 1971; Eisen, et al., 1971; Nagai and Hori, 1972; Abe and Nagai, 1973). The presence of higher levels of collagenase inhibitors in adult urodele limb stump tissues than in larval limb stump tissues would explain why adult limb tissues are less apt to undergo extensive histolysis (Carlson, 1974), and do not regress when amputated and x-irradiated (Schmidt, 1968), or denervated (Singer and Craven, 1948).

The cell type or types producing collagenase in regenerating and regressing limbs were not identified in this study. Cell types found in regenerating and regressing urodele limbs which have been found to have collagenase activity in other systems include epithelium from metamorphosing tadpole tail fin (Eisen and Gross, 1965) and cutaneous

mammalian wounds (Grillo and Gross, 1967), macrophages (Werb and Gordon, 1975), polymorphonuclear leukocytes (Lazarus, et al., 1968; Robertson, et al., 1972), giant cells (Salthouse and Matlaga, 1972), and fibroblast-like cells (Hook, et al., 1973; Reddick, et al., 1974; Werb and Burleigh, 1974; Werb and Reynolds, 1974). Of these, in studies using epithelium and fibroblast-like cells, other cell types may be present with the desired cell type, and hence it is not clear from the methods used which cell types produce collagenase. Epithelium contains macrophages (Weiss and Rosenbaum, 1967) and polymorphonuclear leukocytes (Fischman and Hay, 1962; Norman and Schmidt, 1967; Weiss and Rosenbaum, 1967) in addition to epithelial cells. Similarly, fibroblast-like cells which were assayed for collagenase were either in whole connective tissue, or in primary cell cultures obtained from whole connective tissue. Both sources probably contain both macrophages and fibroblasts, but have not been positively identified as fibroblasts by their ability to synthesize collagen (Vernon-Roberts, 1972). Vernon-Roberts (1972) feels that an aspect of fibroblast identification should be their ability to synthesize collagen. To the extent that these fibroblast-like cells have been characterized, they appear to have many of the properties of macrophages (Vernon-Roberts, 1972). Of particular interest as a potential source of collagenase in regenerating and regressing urodele limbs are macrophages, polymorphonuclear leukocytes, and giant cells, which all appear in regenerating and regressing limbs coincident with limb stump histolysis. In A. maculatum larvae macrophages are found in increased numbers in regenerating limbs during the early stages of regeneration. During dedifferentiation, macrophages were found near cartilage fragments, muscle remnants, and in the wound epithelium.

During blastema formation, large numbers of macrophages accumulate arround cellular and non-cellular debris in the distal region of the limb stump. As the blastema begins to differentiate, macrophage numbers decline (Weiss and Rosenbaum, 1967). Similarly, in adult newts macrophages accumulate subapically prior to the formation of the blastema (Schmidt and Weary, 1962). Polymorphonuclear leukocytes are present in both larval and adult urodeles during the early phases of regenerating, and are found in and under the wound epithelium (Fischman and Hay, 1962; Norman and Schmidt, 1967; Weiss and Rosenbaum, 1967). Chondroclasts, a type of giant cell, are found inside the collapsing perichondrium in early larval A. maculatum regenerates (Butler, 1933), and osteoclasts, another type of giant cell, are found near the ends of resorbing bone, and often are lodged in depressions in bone in adult newt regenerates (Fischman and Hay, 1962)

Macrophages, polymorphonuclear leukocytes, and giant cells are engaged in the same activities during the early phases of experimentally induced larval urodele limb regression (Butler and Schotte, 1941; Weiss and Rosenbaum, 1967). In addition, macrophages and chondroclasts continue to be present during later phases of regression. In denervated, amputated larval A. maculatum limbs, increased numbers of very active macrophages are found in regressing limbs among dedifferentiated cells, along tendons and ligaments, near cellular debris, and in the epidermis (Weiss and Rosenbaum, 1967). Chondroclasts are commonly seen in the denervation-induced regressing limbs of A. mexicanum larvae (Popiela, 1972). The correlation of limb stump histolysis and the presence of macrophages, polymorphonuclear leukocytes, and giant cells described

above suggests that these cell types may produce collagenase in regenerating and regressing urodele limbs, and the collagenase then may be involved in histolysis of the limb stump tissues.

The role of collagenase in regeneration and regression of urodele limbs, as has been suggested by others (Grillo, et al., 1968), is probably to contribute to histolysis of limb stump tissues. In regenerating limbs, this would allow the emergence of a population of cells which becomes the regeneration blastema. In addition, since blastemata and stump tissues were not separated from each other in this study, one can not rule out a role for collagenase in the regenerate itself. It has been suggested (Mailman and Dresden, 1976) that collagenase is active during early cartilage differentiation in the regenerate. In this study cartilage differentiation begins to occur as collagenase activity is falling off (Figures 4, 5, and 6). Since vertebrate collagenases are known to be more active in degrading dermal collagen, $[\alpha 1(I)]_2\alpha 2$, than cartilage collagen, $[\alpha 1(II)]_3$ (Harris and Krane, 1974), during the above mentioned period collagenase could degrade both types of collagen in the developing blastema, and contribute to the preferential differentiation of limb cartilage before the other connective tissues of the limb. This possibility is supported by the fact that cartilage is the first connective tissue to differentiate in the regenerating urodele limb (Hay, 1958). In experimentally-induced regressing larval urodele limbs collagenase probably contributes to the removal of the formed limb structures.

SUMMARY

This investigation has demonstrated that the pattern of collagenase activity, as measured by the radioactive reconstituted collagen fibril assay at 36°C, coincides with the pattern of limb stump histolysis in both regenerating and denervation-induced larval axolotl limbs. This suggests that collagen degradation by collagenase is one of the mechanisms underlying histolysis of amputated larval limbs whether or not they subsequently regenerate.

In addition, the pattern of collagenase activity in regenerating and denervated regressing larval limbs differs. In both cases, collagenase activity rises rapidly following amputation to maximum levels. However, collagenase activity in regenerating limbs then returns to essentially normal limb tissue levels, while collagenase activity in denervated regressing limbs remains high. Since nerves stimulate proliferation of the blastema, these results indicate that the blastema may be inhibiting collagenase activity in regenerating limbs, perhaps by producing a collagenase inhibitor.

Collagenase activity is not directly related to the protein content of crude enzyme extracts, suggesting that specific changes in collagenase activity occur during regeneration and regression of larval axolotl limbs.

Living cells are not required to obtain collagenase from larval tissues, since collagenase activity was obtained from directly

extracted, lyophilized regenerating and regressing limbs.

Larval axolotl collagenase shares attributes with other vertebrate collagenases. It is active at slightly alkaline pH, is inhibited by EDTA and mammalian serum, and is not inhibited by cysteine or soybean trypsin inhibitor. Crude enzyme extract degrades collagen to dialyzable peptides at 36°C. Disc-gel electrophoretic patterns of reaction mixtures run at 25°C show that axolotl collagenase degrades collagen to characteristic TC^{A}_{75} and TC^{B} fragments, as well as to several slightly smaller TC^{A} fragments, perhaps TC^{A}_{67} and TC^{A}_{62}. In addition, crude collagenase extract from regenerating limbs, but not from regressing limbs, contains a neutral protease which converts β to α subunits, indicating that connective tissue may be degraded differently in regenerating than in denervated regressing larval limbs.

LITERATURE CITED

LITERATURE CITED

Abe, S. and Y. Nagai. (1973). Evidence for the presence of a complex of collagenase with α_2 - macroglobulin in human rheumatoid synovial fluid: A possible regulatory mechanism of collagenase activity in vivo. J. Biochem. 73: 897-900.

Bauer, E. A., A. Z. Eisen, and J. J. Jeffrey. (1971). Studies on purified rheumatoid synovial collagenase in vitro and in vivo. J. Clin. Invest. 50: 2056-2064.

Bauer, E. A., G. P. Stricklin, J. J. Jeffrey, and A. Z. Eisen. (1975). Collagenase production by human skin fibroblasts. Biochem. Biophys. Res. Commun. 64: 232-240.

Blum, H. F., E. G. Butler, J. J. Chang, R. C. Maure, and S. E. Schmidt. (1957). Studies on regression and regeneration in the urodele forelimb after localized ultraviolet radiation. J. Cell. and Comp. Physiol. 49: 153-169.

Brewer, J. M., A. J. Pesce, and R. B. Ashworth. (1974). Experimental Techniques in Biochemistry. Prentice-Hall, Inc., Englewood Cliffs, New Jersey.

Burleigh, M. C., A. J. Barrett, and G. S. Lazarus. (1974). Cathepsin B1: a lysosomal enzyme that degrades native collagen. Biochem. J. 137: 387-398.

Butler, E. G. (1933). Effects of x-radiation on the regeneration of the forelimb of Amblystoma larvae. J. Exp. Zool. 65: 271-315.

Butler, E. G. (1935). Studies on limb regeneration in x-rayed Amblystoma larvae. Anat. Rec. 62: 295-307.

Butler, E. G., and W. O. Puckett. (1940). Studies on cellular interaction during limb regeneration in Amblystoma. J. Exp. Zool. 84: 223-239.

Butler, E. G., and O. E. Schotte. (1941). Histological alterations in denervated non-regenerating limbs of urodele larvae. J. Exp. Zool. 88: 307-341.

Carlson, B. M. (1974). Factors controlling the initiation and cessation of early events in the regenerative process. In Neoplasia and Cell Differentiation. (G. V. Sherbet, ed.) pp. 60-105, S. Karger, Basel.

Chung, E. and E. J. Miller. (1974). Collagen polymorphism: characterization of molecules with the chain composition $[\alpha 1(III)]_3$ in human tissues. Science 183: 1200-1201.

Dearlove, G. E. and D. L. Stocum. (1974). Denervation - induced changes in soluble protein content during forelimb regeneration in the adult newt, Notophthalmus viridescens. J. Exp. Zool. 190: 317-328.

Dixon, M. and E. C. Webb. (1964). The Enzymes. Academic Press Inc., New York.

Donoff, R. B., J. E. McLennan, and H. Grillo. (1971). Preparation and properties of collagenases from epithelium and mesenchyme of healing mammalian wounds. Biochim. Biophys. Acta 227: 639-653.

Drake, M. P., P. F. Davidson, S. Bump, and F. O. Schmitt. (1966). Action of proteolytic enzymes on tropocollagen and insoluble collagen. Biochemistry 5: 301-312.

Dresden, M. H. (1969). Denervation effects on newt limb regeneration: DNA, RNA, and protein synthesis. Devel. Biol. 19: 311-320.

Dresden, M. (1971). Evidence for the role of collagenase in collagen metabolism. Nature New Biol. 231: 55-57.

Dresden, M. H. and J. Gross. (1970). The collagenolytic enzyme of the regenerating limb of the newt Triturus viridescens. Devel. Biol. 22: 129-137.

Eisen, A. Z. and J. Gross. (1965). The role of epithelium and mesenchyme in the production of a collagenolytic enzyme and a hyaluronidase in the anuran tadpole. Devel. Biol. 12: 408-418.

Eisen, A. Z., E. A. Bauer, and J. J. Jeffrey. (1971). Human skin collagenase: the role of serum alpha-globulins in the control of activity in vivo and in vitro. Proc. Nat. Acad. Sci., USA 68: 248-251.

Eisen, A. Z., J. J. Jeffrey, and J. Gross. (1968). Human skin collagenase. Isolation and mechanism of attack on the collagen molecule. Biochim. Biophys. Acta 151: 637-645.

Fischman, D. A. and E. D. Hay. (1962). Origin of osteoclasts from mononuclear leucocytes in regenerating newt limbs. Anat. Rec. 143: 329-337.

Fujiwara, K., T. Sakai, T. Oda, and S. Igarshi. (1973). The presence of collagenase in Kupffer cells of the rat liver. Biochem. Biophys. Rec. Commun. 54: 531-537.

Gallop, P. M., O. O. Blumenfeld, and S. Seifter. (1972). Structure and metabolism of connective tissue proteins. Ann. Rev. Biochem. 7: 617-672.

Gospodarowicz, D. (1974). Localization of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature 249: 123-127.

Gospodarowicz, D. (1975). Purification of a fibroblast growth factor from bovine pituitary. J. Biol. Chem. 250: 2515-2520.

Gospodarawicz, D., P. Rudland, W. Seifert, and J. Lindstrom. (1975). Nobel Symposium 28, In Advances in Metabolic Disorders. (Luft, R. and K. Hall, eds.) Vol. 8, 301-335. Academic Press, New York.

Grillo, H. C., and J. Gross. (1967). Collagenolytic activity during mammalian wound repair. Devel. Biol. 15: 300-317.

Grillo, H. C., C. M. Lapiere, M. H. Dresden, and J. Gross. (1968). Collagenolytic activity in regenerating forelimbs of the adult newt (Triturus viridescens). Devel. Biol. 17: 571-583.

Gross, J. (1964). Studies on the biology of connective tissues: Remodelling of collagen in metamorphosis. Medicine 43: 291-303.

Gross, J. and A. B. Bruschi. (1971). The pattern of collagen degradation in cultured tadpole tissues. Devel. Biol. 26: 36-41.

Gross, J. and D. Kirk. (1958). The heat precipitation of collagen from neutral salt solution: Some rate-regulating factors. J. Biol. Chem. 233: 355-360.

Gross, J. and C. M. Lapiere. (1962). Collagenolytic activity in amphibian tissues: A tissue culture assay. Proc. Nat. Acad. Sci., USA 48: 1014-1022.

Gross, J., C. M. Lapiere, and M. L. Tanzer. (1963). Organization and disorganization of extracellular substances: The collagen system. From Cytodifferentiation and Macromolecular Synthesis. Ed. M. Locke in the 21st Symposium of the Society for the Study of Development and Growth. Academic Press, New York.

Gross, J., E. Harper, E. D. Harris, Jr., P. A. McCroskery, J. H. Highberger, C. Corbett, and A. H. Kang. (1974). Animal collagenases: specificity of action, and structures of the substrate cleavage site. Biochem. Biophys. Res. Commun. 61: 555-562.

Harper, E. and J. Gross. (1972). Collagenase, procollagenase and activator relationships in tadpole tissue culture. Biochem. Biophys. Res. Commun. 48: 1147-1152.

Harper, E., K. J. Bloch, and J. Gross. (1971). The zymogen of tadpole collagenase. Biochemistry 10: 3035-3041.

Harris, Jr., E. D., and S. M. Krane. (1974). Collagenases. N. Engl. J. Med. 291: 557-563, 605-609, 652-661.

Harris, Jr., E. D., D. R. DiBona, and S. M. Krane. (1969). Collagenase in human synovial fluid. J. Clin. Invest. 48: 2104-2113.

Hartley, B. S. (1960). Proteolytic enzymes. Annu. Rev. Biochem. 29: 45-72.

Hay, E. D. (1958). The fine structure of blastema cells and differentiating cartilage cells in regenerating limbs of Amblystoma larvae. J. Biophys. and Biochem. Cytol. 4: 583-591.

Hay, E. D. (1966). Regeneration. Holt, New York.

Hayashi, H., K. Udaka, and H. Miyoshi. (1965). Further study of correlative behavior between specific protease and its inhibitor in cutaneous arthus reactions. Lab. Invest. 14: 665-673.

Hook, R. M., C. W. Hook, and S. I. Brown. (1973). Fibroblast collagenase partial purification and characterization. Invest. Ophthal. 12: 771-776.

Jackson, D. S. and J. H. Fessler. (1955). Isolation and properties of a collagen soluble in salt solution at neutral pH. Nature 176: 69-70.

Jeffrey, J. J. and J. Gross. (1970). Collagenase from rat uterus. Isolation and partial characterization. Biochemistry 9: 268-273.

Jeffrey, J. J., R. J. Coffey, and A. Z. Eisen. (1971). Studies on uterine collagenase in tissue culture. I. Relationship of enzyme production to collagen matabolism. Biochim. Biophys. Acta 252: 136-142.

Johnson, M. C. and A. J. Schmidt. (1974). Collagen synthesis in the regenerating forelimb of the adult newt, Diemictylus viridescens. J. Exp. Zool. 190: 185-198.

Kang, A. H., Y. Nagai, K. A. Piez, and J. Gross. (1966). Studies on the structure of collagen utilizing a collagenolytic enzyme from tadpole. Biochemistry 5: 509-515.

Kefalides, N. A. (1971). Isolation of a collagen from basement membranes containing three identical α-chains. Biochem. Biophys. Res. Commun. 45: 226-234.

Lazarus, G. S. (1973). Studies on the degradation of collagen by collagenases. Frontiers in Biology Vol. 29 (Lysosomes in Biology and Pathology 3): 338-364. Ed. A. Neuberger and E. L. Tatum. North Holland Publ. Co., Amsterdam.

Lazarus, G. S. and J. F. Goggins. (1974). Mechanisms of connective tissue degradation. Science 186: 653-654.

Lazarus, G. S., R. S. Brown, J. R. Daniels, and H. M. Fullmer. (1968). Human granulocyte collagenase. Science 159: 1483-1485.

Lebowitz, P. and M. Singer. (1970). Neurotrophic control of protein synthesis in the regenerating limb of the newt Triturus. Nature 225: 824-827.

Linsenmayer, T. F. (1974). Temporal and spatial transitions in collagen types during embryonic chick limb development. II. Comparison of the embryonic cartilage collagen molecule with that from adult cartilage. Devel. Biol. 40: 372-377.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193: 265-275.

Mailman, M. L. and M. H. Dresden. (1976). Collagen metabolism in the regenerating forelimb of Notophthalmus viridescens: Synthesis, accumulation, and maturation. Devel. Biol. 50: 378-394.

Mailman, M. L., D. S. Kirkpatrick, and M. H. Dresden. (1974). A partial characterization of collagens from Notophthalmus viridescens and Rana catesbiana. Comp. Biochem. Physiol. 47 B: 617-630.

McCroskery, P. A., S. Wood, Jr., and E. D. Harris, Jr. (1973). Gelatin: A poor substrate for a mammalian collagenase. Science 182: 70-71.

Mescher, A. L. and R. A. Tassava. (1975). Denervation effects on DNA replication and mitosis during the initiation of limb regeneration in adult newts. Devel. Biol. 44: 187-197.

Miller, E. J. and V. J. Matukas. (1969). Chick cartilage collagen: a new type of $\alpha 1$ chain not present in bone or skin of the species. Proc. Nat. Acad. Sci., USA 64: 1264-1268.

Miller, E. J., E. H. Epstein, Jr., and K. A. Piez. (1971). Identification of three genetically distinct collagens by cyanogen bromide cleavage of insoluble human skin and cartilage collagen. Biochem. Biophys. Res. Commun. 42: 1024-1029.

Miller, E. J., G. R. Martin, K. A. Piez, and M. J. Powers. (1967). Characterization of chick bone collagen and compositional changes associated with maturation. J. Biol. Chem. 242: 5481-5489.

Nagai, Y. and H. Hori. (1972). Vertebrate collagenase: Direct extraction from animal skin and human synovial membrane. J. Biochem. 72: 1147-1153.

Nagai, Y., J. Gross, and K. A. Piez. (1964). Disc electrophoresis of collagen components. Ann. N. Y. Acad. Sci. 121: 494-500.

Nagai, Y., C. M. Lapiere, and J. Gross. (1966). Tadpole collagenase. Preparation and purification. Biochemistry 5: 3123-3130.

Norman, W. P. and A. J. Schmidt. (1967). The fine structure of tissues in the amputated-regenerating limb of the adult newt, Diemictylus viridescens. J. Morph. 123: 271-312.

Pardo, A. and R. P. Tamayo. (1975). The presence of collagenase in collagen preparations. Biochim. Biophys. Acta 392: 121-130.

Popiela, H. (1972). The ultrastructure of regenerating and denervated limbs of the larval salamander, Ambystoma mexicanum. Masters thesis. Ohio State University.

Reddick, M. E., E. A. Bauer, and A. Z. Eisen. (1974). Immunocytochemical localization of collagenase in human skin and fibroblasts in monolayer culture. J. Invest. Dermatol. 62: 361-366.

Robertson, P. B., R. B. Ryel, R. E. Taylor, K. W. Shyu and H. M. Fullmer. (1972). Collagenase: Localization in polymorphonuclear leukocyte granules in the rabbit. Science 177: 64-65.

Rose, S. M. (1948). The role of nerves in amphibian limb regeneration. Ann. N. Y. Acad. Sci. 49: 818-833.

Rudland, P. S., W. Seifert, and D. Gospodarowicz. (1974). Growth control in cultured mouse fibroblasts: Induction of the pleiotypic and mitogenic responses by a purified growth factor. Proc. Nat. Acad. Sci., USA 71: 2600-2604.

Sakai, T. and J. Gross. (1967). Some properties of the products of reaction of tadpole collagenase with collagen. Biochemistry 6: 518-528.

Salthouse, T. N. and B. F. Matlaga. (1972). Collagenase associated with macrophage and giant cell activity. Experientia 28: 326-327.

Samuel, E. P. (1953). Towards controllable silver staining. Anat. Rec. 116: 511-517.

Schmidt, A. J. (1966). The molecular basis of regeneration: Enzymes. Illinois' Monographs in Medical Sciences 6, Urbana: University of Illinois Press.

Schmidt, A. J. (1968). Cellular Biology of Vertebrate Regeneration and Repair. Univ. of Chicago Press. Chicago, Illinois.

Schmidt, A. J., and M. Weary. (1962). The distribution of alkaline phosphatase in the regenerating forelimb of the adult newt, Diemictylus viridescens. (Triturus v.) J. Exp. Zool. 150: 69-81.

Schotte, O. E. and E. G. Butler. (1941). Morphological effects of denervation and amputation of limbs in urodele larvae. J. Exp. Zool. 87: 279-322.

Schotte, O. E., and E. G. Butler. (1944). Phases in regeneration of the urodele limb and their dependence upon the nervous system. J. Exp. Zool. 97: 95-121.

Schotte, O. E., E. G. Butler, and R. T. Hood. (1941). Effects of transplanted blastemas on amputated nerveless limbs of urodele larvae. Proc. Soc. Exp. Biol. Med. 48: 500-503.

Seifter, S. and E. Harper. (1971). The Collagenases. In The Enzymes 3: 649-697. Ed. P. D. Boyer. Academic Press, New York, 3rd Ed.

Senior, R. M., D. R. Bielefeld, and J. J. Jeffrey. (1972). Collagenolytic activity in alveolar macrophages. Clin. Res. 20: 88.

Singer, M. (1965). A theory of the trophic nervous control of amphibian limb regeneration, including a re-evaluation of quantitative nerve requirements. In Regeneration in Animals and Related Problems. Ed. V. Kiortsis and H. A. L. Trampusch, pp. 20-32. Amsterdam, North Holland.

Singer, M. and J. D. Caston. (1972). Neurotrophic dependence of macromolecular synthesis in the early limb regenerate of the newt, Triturus. J. Embryol. Exp. Morph. 28: 1-11.

Singer, M. and L. Craven. (1948). The growth and morphogenesis of the regenerating forelimb of adult Triturus following denervation at various stages of development. J. Exp. Zool. 108: 279-308.

Singer, M., C. E. Maier and W. S. McNutt. (1976). Neurotrophic activity of brain extracts in forelimb regeneration of the urodele, Triturus. J. Exp. Zool. 196: 131-150.

Steven, F. S., A. Torre-Blanco and J. A. A. Hunter. (1975). A neutral protease in rheumatoid synovial fluid capable of attacking the telopeptide regions of polymeric collagen fibrils. Biochim. Biophys. Acta 405: 188-200.

Strawich, E. and M. E. Nimni. (1971). Properties of a collagen molecule containing three identical components extracted from bovine articular cartilage. Biochemistry 10: 3905-3911.

Tassava, R. A., L. L. Bennett and G. D. Zitnik. (1974). DNA synthesis without mitosis in amputated denervated forelimbs of larval axolotls. J. Exp. Zool. 190: 111-116.

Thornton, C. S. (1938). The histogenesis of muscle in the regenerating forelimb of larval Amblystoma punctatum. J. Morph. 62: 17-47.

Thornton, C. S. (1943). The effect of colchicine on limb regeneration in larval Amblystoma. J. Exp. Zool. 92: 281-295.

Thornton, C. S. and D. W. Kraemer. (1951). The effect of injury on denervated unamputated forelimbs of Amblystoma larvae. J. Exp. Zool. 117: 415-440.

Trelstad, R. L. (1973). The developmental biology of vertebrate collagens. J. Histochem. Cytochem. 21: 521-528.

Trelstad, R. L. (1974). Human aorta collagens: evidence for three distinct species. Biochem. Biophys. Rec. Commun. 57: 717-725.

Trelstad, R. L., A. H. Kang, S. Igarashi, and J. Gross. (1970). Isolation of two distinct collagens from chick cartilage. Biochemistry 9: 4993-4998.

Tweedle, C. (1971). Transneuronal effects on amphibian limb regeneration. J. Exp. Zool. 117: 13-30.

Vaes, G. (1972). The release of collagenase as an inactive proenzyme by bone explants in culture. Biochem. J. 126: 275-289.

Vernon-Roberts, B. (1972). The Macrophage. Cambridge University Press, Cambridge.

Wahl, L. M., S. M. Wahl, S. E. Mergenhagen, and G. R. Martin. (1974). Collagenase production by endotoxin-activated macrophages. Proc. Nat. Acad. Sci. USA 71: 3598-3601.

Wahl, L. M., S. M. Wahl, S. E. Mergenhagen, and G. R. Martin. (1975). Collagenase production by lymphokine-activated macrophages. Science 187: 261-263.

Walker, D. B., O. M. Lapiere, and J. Gross. (1964). A collagenolytic factor in rat bone promoted by parathyroid extract. Biochem. Biophys. Res. Commun. 15: 397-402.

Weiss, C. and R. M. Rosenbaum. (1967). Histochemical studies on cell death and histolysis during regeneration. I. Distribution of acid phosphomonaesterase activity in the normal regenerating, and the resorbing forelimb of the larval spotted salamander, Amblystoma maculatum. J. Morph. 122: 203-230.

Werb, Z. and M. C. Burleigh. (1974). A specific collagenase from rabbit fibroblasts in monolayer culture. Biochem. J. 137: 373-385.

Werb, Z. and S. Gordon. (1975). Secretion of a specific collagenase by stimulated macrophages. J. Exp. Med. 142: 346-360.

Werb, Z. and J. J. Reynolds. (1974). Stimulation by endocytosis of the secretion of collagenase and neutral proteinase from rabbit synovial fibroblasts. J. Exp. Med. 140: 1482-1497.