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RESPONSES OF AMPUTATED ADULT NEWT LIMB STUMPS TO
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MORPHOLOGICAL AND HISTOLOGICAL STUDY

The Ohio State University

Ph.D. 1981

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RESPONSES OF AMPUTATED ADULT NEWT LIMB STUMPS TO DENERVATION, REINNERVATION, AND REINJURY: A MORPHOLOGICAL AND HISTOLOGICAL STUDY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Judith Delane Salley, B.S., M.S.

The Ohio State University
1981

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CHAPTER I
INTRODUCTION AND HISTORICAL OVERVIEW

Regeneration, the process by which organisms restore a lost or missing part, has fascinated man since its earliest mention by Aristotle some two thousand years ago. Much has been learned about this phenomenon since Aristotle's era. Abraham Trembley's classic eighteenth century regeneration studies with fresh water Hydra were the first to stimulate great interest in this phenomenon and led to the investigation of the regeneration capabilities in a number of other phyla.

Regeneration can be found to some degree in all animal groups. However, lower invertebrates demonstrate an ability for regeneration that generally exceeds that found in higher vertebrates. Regenerative powers are especially prominent in sponges, coelenterates, flatworms, annelids, and tunicates, many of which have the ability to reconstitute a new organism from a mere fragment of the original body. Among higher vertebrates, the ability to regenerate major body parts is restricted to the urodele amphibians. However, fish can regenerate distal portions of several fins, lizards can regenerate losses to the tail, and anuran tadpoles can regenerate the tail and hindlimbs.

As early as 1768, Spallanzani, a pioneer experimental morphologist, first formerly described regeneration in the Amphibia. A number of Spallanzani's early observations of regeneration have been confirmed by contemporary studies. They include the following: Both larval and adult
salamanders are capable of regenerating lost appendages; however, larvae regenerate much more rapidly than adults. Tadpoles of frogs and toads regenerate both amputated tails and limbs but lose this ability as they metamorphose into adults. Temperature affects regeneration with the regeneration rate slowing considerably during the winter months. Repeated amputations of a limb result in repeated regeneration.

During these exciting early days of discovery, the regenerative abilities of animals attracted great popular interest and led Thomas Hunt Morgan in 1901 to compile a monumental book describing numerous examples of regeneration throughout the animal kingdom. Morgan, in his book, gave a more precise definition to the kinds of regeneration found in the various animal phyla. He noted that the type of regeneration common among invertebrates involved a mere reorganization of residual tissues. He called this type of regeneration morphalaxis. In contrast, regeneration in vertebrates involved the formation of a population of proliferating cells, a regeneration blastema, which eventually gave rise to the duplicate of the original structure. He described this type of restorative regeneration as epimorphic. At present, no other regenerating system has attracted as much attention as epimorphic regeneration in urodele amphibians. Favorite amphibians used to study epimorphic regeneration include predominantly two groups, the larvae of certain Ambystoma species (i.e., A. mexicanum, A. maculatum) and the adult newt, primarily Notophthalmus viridescens. Experiments in the present study deal exclusively with epimorphic limb regeneration in the adult newt, Notophthalmus viridescens.
CHAPTER II
LITERATURE REVIEW

The Process of Normal Limb Regeneration

One of the most popular models for studying the events that bring about regeneration has been the adult newt forelimb. Following the amputation of a limb, the most progressive activity observed is closure of the wound. Blood clotting occurs within minutes after amputation to immediately seal off the wound from the external environment. Cells from the proximal skin epithelium then lose their desmosomal contacts and migrate over the cut surface of the stump to form a thin transparent layer of epidermis that is usually 3 to 4 cell layers thick. This epidermal wound healing is usually completed within 12 to 24 hours post-amputation (Lash, 1955; Thornton, 1968) and does not involve mitosis of the migratory cells themselves. The source of new cells for the wound epithelium resides in a very active proliferating zone in the proximal limb epithelium (Chalkley, 1954; Hay and Fischman, 1961). Cells from this epithelium continue to migrate distally during the early phases of regeneration and eventually give rise to a wound epidermis some 12 to 16 cell layers thick (Singer and Salpeter, 1961). This thickened area of epidermis closely resembles the apical ectodermal ridge of the embryonic limb bud and is sometimes referred to as the apical epidermal cap (Thornton, 1968). The apical epidermal cap is believed to play a very
important role in regeneration and will be discussed in a later section. Unlike normal skin epidermis that is underlain by a basement membrane and dermis, the wound epidermis is completely free of both dermis and basement membrane and is thus in direct contact with the mesodermal tissues of the stump (Schmidt, 1968; Singer and Salpeter, 1961). This intimate contact between the wound epidermis and the underlying mesodermal tissues has been shown to be an important prerequisite for normal regeneration (reviewed by Singer and Salpeter, 1961).

Following wound epidermis formation, a series of complex changes occur in the underlying mesodermal tissues of the distal stump. The injured stump tissues become edematous and a number of white blood cells and macrophages invade the wound area and begin to phagocytize the necrotic and extraneous cellular debris created by the injury of amputation. The wound epidermis has been shown to also actively participate in this initial clean-up phase. Singer and Salpeter (1961) have described epidermal tongues that extend into the underlying damaged tissues to the stump. The epidermal tongues surround pockets of cellular debris and extrude this material through the wound epidermis to the outside.

An increase in lactic acid and proteolytic enzymes, i.e., acid phosphatase, also occurs during this demolition period and adult tissues begin to break down (Schmidt, 1960). Mononucleated cells, closely resembling embryonic cells, are released and accumulate in the area beneath the wound epidermis in the distal stump. The process by which these cells lose their differentiated characteristics and revert to a more mesenchymatous state is called dedifferentiation. The origin of
these dedifferentiated cells was a major point of controversy for many years. However, most of the evidence suggests that all of the mesodermal tissues of the stump, except for endothelial cells of the vasculature and pigment cells, are capable of giving rise to these mesenchyme-like cells (Butler, 1935; Chalkey, 1954; Steen, 1968).

As histolysis of the stump sweeps proximally (involving a few millimeters of the adult tissues), the number of dedifferentiated cells increases. Autoradiographic evidence has shown these cells to be actively involved in synthesis of DNA by 4 days after amputation in the adult newt (Hay and Fischman, 1961), and both RNA (Bodemer, 1962; Kelly and Tassava, 1973) and protein synthesis (Bodemer and Everett, 1959) as early as 2 days post-amputation. The continued proliferation of these dedifferentiated cells during the first few weeks post-amputation results in a homogeneous cellular mass called the regeneration blastema. It is from this blastema that the missing parts of the limb are reconstructed.

At the end of the third week, histolysis of the stump tissues declines and a cone-shaped blasternal outgrowth appears at the end of the stump. It is during this period (cone-stage) that proliferation of blastema cells reaches a maximum (Chalkley, 1959). Shortly after this rapid growth phase, the cone-shaped blastema becomes flattened to form a paddle. The growth rate slows and the first signs of tissue differentiation become evident. Chondrogenesis of the long bones occurs first and later extends distally to the hand and fingers. After the fingers are well formed, the cartilage is gradually transformed into bone (in the adult newt). Both nerves and blood vessels regrow into the regenerate and eventually reproduce their original innervation and vascular
patterns. New muscle fibers form de novo from the mesenchymatous cells surrounding the cartilage and functional interactions between muscles and nerves are re-established (Lentz, 1969). Other signs of tissue differentiation include the formation of skin glands, at first proximally and then distally, along the regenerate. In the final stages of regeneration, the wound epidermis is gradually lost and is replaced by normal skin complete with skin glands, a basement membrane, and a dermal layer (Singer and Salpeter, 1961).

Regardless of the level of amputation, the sequence for regeneration described above, wound healing, dedifferentiation, and blastema formation is about the same. The difference involves the speed of regrowth, with limbs amputated proximally regenerating faster than those amputated at a more distal level (Iten and Bryant, 1973). Regeneration events in larval limbs closely resemble those described for the adult newt except that amputated larval limbs regenerate faster than adult limbs and histolysis of the stump tissues during dedifferentiation is much more extensive (Schotte and Butler, 1944). Three important factors, nerves, injury, and wound epidermis are known to regulate normal regeneration. Their exact roles in initiating limb regeneration, however, are still not fully understood. The following is a review of the roles of the wound epidermis, injury, and nerves.

Role of the Wound Epidermis

There is little question that the formation of a wound epidermis over the cut surface of an amputated limb is an indispensable condition for regeneration. The importance of the wound epidermis in regeneration
was established years ago from studies that involved covering a fresh wound with full thickness skin. No wound epidermis formed and regeneration did not occur (Tonier, 1906; Godleski, 1928). Since skin consists of a thick layer of connective tissue, i.e., dermis, upon which the epidermis lies, grafts of skin prevent wound epidermis contact with the underlying wound tissues. This important wound epidermis-wound tissue relationship is normally established early in regenerating limbs since only epidermis migrates over the amputation surface to close the wound. The exact nature of the epidermal role in regeneration is still unknown, however, several possibilities have been suggested.

Thornton (1960; 1968) proposed the theory that the apical epidermal cap was responsible for accumulation and aggregation of dedifferentiated mesenchymatous cells beneath the epidermis for blastema formation. His conclusions were based on the following observations: Daily removal of the epidermal cap from the amputated limbs of *Ambystoma* completely inhibited regeneration as did daily irradiation of the apical cap with ultraviolet light. Shifting the epidermal cap to an eccentric position at the end of a larval limb stump caused mesenchymal cells to accumulate beneath the shifted cap and resulted in asymmetrical regenerates. It was once believed that nerves and not the epidermal cap might be responsible for the shifted accumulation of blastema cells. This possibility was eliminated when the epidermal caps of aneurogenic salamander limbs (limbs never innervated) were shifted to an eccentric position and eccentric blastemas still formed.

Faber (1971) postulated a growth promoting and morphogenetic role for the wound epidermis noting that the epidermal cap controlled the
proliferation tendencies of the blastema. Whenever intimate contact existed between the wound epidermis and the underlying mesenchymal cells, outgrowth of a blastema occurred. However, in the absence of this epidermal contact no blastemal outgrowth formed.

Additional insight into the role of the wound epidermis in regeneration has been gained from studies involving preventing its formation and examining the regeneration events that occur or fail to occur in its absence. Polezhaev and Faworina (1935) inserted the cut ends of freshly amputated limbs of axolotls under the skin of the flank and found that such limbs failed to regenerate. Lack of regeneration was attributed to the fact that immediate insertion under the flank prevented closure of the amputation surface with a wound epithelium. A similar experimental method was employed by Goss (1956) and more recently by Loyd (1980) whereby the cut ends of amputated forelimbs of adult newts were immediately inserted into the coelomic cavity. These inserted limbs did not form a wound epidermis and failed to regenerate. Loyd (1980), in a more extensive study, examined the cellular events that occurred in these non-regenerating inserted limb stumps and found that some cells dedifferentiated, entered the cell cycle, replicated DNA, but did not accumulate to form a blastema. Mescher (1976), and Tassava and Garling (1979), blocked wound epidermis formation and subsequent regeneration in the adult newt and the axolotl by placing flaps of whole skin over the amputation surface. In these skin graft limbs, cells entered the cell cycle, underwent DNA synthesis and mitosis, all in the absence of a wound epidermis, however levels needed for blastema formation were never reached. The mesodermal tissues of the stump were merely repaired as
cells left the cycle and tissue redifferentiation occurred but not
epimorphic regeneration. It would appear from the results of these
studies that the wound epidermis is not involved in initial dedifferen­
tiation of cells. These results led Mescher (1976) to conclude that
the wound epidermis was needed to prevent immediate redifferentiation of
post-mitotic cells. Thus cells could remain in the cycle to undergo
additional rounds of DNA replication and mitosis and form a blastema.

A wound epidermis, although present, might not be functional. For
instance, a wound epidermis forms on the limb stumps of completely
denervated limbs which do not regenerate (Tassava et al., 1974; Mescher
and Tassava, 1975), and non-regenerating post metamorphic frogs also form
a wound epidermis over their amputated limb stumps (Schotte' and Harland,
1943; Schotte' and Smith, 1961).

It has been well documented that the wound epidermis is involved in
the dissolution and disposal of cellular debris from underlying tissues,
including the stump and the regenerating tissues (Singer and Salpeter,
1961; Riddiford, 1960). Observations with both the light microscope and
the electron microscope have shown cellular debris in various stages of
dissolution in vacuoles of the wound epidermis during the early wound-
healing stages of regeneration. Singer and Salpeter (1961) have shown
that there is a continuous stream of detritus and wandering phagocytes
entering the wound epidermis from the underlying tissues during wound
healing. It was suggested that the wound epidermis acts as an organ of
disposal since it discharges this debris to the outside.
Role of Injury

Injury has long been recognized as a necessary stimulus for regeneration, however, it is not known how injury exerts its influence. A number of studies have been done which suggests that injury alone is instrumental in initiating the dedifferentiation of stump tissue cells needed for blastema formation. It is important to note that wound epidermis and nerves have also been implicated in the dedifferentiation process. Experiments have therefore been designed to evaluate the extent of dedifferentiation in the absence of nerves, wound epidermis, or injury, singly or in combination.

Thornton and Kramer (1951) and Thornton (1953) found that, after denervating larval Ambystoma limbs, excessive dedifferentiation or regression could be stimulated by piercing or crushing the internal tissues of the limb. Although these types of injuries did not cause a break in the skin and no wound epidermis formed, stump tissues nevertheless underwent dedifferentiation. That nerves were not important for dedifferentiation was also shown in the early experiments of Schotte and Butler (1941) wherein tissues of denervated, amputated limbs of both Ambystoma and Triturus larvae underwent excessive dedifferentiation ultimately leading to complete limb stump resorption. On the other hand, if these denervated limbs were not injured or amputated, resorption and dedifferentiation did not occur.

A number of convenient methods have been employed to prevent the formation of a wound epidermis over an amputated limb stump. These methods have been very instrumental in investigating the regeneration
events that occur in the presence of injury alone. Goss (1956) found that if the amputated end of a newt limb was inserted into the coelomic cavity before a wound epidermis formed, stump tissue cells nevertheless underwent dedifferentiation in these non-regenerating limbs. However, nerves were also present in those limbs. More recently, Loyd (1980) extended the findings of Goss by denervating amputated coelom inserted limbs. This experimental system of Loyd was an important one because it provided a means to analyze the effects of injury alone on the initiatory events of regeneration. Although these amputated, denervated, inserted limbs never regenerated, cells underwent dedifferentiation and entered the cell cycle as evidenced by their histological appearance and their ability to incorporate H\(^3\)-thymidine. Using another method, Mescher (1976) placed whole skin flaps over the amputation surface of adult newt forelimbs to prevent wound epidermis formation and found that dedifferentiation could also be seen in these non-regenerating limbs. The results from all of these studies suggest the following role for injury in regeneration. The injury of amputation is the necessary stimulus to cause stump tissue cells to dedifferentiate, whether in the presence or absence of nerves and a wound epidermis.

The extent of injury has also been shown to be important in initiating regeneration. Orechowitsch and Bromley (1934) and Polezhaev (1933) placed whole skin flaps over the amputated limb stumps of axolotls and prevented regeneration from occurring. They noted that if the skin flaps were carefully removed from the distal tip 8 to 10 weeks later, imparting only minimal injury to the underlying stump tissues, a wound epidermis formed over these limb stumps but regeneration still did not occur.
However, if the mesodermal tissues were damaged extensively with a needle, these limbs were stimulated to regenerate. More recently, Tassava and Loyd (1977) undertook a study to quantitate the amount of injury necessary to initiate regeneration in non-regenerating skin-graft newt limbs. They found that various types of re-injuries were successful in restoring regeneration capabilities to these skin-flap limbs at 5 weeks post-skin grafting. Re-injuries which stimulated regeneration included: 1) re-amputation 1 mm into the stump; 2) a single razor incision 1 mm into the stump; and 3) removal of either a portion of or the entire skin graft. Needle penetrations through the skin graft were the only unsuccessful means for stimulating regeneration.

Although the importance of injury to regeneration has been established, the exact means by which injury initiates regeneration remains unknown and is open to further investigation.

The Role of Nerves in Regeneration

The necessity of nerves for salamander limb regeneration has been recognized since the experiments of Tweedy John Todd in 1823. However, a detailed analysis of the nervous influence in limb regeneration was not begun until the 1940s by Marcus Singer (reviewed by Singer, 1952). Since that time much has been learned about the neurotrophic influence during regeneration. For example, 1) all neurons are trophic; motor, sensory, and sympathetic neurons can supply the needed stimulus for regeneration; 2) the trophic effect is centrifugal in direction and is therefore independent of polarization of the neuron; 3) neither central connections or reflex circuitry is required for trophic activity; and
4) a threshold number of nerve fibers must be present at the amputation surface for limb regeneration to occur.

Much experimental evidence suggests that the primary role of the nerve is the stimulation of mitosis (Singer and Craven, 1948; Singer, 1952; Thornton, 1968; Mescher and Tassava, 1975); however, the manner in which the mitogenic activity is implemented is not understood. Spinal nerves, 3, 4, and 5 supply the innervation to the forelimb of the adult newt. Cutting these nerves in the brachial plexus, i.e., the shoulder level, at the time of the amputation, effectively blocks regeneration. In larval salamanders, denervation at the time of amputation not only suppresses regeneration but also causes the entire limb to resorb (Schotte and Butler, 1941). If denervation occurs during the early bud or mound stage, after the blastema has formed, resorption of the blastema occurs (Schotte and Butler, 1944; Singer and Craven, 1948). However, if denervation is delayed until a later stage of regeneration (late bud, paddle stage), the nervous influence is seen to be of lesser importance and differentiation of blastemal cells and morphogenesis of the regenerate into a small but normal limb occurs despite the lack of nerves (Schotte and Butler, 1944; Singer and Craven, 1948; Powell, 1969). This suggests that the neurotrophic factor is needed for the cellular proliferation involved in blastema formation but not for the later events of regeneration.

The effects of denervation on regeneration have also been examined biochemically. Cutting the nerves to an early regenerate results in a 40 percent reduction in protein, RNA, and DNA synthesis after two days (Lebowitz and Singer, 1970; Dresden, 1969). However, preceding this
decline in synthesis, initial outbursts in RNA synthesis (4 hours), protein synthesis (6 hours), and DNA synthesis (12 hours) have been seen (Singer and Caston, 1972). These results suggest that at 48 hours following denervation, synthesis of macromolecules does not completely stop; however, a large decrease (60 percent) is seen, presumably, that which is nerve-dependent. Other biochemical studies have shown that synthesis of mRNA and rRNA are also adversely affected within 2 to 3 days following denervation of nerve-independent regenerates (Morzlock and Stocum, 1971; Bantle and Tassava, 1974). Unfortunately these biochemical studies give little clue as to the nature of the neurotrophic agent.

There is much evidence which suggests that the neurotrophic factor is a chemical emitted from the ends of axons. Support for this view was published in 1970 by Lebowitz and Singer. They found that infusions of crude homogenates of nerves into denervated blastemas could recover most of the nerve-dependent protein synthesis lost as a result of denervation. More recent attempts to extract and characterize the neurotrophic factor have met with limited success. A major problem in the identification and purification of the trophic factor has been the lack of a rapid, convenient and quantitative regeneration bioassay. Denervated limb stumps and denervated nerve-dependent and nerve-independent blastemas have all served as in vivo bioassays. Brain extracts and recently various growth promoting substances (i.e., fibroblast growth factor, FGF) have been infused into denervated blastemas in vivo and have been found to stimulate mitosis (Mescher and Gospodarowicz, 1979) and to
increase the levels of protein and DNA synthesis to levels of controls (Singer et al., 1976; Jabaily and Singer, 1977). The major disadvantage of such in vivo bioassays have been: 1) the leakage of the infusate out of the limb through time, thereby requiring the use of very high concentrations of extracts to get significant results, and 2) damage to the regenerate resulting from continued daily injections. Such limitations have been avoided by using blastemas maintained in organ cultures. Recent studies suggest that this approach may provide a sensitive test for factors thought to have neurotrophic qualities (Globus et al., 1978; Carlone and Foret, 1979; Rathbone et al., 1979; Rathbone et al., 1981; Mescher and Loh, 1981). Such a system could lend further insight into how these factors influence the initiatory and proliferative events of urodele limb regeneration.

Regeneration and the Cell Cycle

Salamander limb regeneration is dependent on the accumulation of a mass of mesenchymal cells at the tip of the stump beneath a wound epidermis. Proliferation of these cells results in the formation of a blastema which eventually gives rise to the missing parts of the limb (Thornton, 1970). In terms of the cell cycle, the early events which lead to blastema formation can be described as follows: Differentiated (G₀) mesodermal cells undergo dedifferentiation and enter the cell cycle in G₁, they then replicate their DNA in S, proceed through G₂, and finally undergo division in the M phase.
Three factors have been shown to be essential for blastema formation. The limb must be injured, a sufficient number of nerves must be present at the amputation surface, and a dermis-free wound epidermis must cover the amputation surface (reviewed by Thornton, 1968; Singer and Salpeter, 1961; Carlson, 1974; Singer, 1978; Tassava and McCullough, 1978). The precise roles for each of these three requirements are still not understood and only recently have the roles of these factors been investigated in terms of their possible influence on cell cycle events.

Tassava and Mescher in 1975 proposed a cell cycle model to explain the roles of injury, nerves and a wound epidermis that is consistent with much of the regeneration literature. In terms of the cell cycle, injury (i.e., amputation) initiates dedifferentiation and entry of limb stump cells into the G1 phase of the cycle. The cells then move through the S phase and into G2. Nerves regulate one or more G2 events so that the cells can proceed through G2 into M where they undergo mitosis. The wound epidermis functions to keep these post-mitotic cells in the cycle so that they may undergo additional cycles to establish a blastema. Evidence supporting each of these roles has been described previously (see literature review) and will be discussed only briefly in this section.

The view that injury is instrumental in triggering cells to dedifferentiate is supported from the results of the following types of experiments: 1) limbs were denervated and injured without amputation (Thornton, 1953; Thornton and Kramer, 1951); and 2) limbs were amputated and not allowed to form a wound epidermis; i.e., skin-graft limbs and coelom-inserted limbs (Mescher, 1976; Goss, 1956; Loyd, 1980). A number
of these methods allowed the influence of injury alone to be examined during the initiatory phase of regeneration. The results from all of these studies led to the following conclusion: When a limb has been injured, whether or not nerves and wound epidermis are present, cells undergo dedifferentiation and enter the cell cycle.

Evidence supporting the role of the wound epidermis in maintaining cells in the cycle came mainly from studies that prevented the formation of a wound epidermis. When the wound epidermis is not present on an amputated limb stump, as when the limb tip is coelom-inserted (Goss, 1956; Loyd, 1981) or covered with a flap of skin (Tonier, 1906; Godlewski, 1928; Mescher, 1976; Tassava and Garling, 1980), regeneration fails to occur. Cells nevertheless dedifferentiate, move through the cell cycle, and undergo mitosis, as evidenced by early increases in $^{3}$H-thymidine labeling and mitotic indices. However, these cells immediately leave the cycle to redifferentiate into stump tissues (i.e., cartilage, muscle and connective tissue) and thus never undergo the necessary rounds of DNA replication and mitosis needed to form a blastema.

That nerves also influence certain cellular events during the initiatory phase of regeneration was demonstrated mainly from experiments involving autoradiographic analysis of DNA synthesis, RNA synthesis and mitotic indices in amputated, denervated limbs which do not regenerate (Butler and Schotte, 1941; Singer, 1952). Tassava and Mescher (1975) designed experiments to determine, if in fact, denervated limbs initiate regeneration even though they never form blastemas. They also wanted to identify which cellular event was limiting after denervation. Their
results were similar for both adult newts and axolotl larvae and showed that \(^3\text{H}\)-thymidine incorporation in denervated limbs paralleled that of innervated control limbs for approximately 10 days post-amputation. However, while the mitotic index of innervated limbs increased significantly during this time, the mitotic index in denervated limbs remained near zero. These results led Tassava and Mescher (1975) to propose the following view concerning the neural control of cell cycle events:

Denervated limbs do initiate regeneration in the absence of nerves; however, dedifferentiated cells are blocked in the G\(_2\) phase of the cell cycle and are thus restricted from proliferating to form a blastema.

Recently two new models have been proposed which contradict the G\(_2\) block proposed by Tassava and Mescher (1975).

Maden (1978) examined cellular events in amputated denervated axolotl limbs during the first week post-amputation and found that the mitotic index in these denervated limbs increased significantly when compared to unamputated controls. It was also observed that the labeling index in these limbs paralleled the control innervated limbs for the first week post-amputation. These findings prompted Maden to suggest that cells in a denervated limb block in G\(_1\) instead of G\(_2\). Globus (1978) utilized a transfilter culture system to study the effects of nerves on cone stage blastemas from adult newts. His model suggests that nerves may be needed to facilitate the progression of cells from the G\(_0\) state through G\(_1\) and S and may also serve to remove constraints during the G\(_2\) phase to allow cells to undergo division. Thus, according to Globus, the neurotrophic factor may act in the G\(_1\), S, and/or the G\(_2\) phases of the cell cycle. Much work is still needed before a complete
understanding of the role of nerves during regeneration can be made.
CHAPTER III
FOCUS OF THE DISSERTATION

Initially, I attempted to establish a system which would provide a means by which cellular events could be examined when regenerating nerves reached the tip of a denervated limb stump. By denervating limbs which were amputated at different levels, nerves had various lengths of stump to reinnervate, i.e., nerves would reach the tip of short limb stumps earlier.

Unfortunately, none of the denervated limb stumps regenerated regardless of the amputation level, even though threshold reinnervation occurred. I, therefore, became interested in determining what inhibitory events occurred in denervated newt limb stumps that prevented regeneration after reinnervation. Experiments were designed to examine denervated limb stumps, first of all morphologically, for changes such as the healing over of the amputated limb stump with skin rather than wound epidermis. Whole skin has been shown to block regeneration of amputated adult newt limb stumps (Mescher, 1976). Other changes in the wound epidermis which might be responsible for inhibiting regeneration were also possibilities. Secondly, experiments were designed to examine denervated newt limb stumps histologically for the presence of a marker or feature common to all denervated limb stumps which overtly signaled that regeneration was blocked, i.e., rapid scar tissue formation around
the cut edges of the bone. Such histological features are commonly found in amputated non-regenerating limbs of higher vertebrates (i.e., mammals) so it was also of interest to see if long-term denervated newt limbs histologically resembled non-regenerating limbs of other vertebrates. A detailed histological description of amputated/denervated adult newt limb stumps is lacking in the literature. The few micrographs available do not adequately portray the cellular and histological features of long-term denervated limb stumps (see Rose, 1948; Singer and Inoue, 1964; Kamrin and Singer, 1959).

Specifically, experiments were designed to answer the following questions concerning amputated/denervated newt limbs: 1) Will amputated/denervated limb stumps of adult newts initiate regeneration upon reinnervation without additional injury? 2) If so can a potential system be developed using denervated newt limbs for analyzing the cell cycle events (labelling index and mitotic index) that occur upon reinnervation? 3) If denervated limbs do not regenerate upon reinnervation, what inhibitory events occur to prevent regeneration? 4) When does the denervated limb become committed to tissue regeneration versus epimorphic regeneration? 5) What kinds of histological changes occur in denervated newt limb stumps? 6) What effect does long-term denervation have on cellular events? 7) For example, does dedifferentiation occur in the absence of nerves? 8) Do cells undergo cellular proliferation in long-term denervated newt limbs? 9) Do cells replicate DNA in the absence of nerves? 10) What are the regeneration limiting factors in long-term denervated newt limb stumps? Each experiment in this study was based on the results of the preceding experiment and the various questions asked concerning
amputated/denervated newt limbs also arose in a similar sequence.

It was hoped that the results of this study would further clarify the roles of injury, nerves, and the wound epidermis in initiating regeneration.
General Care of Newts

Adult newts (Notophthalmus viridescens), collected in southern Ohio, were used in all of the experiments in this study. Newts were maintained without feeding in plastic containers of aerated tap water at 4°C until the time of operations. Experimental animals were kept in aerated water in incubators at 24°C under constant light for the duration of each experiment.

Before each operation, newts were always anesthetized in a 1:1000 solution of MS222 (ethyl-m-amino benzoate methanesulfonate, Eastman). After operations, newts were treated in a solution of aqua-aid (a fungicide) for a period of 24 to 48 hours to avoid infection of the wounds by fungi.

Denervations

The limb of the adult newt is innervated by spinal nerves 3, 4, and 5 that together form a brachial plexus just before entering the forelimb. Complete denervation involves the transection of all the nerves in the brachial plexus at the scapular region. Cutting a nerve results in degeneration of all the cut axons distal to the cut so that the area to which these nerve fibers distribute becomes paralyzed and
insensitive. Axons proximal to the cut remain connected to their cell bodies and are able to regenerate and regrow along the original paths of the nerve to reconnect with the periphery, a process called reinnervation.

For all of the experiments in this study, complete denervations were performed on the left forelimb, either 1 day post-amputation, 7 days prior to amputation, or 14 days prior to amputation. Newts were always observed for movement and sensitivity in the denervated limb. Those few newts which showed signs of nerve function in the left limb after denervation were removed from the experimental group.

In those experimental series where redenervations were performed, limbs were first completely denervated 1 day post-amputation. Then, 14 days later, the wound was reopened at the shoulder level and carefully examined for regenerated nerve fibers. Any nerve fibers found were cut in order to increase the amount of time the limb remained in the completely denervated state.

Histological Procedures

All limbs to be examined histologically were fixed in Bouin's solution for 24 hours with subsequent decalcification in 0.5 m EDTA (ethylene-diamine-tetra acetic acid - tetra Na salt) for 4 days. Left and right limbs from each newt were kept together to allow direct comparison of the control with the experimental forelimb of each newt. Limbs were prepared by routine histological processing and embedded in paraffin (Humason, 1972). All limbs were sectioned longitudinally at 10 μM. Sections from each limb were either 1) distributed serially on
a set of four slides, or 2) distributed so that each of four slides had equal representation of the entire limb. Different slides from each limb were used for either nerve staining (Samuel, 1953), hematoxylin-eosin (general histological survey), Mallory's triple stain (connective tissue), or for autoradiography.

**Autoradiography**

These experiments were done to determine 1) whether cells that synthesize DNA on day 7 in once-denervated and re-denervated limb stumps are maintained in the stump and/or proliferate during the 5 weeks post-amputation/denervation time, and 2) the extent of cellular activity (cycling cells) on day 35 post-amputation in once-denervated and re-denervated limb stumps.

Newts were injected intraperitoneally with the radioisotope (methyl-\(^{3}\)H) thymidine (New England Nuclear Corporation). The specifics on the amount of isotope used and the incorporation period are discussed below for the specific experiments (see Experiments 8 and 10).

Slides containing sections from each limb were deparaffinized, hydrated, and allowed to air dry. In the darkroom, the slides were dipped into a 3:1 solution of water:Kodak NTB-2 Nuclear Track Emulsion (45°C) and hung perpendicularly until dry. The emulsion-coated slides were stored at 5°C for two weeks in light-proof plastic slide boxes containing dessicant. After a two-week exposure period, the autoradiographs were developed in the darkroom for 3 minutes in Kodak D-19 developer at 21°C, fixed in Kodak F5 fixing bath for 5 minutes at the same temperature, and rinsed thoroughly in running
tapwater. The sections were finally stained in hematoxylin-eosin, dehydrated, cleared, and mounted with Piccolyte.

Experimental Design for the Morphological Study

Experiment 1: A Test of Whether Once-Denervated Newt Limb Stumps Will Regenerate Upon Reinnervation.

Rationale

Since it is known that denervated, amputated limbs of larval Ambystoma regenerate upon reinnervation without further injury (Petrosky et al., 1980), it became important to establish whether an adult newt limb that has been amputated at different levels and completely denervated will regenerate without additional injury after nerves return to the limb stump, since the system could provide a means for studying the cellular events that occur in denervated newt limbs after nerves grow back in.

Questions

Will an amputated denervated adult newt limb regenerate upon reinnervation? Will the level of amputation affect the timing of reinnervation and subsequent regeneration? The following experiment was designed to specifically answer these questions.

Both the right and left forelimbs of 18 adult newts were amputated through either the proximal humerus, distal humerus, or through the middle portion of the radius and ulna. One day post-amputation, the left forelimb of each newt was completely denervated. Left limbs were denervated only once to allow complete reinnervation (Fig. 1).
LEVELS OF AMPUTATION AND DENERVATION

Figure 1. This figure diagrams the innervation pattern as seen from a dorsal view of the limb and a side view of the body wall. Levels of amputation include I, mid-radius and ulna, II, distal humerus, and III, proximal humerus. Spinal nerves 3, 4, and 5 were transected at the shoulder level, IV.
Right limbs were not denervated but were left as amputated, innervated, regenerating controls. Both right and left limbs were observed every 2 to 3 days for the presence or absence of a blastema. All regenerating limbs were staged according to the system of Tank et al. (1976).

Experiment 2: The Timing of Stump Healing in Relation to Reinnervation

Rationale

The rationale of the following experiment was derived from the results of Experiment 1. The results of Experiment 1 showed that amputated completely denervated limbs of adult newts remain "stumped" and do not regenerate upon reinnervation regardless of the level of amputation. These results represent a fundamental difference between larval and adult newt limbs, a difference worthy of analysis. It was important first to determine when and to what extent reinnervation of the limb stump occurred.

Question

When and to what extent does reinnervation occur in a completely denervated adult newt limb stump? If once-denervated limb stumps are reamputated at various times post-denervation, what is the frequency of regeneration?

Using reinjury (reamputation) and subsequent regeneration as a morphological test for threshold innervation, the following experiment was designed.
RELATIONSHIP OF TIME OF REAMPUTATION TO REINNERVATION OF NEWT LIMB STUMPS

Figure 2. This diagram depicts the level of reamputation, 1 mm proximal to the original amputation surface, and the reinnervation pattern of denervated newt limb stumps at the time of reamputation, 7, 14, 21, 28, and 35 days post-amputation/denervation.
The left and right forelimbs of 97 newts were amputated through the middle portion of the radius and ulna. One day post-amputation the left limb was completely denervated. The right limb served as the amputated, innervated control. No redenervations were done on the left limb and on each of the following days post-amputation, 7, 14, 21, 28, and 35, a group of animals was anesthetized and a fresh amputation was made 1 mm proximal to the original amputation surface of both left and right forelimbs (Fig. 2). Limb stumps were thus reinjured and new wound epidermis formed. If nerves had returned to the distal limb stump in threshold quantities by the time of the second amputation, then denervated left, and innervated control right limbs should initiate regeneration at the same time. Limbs were observed for signs of regeneration every three days.

The 1 mm portions (distal tips) removed from each limb, were fixed and examined histologically.

Experiment 3: Delayed Amputation of Once-Denervated Newt Limbs

Rationale

If nerves are given a headstart back into the denervated limb stump will epimorphic regeneration result? Can regeneration of a completely denervated newt limb be initiated without additional injury? The results of Experiment 2 suggest that certain inhibitory healing events occur before nerves reach the amputation surface and even though reinnervation occurs, suitable conditions for regeneration no longer exist. The next three experiments (3, 4, and 5) were attempts designed to initiate regeneration upon reinnervation but without additional injury.
by: 1) denervating and delaying amputation so as to get nerves back into the limb before the limb stump heals over, 2) increasing the amount of cartilage at the amputation surface of denervated newt limbs, since larvae *Ambystoma* have cartilaginous skeletons and are able to regenerate after denervation and reinnervation, and 3) amputating and denervating a regenerate (70 days) of an adult newt limb since young newt regenerates manifest many of the same properties as larval *Ambystoma* regenerates.

Question

Will regeneration occur in a denervated forelimb that is amputated at different levels and at various times post-denervation?

The left limbs of 20 adult newts were completely denervated. The right limbs were left as innervated controls. On days 7 and 14 post-denervation, both the left and right forelimbs were amputated (Fig. 3). Five limbs were amputated through the proximal part of the humerus and 5 limbs were amputated through the middle portion of the radius and ulna. Thus, on the day of amputation, left limbs were either 7 or 14 days post-denervation.

All limbs were observed for signs of regeneration every three days for a period of 10 weeks.

Experiment 4: Cartilage Grafts in Completely Denervated Newt Limbs

Question

Will cartilage grafts at the amputation level of denervated newt limb stumps result in regeneration upon reinnervation?
RELATIONSHIP OF TIME OF DENERVATION TO AMPUTATION OF NEWT LIMB STUMPS

Figure 3. Newt limb stumps were denervated (day 0) and amputated through the proximal humerus or radius and ulna, 7 and 14 days post-denervation.
The right and left forelimbs of 5 larval axolotls (*Ambystoma mexicanum*) were amputated just distal to the elbow. Under the dissecting microscope the cartilaginous elements of the amputated portion of the limbs, particularly the radius, ulna, and digits, were carefully separated and cleaned free of skin, connective tissue, and muscle, and cut into equal sizes.

Limbs of 28 adult newts were amputated through the middle portion of the radius and ulna (both left and right forelimbs). Using watchmaker's forceps, a tunnel was made approximately 1 mm in depth between the radius and ulna of only the amputated left limbs. One piece of cartilage was carefully placed in the tunnel. Care was taken to assure that the cartilage graft was at the level of amputation and did not protrude beyond the amputation surface. Newts were kept immobile for approximately 3 hours on crushed ice to facilitate healing of the graft into the amputation wound.

One day post-amputation and grafting left limbs were completely denervated. Right limbs were left as amputated, innervated controls. Therefore, all 28 newts in this study had an amputated, innervated right limb and an amputated, completely denervated left limb containing a cartilage graft. Limbs were observed for signs of regeneration for a 10-week period.

Experiment 5

The response of 70 day newt *regenerates* to amputation, denervation, and subsequent reinnervation.
Question

If an adult newt regenerate (70 days post-amputation) is amputated and denervated, will regeneration occur after the stump becomes reinnervated? Can this system be used to analyze the cell cycle events that occur in denervated newt limbs upon reinnervation and subsequent regeneration?

The right and left forelimbs of 10 newts were amputated through the mid-radius and ulna while 9 newts were amputated through the proximal part of the humerus. The limbs were allowed to regenerate for 70 days. By 70 days post-amputation morphological regeneration is considered complete; most of the skeletal structures have been individualized and further changes involve only growth and histogenesis (Schotte and Liversage, 1959). At this time, (70 days), both left and right limbs were amputated through the mid-radius and ulna. One day post-amputation the left limbs were completely denervated. Right limb regenerates were left as amputated, innervated controls.

During the first 2 weeks post-amputation, denervated left limbs were observed daily under the dissecting microscope for 1) any signs of regression, and 2) the earliest signs of regeneration.

Experiment 6: Responses of Denervated Newt Limb Stumps to Reinjury at Five Weeks Post-Amputation

Rationale

Since reinnervation alone did not result in the regeneration of denervated adult newt limb stumps, experiments were designed to identify the regeneration limiting factors in these inhibited limb stumps by
subjecting denervated limbs to various types of injury at 5 weeks. The 5 week time was chosen in order to compare the results with those from the only other reinjury study involving adult newts (Tassava and Loyd, 1977).

Question

What kinds of injury will initiate regeneration in long-term denervated adult newt limbs?

Both left and right forelimbs of 156 newts were amputated through either the middle portion of the radius and ulna or the proximal part of the humerus. One day post-amputation the left limbs were completely denervated. Right limbs were not denervated and served as amputated, innervated controls.

At 5 weeks post-amputation/denervation, newts amputated through the R/U were divided into 6 groups. Left limbs were either maintained as controls, without reinjury for up to 10 weeks post-amputation (Group 1RU), or were reinjured using the methods employed by Tassava and Loyd (1977) in the following ways. A new amputation was made at a depth of 1 mm into the distal stump (Group 2RU, Fig. 4c). Using watchmaker's forceps, the layers of the epidermis and healed tissue covering the tip of the limb were carefully removed to expose the entire amputation surface (Group 3RU, Fig. 4a). A single razor incision was made to a depth of 1 mm into the stump between the radius and ulna, through the dorsal-ventral plane of the limb (Group 4RU, Fig. 4b). Using watchmaker's forceps, the epidermal flap covering the entire amputation surface was carefully loosened, peeled back to expose the amputation surface, and
then returned to its original position (Group 5RU, Fig. 4e). Finally 15 penetrations were made through the epidermis covering the distal end of the limb stump with a sharp, sterile needle to a depth of 1 mm into the stump tissues (Group 6RU, Fig. 4d). In those newts where amputation was through the proximal humerus, denervated left limbs were either maintained as controls (Group 1H) or were reinjured by a new amputation as described above (Group 2H) or by removal of the epidermis and healed tissues covering the amputation surface as described above (Group 3H). All regenerating contralateral right limbs of newts used for reinjury were amputated 1 mm proximal to the original amputation surface at the same time left limbs were reinjured.

Reinjured limbs were observed at 4 day intervals under a dissecting microscope for the presence or absence of regeneration. Regenerates were staged according to the system of Tank et al. (1976). Immediately following injury, 3 left limbs from groups 1RU, 2RU, 3RU, and 4RU were prepared for paraffin histology (hematoxylin/eosin, Mallory's triple stain).

Materials and Methods and Experimental Design for the Histology Study

Experiment 7: Histological and Cellular Studies of Once-Denervated and Redenervated Newt Limbs

Rationale

A thorough histological study of long-term denervated adult newt limbs has not been previously reported in the literature. The following experiments were designed to 1) describe the histology of denervated
METHODS USED TO REINJURE 35 DAY DENERVATED NEWT LIMB STUMPS

a. Flap Removal
b. Razor Incision
c. Reamputation
d. Needle Pricks
e. Flap Removed and Replaced

Figure 4. Diagram of various types of reinjuries employed 35 days post-amputation/denervation.
newt limbs from 1 through 5 weeks post-amputation/denervation, 2) determine what effect long-term denervation has on cellular events (i.e., dedifferentiation, cellular proliferation, and DNA synthesis), and 3) determine which histological and cellular changes observed in once-denervated limbs are the result of reinnervation.

Questions

What kind of histological changes occur in completely denervated limb stumps through time (1 to 5 weeks post-amputation/denervation).

Both left and right forelimbs of 20 newts were amputated through the middle portion of the radius and ulna. One day post-amputation, left limbs were completely denervated. Right limbs were not denervated and served as regenerating controls. On the following days post-amputation, 7, 14, 21, 28, and 35, 4 animals were fixed and the limbs were prepared for routine paraffin histology. Right and left forelimbs were kept together to allow direct comparison of denervated and innervated control limbs. Sections of each limb were distributed on 4 slides as previously described. One slide from each limb was stained with hematoxylin and eosin, one with Mallory’s triple stain, and one was stained for nerves (Samuel, 1953).

Experiments 8, 9, 10, and Rationale: Analysis of Histological Changes Seen in Long-Term Denervated Limb Stumps

The results of Experiment 7 showed that several cellular and histological changes occur in long-term denervated newt limbs. As a result, the following parameters in these denervated limbs became important to
quantify: 1) the formation of skin glands across the entire amputation surface; 2) changes in the thickness of the wound epidermis; 3) the total number of dedifferentiated cells remaining in the limb stump on day 35; 4) if these dedifferentiated cells were among the initial population seen on day 7; 5) changes in the cell density during the 5-week period; and 6) the degree of tissue regeneration (amounts of cartilage and layered cells) present in the limb stump.

The parameters listed above were examined in once-denervated, redenervated and control limbs and were determined in the following ways:

**Skin glands.** All limbs fixed for histological and cellular studies from 1 through 5 weeks (post-amputation) for once denervated and redenervated limbs were examined for the formation of skin glands across the entire amputation surface. The number of limbs for each time period was recorded in Table 7.

**Wound epidermis thickness.** The changes in the thickness of the wound epidermis were determined in two ways: 1) counting the number of cell layers in the wound epidermis from weeks 1 through 5, and 2) using individual grid units as a measure for determining the percentage of area (depth) occupied by the wound epidermis (Table 5).

**Cell counts.** The number of cells in denervated newt limbs was observed to change through time, and it became important to assess whether this change in cell number was related to nerves (i.e., reinnervation). This change was quantified by determining total cell numbers in pre-determined sampled areas of once-denervated, redenervated, and control, innervated (regenerating) limbs. Two sample areas were
selected. Area I was 2 grid depths proximal to the amputation surface (2 grid depths proximal from the cut ends of the radius and ulna), and Area II was a more distal area, that distance from the cut ends of the radius and ulna to the base of the wound epidermis. In each of these areas the total number of cells, excluding wound epidermis, bone and red blood cells, was counted for at least 2 sections per limb and 3 limbs per time period. The 2 sections chosen always included both the radius and ulna in the section but were at least 30 uM apart. For once-denervated limbs, weeks 1 and 5 post-amputation were examined; for redenervated limbs, week 5 and 7 post-amputation were examined; and for control, regenerating limbs, weeks 1, 3, and 5 post-amputation were examined. The total number of cells from both sample areas of each limb was combined and a mean cell count was determined for each limb.

Cell density counts. Distal accumulation of dedifferentiated cells in a regeneration blastema occurs as a result of dedifferentiated cells traversing the cell cycle and dividing. Even though cells in completely denervated limb stumps initially dedifferentiate, enter the cell cycle, and undergo a few divisions, little or no distal accumulation of cells occurs through time (5 weeks) and no regeneration blastema ever forms. This latter feature represents one of the most obvious histological differences between regenerating limbs and denervated limb stumps. It therefore became important to examine the change in cell density through time in denervated limb stumps to compare with the cell density in regenerating control limbs.
The same sample areas used for total cell counts were also chosen to examine the cell density. Full grids were used to sample each area and the total number of cells per grid was determined. At least 3 sections per limb were counted for as many as 3 limbs per sample time. A mean cell density (mean number of cells/grid area) was determined for each limb. Limbs from weeks 1 and 5 were sampled for both once-denervated and redenervated limbs and limbs from weeks 1, 3, and 5 were sampled for controls.

**Degree of tissue regeneration.** Histology of denervated newt limb stumps 5 weeks post-amputation/denervation revealed that various degrees of tissue regeneration had occurred. The relative amounts of cartilage and layered cells seen in these limbs were categorized from the largest amount observed to the smallest amount of tissue regeneration seen. Twenty-two once-denervated and 15 redenervated limbs 5 weeks post-amputation examined for the presence of cartilage and layered cells were scored in the following way: 1) +++ - largest amount observed, 2) ++ intermediate (average), and 3) + - smallest amount observed. The same comparison was made for once-denervated and redenervated limbs 4 weeks post-amputation (4 limbs for each group). Only redenervated limbs were examined for tissue regeneration and scored as mentioned previously 7 weeks post-amputation.

Since it was observed that the total number of cells in denervated limbs changed throughout the 5-week period (tissue regeneration occurred), it was of interest to compare the percentage of cells found in the various tissue types (i.e., cartilage, layered cells, dedifferentiated and other cell types) of once-denervated and redenervated limb stumps.
The distal tip of the limb stump was chosen as the sample area since most of the tissue regeneration occurred in this area. The total number of cells in cartilage, layered cells, dedifferentiated and other cell types were counted and expressed as a percentage of the total cells found in the distal tip. Two limbs were sampled for each time period (5 weeks post-amputation).

The following experiment was designed to examine parameter 6 (the number of cells that dedifferentiated on day 7 and remained in the limb stump on day 35).

Experiment 8 and Questions

For how many days will cells that synthesize DNA on day 7 be maintained in denervated limb stumps? What is the fate of cells that synthesize DNA on day 7 in denervated limb stumps? What is the extent of cellular activity (cycling cells on day 35) in once-denervated limbs?

Experiment 8: Series I

The left and right forelimbs of 18 newts were amputated through the mid-radius and ulna. One day post-amputation all 18 left limbs were completely denervated, right limbs served as amputated, innervated controls. On day 7 post-amputation, 14 newts were given an intraperitoneal injection of 10 uci (methyl-3H) thymidine (ICN Pharmaceuticals: specific activity 60 Ci /mM) in 0.1 ml of sterile water. Four newts were not labelled at this time (Series II). After a 6-hour incorporation period, 14 newts were give a 1000 X chase of cold thymidine. Immediately after the chase, limbs of 3 newts were fixed and prepared for paraffin histology and autoradiography for day 7 analysis. The limbs of the 11
remaining labeled newts were fixed in the same manner described above on
days 14 (3 newts fixed), 21 (3 newts fixed) and day 35 (5 newts fixed).

Experiment 8: Series II

On day 35 post-amputation/denervation, 4 newts were pulse labeled
with 10 uci of (methyl-\(^{3}\)H) thymidine and after a 6-hour incorporation
period, limbs were fixed for 24 hours and both left and right limbs were
prepared for routine paraffin histology and autoradiography. Thus in
experiments of Series I, 3 newts were pulse labeled and fixed on day 7
post-amputation, 11 additional newts were also labeled on day 7, chased,
but not fixed until days 14 (3 newts), 21 (3 newts), and 35 (5 newts)
post-amputation, and in Series II, 4 newts were pulse labeled and fixed
on day 35 post-amputation.

Experiment 8: Autoradiographical Analysis of Once-Denervated Limbs of
Series I and Series II

To determine if cells labelling on day 7 remain in the denervated
limb stump through time (5 weeks post-amputation/denervation) autoradi-
ographs of limbs fixed on days 7, 14, 21, and 35 (Series I) were examined.
Any nucleus with 5 or more silver grains was considered labelled. The
distal end of the limb was the area selected to do all autoradiographical
analysis. On all days sampled, the distal end of the denervated limb was
considered to be 5 full grid depths proximal to the wound epidermis.
(The number of labelled cells in the sample areas was counted, the average
number of silver grains per nucleus was counted, and the distribution of
labelled cells with regard to cell type was also examined.) For control
limbs, the entire blastema, when present, was the area used to count
labelled cells. All counts were done at 430 X with a binocular microscope and at least 3 limbs per time period were examined.

To determine the extent of cellular activity on day 35, autoradiographs from the 4 limbs pulse labelled on day 35 were examined for the presence of labelled nuclei (nuclei with 5 or more silver grains) in the distal end of the limb, and a labelling index was determined for each limb. Labelling indices were expressed as a percent of labelled nuclei per total nuclei: 

\[ LI = \frac{\# \text{ of labelled nuclei}}{\text{total \# of nuclei}} \times 100. \]

Distribution of labelled cells with regard to cell type (i.e., cartilage, layered cells, dedifferentiated cells) was also noted.

To determine the number of cell divisions that occurred during the 5-week period in once-denerivated limbs, the autoradiographs from Series I were used, and since cell division results in dilution of the number of silver grains above labelled cells, labelled nuclei in the area of dedifferentiation on day 7 and the distal limb tip on day 35 were scored according to the average number of silver grains per nucleus to determine if any dilution of silver grains occurred during the 5-week period. Labelled cells were categorized according to the number of silver grains above their nuclei. Silver grain categories were based on the following: After each cell division, a labelled cell gives rise to two daughter cells each containing approximately one-half the total number of silver grains of the parent cell. The average number of silver grains above a heavily labelled nucleus was determined to be 100 in day 7 denervated and innervated limbs. Therefore, after one cell division, these 100 silver grains would be diluted to 50 for each daughter cell.
cell division would result in two daughter cells with approximately 25 silver grains; a third cell division would result in two daughter cells with 13 silver grains; a fourth cell division would result in two daughter cells with 7 silver grains; and finally, 5 cell divisions would result in two daughter cells with 4 silver grains (the background number of silver grains). Thus, a heavily labelled nucleus with 100 silver grains on day 7 would have to undergo at least 5 cell divisions to give rise to a labelled cell with only 4 silver grains above its nucleus. At least 3 limbs per time period were examined.

Experiment 9 and Questions

Since considerable cellular proliferation is seen in denervated limbs by day 35 (i.e., tissue regeneration occurs) the following questions were asked: Does this cellular proliferation occur before or after reinnervation? Is tissue regeneration in denervated limb stumps related to nerve ingrowth (reinnervation)?

Both the left and right forelimbs of 13 newts were amputated through the middle portion of the radius and ulna. One day post-amputation the left limb was completely denervated. Right limbs served as innervated, amputated controls. Fourteen days post-amputation, the left limb only of all 13 newts was redenervated (see section on denervations). On day 28 post-amputation (time period when tissue regeneration is first observed in once-denervated limbs), limbs of 4 newts were fixed and prepared for routine paraffin histology. Also, on day 35 and on day 49 post-amputation, limbs of 5 and 4 newts respectively, were fixed and prepared for paraffin histology. Even though redenervated limbs were of
the same amputation age as once-denervated limbs from Experiment 7, their denervation age differed. Therefore, redenervated limbs fixed on day 28 post-amputation were only 14 days post second denervation and redenervated limbs fixed on day 35 post-amputation were 21 days post-second denervation. Limbs fixed on day 49 post-amputation were 35 days post-second denervation.

Slides from each limb were treated with 1) Samuel's nerve stain to confirm denervation and to determine the extent of reinnervation, 2) hematoxylin-eosin for a general histological survey, and 3) Mallory's triple stain to confirm the presence or absence of cartilage and connective tissue.

Total cell counts and density counts were also determined for at least 3 limbs per time period to compare with the total cell counts and density counts in once-denervated limbs. These counts were made in the same manner as used for once-denervated limbs.

Experiment 10 and Questions

How does the level of DNA synthesis (labelling indices-silver grain densities) seen in in once-denervated limb stumps compare with the level seen in redenervated limbs?

Both the right and left forelimbs of 18 newts were amputated through the mid-radius and ulna. One day post-amputation, all left limbs were completely denervated. Seven days post-amputation, 15 newts were injected with 10 uCi of (methyl-3H) thymidine. After a 6-hour incorporation period, all 15 newts were given a chase of cold thymidine (1000 X). Limbs of 4 newts were immediately fixed (day 7). Fourteen
days post-amputation, 14 denervated newts (11 labelled on day 7, Series I, and 3 not labeled on day 7, Series II) were redenervated. On day 28 post-amputation, 4 newts (Series I) were fixed and prepared for histology and autoradiography. On day 35 post-amputation, the remaining 7 newts from Series I were fixed and also prepared for histology and autoradiography.

The 3 newts in Series II (amputated and redenervated) were labeled with 10 uCi of (methyl-\(^3\)H) thymidine on day 35 post-amputation, and after a 6-hour incorporation period were fixed and prepared for histology and autoradiography.

The autoradiographs from Series I and II of this experiment were analyzed using the same procedures described for once-denervated limbs.
CHAPTER V
RESULTS

Morphological Study

Regeneration of Once-Denervated Newt Limb Stumps After Reinnervation

Amputated, completely denervated limbs of adult newts did not regenerate even after reinnervation of the limb stump occurred. The level of amputation had no effect on the timing of reinnervation and subsequent regeneration. All amputated denervated limbs remained "stumped," even after 10 weeks post-amputation/denervation, while their amputated, innervated counterparts regenerated normally.

Morphological examination of denervated limb stumps through the 10-week period revealed, beginning at 3 weeks post-amputation/denervation, significant differences between the innervated and denervated limb stumps existed which became more pronounced by 4 and 5 weeks. The denervated limb stumps were almost completely "healed over" with skin and were reduced in diameter, when compared to their innervated counterparts, in agreement with earlier findings (Singer and Craven, 1948; Singer and Egloff, 1949).

Blastemal outgrowths were absent but each limb exhibited a small, clear, pigment-free area over the distal end which had the morphological appearance of wound epidermis. Histology later showed that this clear area did not resemble a functional wound epidermis but instead looked
very similar to skin-like epidermis (see results in histological section that follows).

The amputated, innervated right limbs had early to mid bud blastemas by 21 days, which continued to develop, and by 5 weeks had all reached the 4-digit stage of regeneration.

The failure of amputated denervated adult newt limb stumps to regenerate after reinnervation represents a fundamental difference between larval Ambystoma and adult newt limbs, since denervated larval limbs are capable of regeneration without additional injury, upon reinnervation (Petrosky et al., 1980).

The exact time of reinnervation of denervated newt limbs has not been clearly established in the literature; therefore, it became important to determine when reinnervation of the limb stump occurred in order to find out if denervated limb stumps "heal up" in such a way that the conditions for regeneration no longer exist when nerves return.

The Timing of Stump Healing in Relation to Reinnervation

The results of the morphological test for threshold reinnervation of the limb stump, reinjury by reamputation of the denervated limb at various times post-amputation/denervation, are reported in Table 1. These results indicated that all amputated, denervated limb stumps reamputated 7 days post-original amputation/denervation, remained inhibited from regeneration throughout the 10-week observation period. They also suggest that threshold reinnervation of the limb stump has not yet occurred as early as 7 days post-amputation/denervation. Thus, even though a fresh injury was imparted on the limb stump, by the time nerves
returned to the distal limb tip, the conditions for regeneration no longer existed (the stump healed over), and none of the limbs regenerated (Table 1).

Reamputation of amputated denervated limb stumps 14 days post-amputation/denervation resulted in 43 percent of the limbs regenerating but only after a delay of one week. Fifty-seven percent of these limbs, like those reamputated on day 7, had healed up in such a way that by the time nerves returned to the limb stump, the conditions for regeneration no longer existed. However, threshold reinnervation did occur in 43 percent of the limbs reamputated on day 14 before these critical healing events occurred and thus regeneration resulted, even though delayed when compared to contralateral innervated control limbs reamputated at the same time.

A much higher percentage of reamputated denervated limb stumps regenerated if reamputation (reinjury) was delayed until 21 days post-original amputation/denervation. Eighty-six percent of these reinjured limbs regenerated and with only a 2 to 3 day delay when compared to their reamputated, innervated control counterparts. Because of this short delay in initiating regeneration after reamputation, these results suggested that a threshold number of nerves had returned to the distal limb stump by 21 days post-amputation denervation, before the limb healed over in such a way that regeneration could not occur. Only a small percent (14 percent) of these limbs remained inhibited after reinjury.

Reamputation of denervated limb stumps 28 days post-original amputation denervation resulted in 92 percent of reinjured denervated
limbs regenerating with no delay in initiating regeneration when compared to control limbs. Therefore, more nerve fibers had reached the distal limb stump by the time reamputation of the stump occurred, and only one limb failed to regenerate.

Amputated denervated limb stumps reamputated 35 days post-original amputation/denervation resulted in 100 percent of the limbs regenerating with no delay. These results indicate that complete reinnervation of the denervated limb stump had occurred by 35 days.

Delaying reamputation of denervated limb stumps allowed more time for nerve fibers to reach the distal tip of the limb and effectively increased the number of denervated limbs that regenerated after reinjury.

Response of Completely Denervated Limbs to Delayed Amputation

The regeneration responses of completely denervated limbs amputated one and two weeks post-denervation, were dependent upon both the level of amputation and the length of the delay post-denervation before amputation was done. The results are reported in Table 2.

Denervated limbs that were amputated at the proximal humerus levels 7 days post-denervation, regenerated (4 out of 4 limbs) but were delayed by 1 week in initiating regeneration when compared to innervated controls amputated at the same time. Denervated limbs were observed to initiate regeneration 28 days post-amputation (35 days post-denervation) while controls initiated regeneration 21 days post-amputation. No regeneration was seen from denervated limbs amputated through the radius and ulna 7 days post-denervation. Reinnervation to the amputation surface of the proximal humerus limb stumps occurred before reinnervation of the R/U
<table>
<thead>
<tr>
<th>Reinjury Date (post-amp/den)</th>
<th>Total number of limbs</th>
<th>Yes</th>
<th>%</th>
<th>No</th>
<th>%</th>
<th>Number of days post-reamputation that regeneration was first observed*</th>
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</thead>
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<tr>
<td>7 days</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>14 days</td>
<td>28</td>
<td>12</td>
<td>43</td>
<td>16</td>
<td>57</td>
<td>28</td>
</tr>
<tr>
<td>21 days</td>
<td>14</td>
<td>12</td>
<td>86</td>
<td>2</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>28 days</td>
<td>12</td>
<td>11</td>
<td>92</td>
<td>1</td>
<td>6</td>
<td>21</td>
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<td>35 days</td>
<td>20</td>
<td>20</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

*Regeneration was based on the presence of an early-bud stage blastema. Control reamputated limbs consistently showed early bud stages at 21 days post-reamputation.
limb stumps. These results were expected since the proximal humerus stumps were shorter. Therefore, threshold numbers of nerve fibers were able to return to the limb stump while the prerequisites for regeneration (wound epidermis, injury, and dedifferentiated cells) still existed. Nerve fibers had a longer distance to travel to reinnervate the distal (R/U) limb stump and by the time threshold reinnervation occurred, inhibitory healing events had already taken place and regeneration was prevented.

When a time of 14 days post-denervation elapsed before the limbs were amputated, 7 of 8 limbs initiated regeneration regardless of the level of amputation. Three out of four limbs regenerated from the proximal humerus level while all 4 limbs amputated through the radius and ulna regenerated. Proximal humerus regenerates were at the early bud stage 19 days post-amputation (33 days post-denervation) while early bud blastemas were present 22 days post-amputation (36 days post-denervation) at the radius and ulna level. Therefore, no delay in initiating regeneration was seen of any of the limbs amputated 14 days post-denervation when compared to control limbs that had early bud blastemas on day 21 post-amputation.

The delay of 14 days before amputation allowed nerves to reach the distal limb tip in threshold numbers before the limb stumps "healed" in such a way that prevented regeneration from occurring upon reinnervation.


**TABLE 2**

REGENERATION RESPONSES AFTER DELAYED AMPUTATION OF DENERVATED NEWT LIMBS 7 AND 14 DAYS POST-DENERVATION

<table>
<thead>
<tr>
<th>Day of Amputation (post-denervation)</th>
<th>Level of Amputation</th>
<th>Regeneration</th>
<th>Number of days post-amputation regeneration first observed</th>
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<td></td>
<td></td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>7 D</td>
<td>proximal humerus</td>
<td>4</td>
<td>1</td>
</tr>
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<td>radius and ulna</td>
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</tr>
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<td>14 D</td>
<td>proximal humerus</td>
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</tr>
<tr>
<td>14 D</td>
<td>radius and ulna</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

*Post-denervation age of limb.
Cartilage Grafts in Completely Denervated Newt Limbs

Denervated-amputated limbs with cartilage grafts at the amputation surface never regenerated upon reinnervation (even after 10 weeks post-amputation).

All 28 amputated, denervated limbs with cartilage grafts healed over and were very similar morphologically to those non-regenerating amputated, denervated limbs in Experiment 1.

Since histology was not done on a representative number of these denervated limbs to confirm the presence of the cartilage grafts in the limb stump, the possibility that regeneration did not occur because the cartilage graft was extruded, cannot be ruled out. On the other hand, if the grafts remained in the denervated limb stumps, these results mean that 1) cartilage had no effect in preventing "inhibitory healing events" from occurring in denervated limb stumps, and 2) simply the presence of a cartilaginous skeleton is not the reason larval *Ambystoma* denervated limbs regenerate upon reinnervation. However, another factor that must be considered is that an insufficient amount of cartilage was grafted to these amputated denervated limbs.

Amputation and Denervation of 70-day Adult Newt Regenerates

Amputated adult newt forelimbs regenerated normally and were at the 4-digit stage of regeneration 70 days post-amputation. The stem regenerates had two different stump lengths with the longer regenerate being those limbs originally amputated through the proximal humerus. Regardless of the stump length the new regenerates were all amputated
through the proximal radius and ulna. Denervation of these amputated regenerates 1 day post-amputation resulted in resorption of all of the stem regenerate (19 limbs). Resorption always resulted in a loss of the newly regenerated limb back to the original level of amputation (adult limb stump) and was complete by 3 weeks post-amputation/denervation. The length of the stem regenerate had no bearing on the amount of resorption that occurred. Resorption of the adult limb stump was never observed.

These results are not in agreement with those of Schotte and Liversage (1959) since they reported very little resorption of 70-day newt regenerates which were amputated and denervated simultaneously. The resorption of the amputated/denervated regenerates in this study does, however, show similarities to the larval system since amputated larval limbs also resorb after denervation.

The amputated denervated stem regenerates in this study were also observed for signs of regeneration upon reinnervation. Only after 53 days post-amputation/denervation were any signs of regeneration seen. Three of the proximal humerus stem regenerates and 3 of the radius and ulna stem regenerates initiated and completed regeneration. Two of the six regenerates were observed to be heteromorphic (regenerates with fused digits).

This long delay (52 days post-amputation/denervation) in initiating regeneration was surprising and cannot be easily explained since complete reinnervation of the adult newt limb stump occurs by 5 weeks (35 days) post-amputation/denervation (see results of Experiment 1).
The 13 limbs that failed to regenerate upon reinnervation were morphologically "healed over" like those nonregenerating limbs in Experiment 1. The length of this experiment was approximately 22 weeks and resulted in extreme weight loss in all of the animals. This factor, therefore, cannot be ruled out as a reason for explaining the lack of regeneration of the majority of limbs (13/19) in this experiment.

Because of the length of this experiment and the small percentage (32 percent) of stem regenerates that successfully initiated regeneration upon reinnervation, this experimental system provides very little potential for analyzing the cellular events occurring in denervated newt limbs upon reinnervation.

Responses of Denervated Newt Limb Stumps to Reinjury at Five Weeks Post-Amputation

The responses of 5-week amputated/denervated limb stumps to various types of reinjury can be found in Table 3. Reamputation 1 mm into the limb stump (Group 2RU) elicited a regeneration response in every case from both the radius and ulna level and the proximal humerus level (Group 2H) of amputation (Table 3). No delay or enhancement of the regeneration rate was observed compared to contralateral controls. These results confirm that threshold reinnervation to both the R/U and proximal humerus levels had occurred by 35 days. It should be noted that this reamputation imparted a new injury on the limb stump tissues, allowed new wound epidermis to form, and removed the cartilage, layered cells, and the dedifferentiated cells in the distal limb stump (Fig. 5A).
Merely removing the epidermis and most of the layered cells from the distal limb tips (Group 3RU and 3H) (Figs. 5C and 5D), with minimal injury to underlying tissues, elicited regeneration in 21 of 26 cases (Table 3). This type of injury exposed the entire amputation surface and allowed new wound epidermis formation. Approximately 80 percent of these limbs regenerated from the radius/ulna level (Group 3RU) and 100 percent regenerated from the proximal humerus level (Group 3H). Many of the limbs reinjured in this manner regenerated faster than contralateral controls. For example, as early as 16 to 21 days after injury, over 50 percent of the minimally reinjured limbs in Group 3RU had already reached the 3- or 4-digit stage of regeneration (R/U level of amputation), a stage not reached by control right limbs until 4 to 5 weeks post-reamputation (Table 4).

All of the proximal humerus limb stumps reinjured in this manner resulted in limbs regenerating at least 2 stages ahead of their reamputated control right limb counterparts. These findings suggest that dedifferentiated cells are still present in these denervated limb stumps through 5 weeks post-amputation/denervation and rapidly participate in blastema formation after reinjury. Histological evidence of dedifferentiated cells in these denervated limb stumps (Fig. 5A, 5B, and 5C) is consistent with this view.

The third type of injury, a single razor incision 1 mm into the stump between the radius and ulna (Group 4RU) (Fig. 5B), stimulated regeneration in approximately 60 percent of the limbs (Table 3). Within 4 to 5 days after reinjury to this latter group of limbs, a ridge formed along the razor incision as observed by Tassava and Loyd (1977) after
razor reinjury to skin graft limbs. However, in each of the reinjured razor incision limbs which initiated regeneration, an increase in the size of the wound area was seen. The regenerates were not restricted to the razor incision and did not develop in the plane of the incision. The regeneration rate was equal to that of their control reamputated counterparts, unlike the results reported by Tassava and Loyd (1977) for skin flap limbs reinjured in the same manner. Reinjured skin flap regenerates always formed in the plane of the razor incision and blastema formation was observed as early as 1 week post-reinjury. Razor incisions were not done on humerus-level limb stumps because of the difficulty of cutting into the humerus.

The fourth type of injury involved peeling back the epidermis and healed tissues covering the distal limb tip to expose the entire amputation surface and then returning the flap to its original position to heal and to prevent new wound epidermis formation over the limb stump (Fig. 4E). This method of injury was an important one to use since it provided a means to investigate the effects of injury alone in stimulating denervated limbs to regenerate and also tested the competence of the "old" wound epidermis for participation in regeneration after injury. Fifty percent of the limbs reinjured in this manner regenerated (Table 3). This type of injury imparted only minimal injury to the underlying stump tissues as did complete removal of epidermis and healed tissues (Group 3RU and 3H). However, unlike the injured limbs in groups 3RU and 3H, new wound epidermis formation after injury was not observed to occur and these limbs did not regenerate at a rate faster than their contralateral controls. Of the 4 injured limbs that failed to regenerate in
this group, only 2 limbs were observed to lose the flap of epidermis after injury. It is therefore likely that new wound epidermis formation did occur in these limbs. However, the presence of a new wound epidermis was still insufficient to elicit a positive regeneration response. That these 4 limbs require more than a minimal amount of injury to initiate regeneration is also likely. The possibility that new wound epidermis formation also occurred in all of the other injured regenerating limbs in this group cannot be ruled out completely since these limbs were not examined histologically.

Denervated limbs responded to all of the various types of injuries employed except the 15 needle penetrations (Group 6RU, Table 3). All limbs reinjured in this manner failed to regenerate. Although the mesodermal stump tissues were clearly injured by the 1 mm penetrations of the needle, these limbs remained inhibited from regeneration. The 15 individual small wound areas were never observed to fuse into a single wound epidermis. It is therefore likely, as Tassava and Loyd (1977) proposed for skin flap limbs injured in the same manner, that the small areas of wound epidermis created by the needle penetrations were insufficient to allow regeneration to occur.

Histological and Cellular Analysis of Once-Denervated and Redenervated Limbs

Amputated, denervated limbs of adult newts did not regenerate even upon reinnervation. However, a number of histological and cellular changes occurred in these limbs through time. The following results describe the histological and cellular features of once-denervated newt
Figure 5. Micrographs of a longitudinal section through three completely denervated forelimb stumps at 35 days post-amputation/denervation illustrating the most typical histological features seen after long-term denervation (Fig. 5A), and immediately following reinjury (Fig. 5B, 5C, and 5D). Differentiated cartilage (C) is present closely associated with the cut ends of the radius (R) and ulna (U). Layered cells (L) can be seen underneath the thin wound epidermis (WE). Dedifferentiated cells are still present in the distal tip of the limb. Skin glands (S) rarely covered the entire amputation surface of denervated limbs at 35 days. Muscle fibers (M) are also present in the distal area of the limb stump. The arrows in Figure 5B (35X) point to the razor incision and the depth of the incision between the radius (R) and ulna (U). Note that razor incision reinjury removed no tissues from the distal limb stump (compare Fig. 5A). Cartilage (C), dedifferentiated cells, (DE) and a few layered cells (L) remain present in the distal limb tip after removal of healed tissues (Figure 5C). The flap of tissue peeled away from the limb stump in 5C is shown in Figure 5D and contains mostly wound epidermis (WE), some connective tissue (CT) and a few layered cells (arrow). Removal of this small amount of tissue was typical for all denervated limbs reinjured in this manner. Hematoxylin and eosin (Figures 5A, 65X; 5B, 35X; 5C, 50X; and 5D, 75X)
Figure 5.
#### TABLE 3

**REGENERATION RESPONSES AFTER REINJURY OF 5-WEEK, ONCE-DENERVATED NEWT LIMBS**

<table>
<thead>
<tr>
<th>Type of Reinjury at:</th>
<th>Total Number</th>
<th>Regeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td><strong>A. Radius and ulna level of amputation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1RU; Control: no reinjury</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Group 2RU; Reamputation 1 mm into stump</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Group 3RU; Removal of healed tissues</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>Group 4RU; Single razor incision</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Group 5RU; Needle penetrations</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Group 6RU; Flap removed and replaced</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>B. Proximal humerus level of amputation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1H; Control: no reinjury</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Group 2H; Reamputation 1 mm into stump</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Group 3H; Removal of healed tissues</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

*All reamputated, innervated control right limbs regenerated.*
# TABLE 4

**ENHANCED REGENERATION OF DENERVATED LIMBS REINJURED BY REMOVAL OF HEALED TISSUES COMPARED WITH CONTROL REAMPUTATED LIMBS**

<table>
<thead>
<tr>
<th>Type of Reinjury</th>
<th>Days Post Reinjury</th>
<th>Total # of Animals</th>
<th>Wound Healing</th>
<th>Early Bud</th>
<th>Mid Bud</th>
<th>Late Bud</th>
<th>Paddle</th>
<th>Digit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Removal of healed tissues</td>
<td>11</td>
<td>12</td>
<td>6</td>
<td>4</td>
<td>2</td>
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<td>0</td>
<td>0</td>
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<tr>
<td>Reamputated control</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Removal of healed tissues</td>
<td>16</td>
<td>12</td>
<td>0</td>
<td>7</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Reamputated control</td>
<td>16</td>
<td>12</td>
<td>0</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Removal of healed tissues</td>
<td>21</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Reamputated control</td>
<td>21</td>
<td>12</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Removal of healed tissues</td>
<td>25</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>3</td>
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<td>5</td>
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<tr>
<td>Reamputated control</td>
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<td>12</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Removal of healed tissues</td>
<td>29</td>
<td>12</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>7</td>
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<tr>
<td>Reamputated control</td>
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<td>0</td>
<td>4</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Reamputated control</td>
<td>34</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

*Only 5-week amputated denervated left limbs whose original amputation was through the radius and ulna are recorded in this table, even though denervated left limbs amputated at the proximal humerus level and reinjured in the same manner as described above, also showed enhanced regeneration. Control limbs were reamputated at the time reinjury to left limbs.
limbs from one through five weeks post-amputation/denervation and in redenervated limbs at 4, 5, and 6 weeks post-amputation. Comparisons were made with innervated, regenerating controls at each time.

**Day 7 Post-Amputation/Denervation**

Histological examination of denervated and innervated limb stumps at 7 days post-amputation did not reveal significant differences between the two limb stumps. Both types of limb stumps were healed over with a dermis-free thickened wound epidermis that was 12 to 14 cell layers thick (Fig. 6A and 6B). Dedifferentiation of limb stump tissues had begun but no distal accumulation of dedifferentiated cells had yet occurred (Fig. 6A and 6B). The cut ends of the radius and ulna were still in contact with the wound epidermis except in some cases where extra cellular matrix was found immediately beneath the wound epidermis (Fig. 6A and 6B). This matrix was found in all denervated and innervated limbs examined and was not different between the two groups of limbs. Cellular debris and numerous blood cells could also be seen directly beneath the wound epidermis and sometimes suspended in the extra-cellular matrix.

Examination of nerve stains from control innervated limbs showed typical nerve bundles full of dark staining nerve fibers (Fig. 6C). Nerve bundles were characteristically smooth and wavy in appearance. When nerve stained sections from control limbs with blastemas (Days 21, 28, and 35) were examined, individual fibers could be seen coarsing among blastema cells. Since the appearance of control nerve bundles did not change through time, they will not be described additionally below.
Nerve bundles in denervated limbs one week post-amputation in all 4 cases showed typical degenerating nerve fibers (Fig. 6B). The smooth wavy appearance of the bundles (as displayed by controls) was lost and instead bundles contained disrupted and fragmented nerve fibers that were degenerating. It is important to note that complete degeneration of nerve fibers in denervated limbs had not yet occurred by 7 days post-denervation and also that no new ingrowth of nerve fibers into the limb stump had occurred as early as one week post-denervation.

Day 14 Post-Amputation/Denervation

The histology of both innervated and denervated limb stumps did not change considerably by 2 weeks post-amputation/denervation (compare Figs. 6A and 6B with Figs. 7A and 7B). A thickened wound epidermis was still present over the amputation surface of both control innervated limbs; these results were surprising since after examining denervated limb stump healing in relation to reinnervation (results discussed previously), critical healing events occurred in denervated limb stumps as early as 14 days post-amputation/denervation. However, none of the 14-day denervated limbs examined had reformed skin glands across the amputation surface. Tissue dissociation and dedifferentiation of stump tissues were more extensive than seen on day 7 for both types of limbs; however, only minimal distal accumulation of these dedifferentiated cells had occurred.

By 14 days post-amputation/denervation nerve fibers were completely degenerated. Nerve bundles were filled with a dark grainy particulate matter and with light staining cells (Fig. 7D). No new nerve fibers
Figure 6. Micrographs of a longitudinal section through a control innervated limb 7 days post-amputation (Fig. 6A, 65X) and a completely denervated limb (Fig. 6B, 65X) 7 days post-amputation/denervation showing histological similarities between the two limbs. A thickened wound epidermis (WE) covers the distal tip of both limbs. The cut edges of the radius (R) and ulna (U) are still in contact with the wound epidermis. Dedifferentiation is underway proximal to the amputation surface (arrows). Extracellular matrix (E) can be seen beneath the wound epidermis in both limb stumps (compare 6A and 6B). Hematoxylin and eosin. Figure 6C shows a nerve bundle (N) typical of control innervated limbs at the radius and ulna level. Note the dark staining, smooth wavy appearance of the nerve fibers. This micrograph will be repeated in the figures that follow to allow direct comparison between denervated and innervated nerve bundles at different times post-denervation (50X). Figure 6D shows at a higher magnification (250X) nerve fibers undergoing degeneration at the humerus level in a denervated limb 7 days after amputation/denervation. Degenerating nerve fibers (DN) lose their smooth appearance and appear fragmented (compare Fig. 6C). Samuel's nerve stain.
Figure 6.
Figure 7. Micrographs of a longitudinal section through a control innervated limb (Fig. 7A), 14 days post-amputation, and a completely denervated limb (Fig. 7B), 14 days post-amputation denervation, illustrating similarities in histological features. A thickened dermis-free wound epidermis (WE) covered the amputation surface of both control and denervated limbs (compare Figs. 7A and 7B). Skin glands (S) never covered the entire amputation surface of denervated limb stumps. More dedifferentiated cells (DE) were present in both limbs, however, the distal accumulation of these cells beyond the radius (R) and ulna (U) was minimal (Figs. 6A and 6B). A small amount of extracellular matrix (E) could still be seen in control limbs (6A). Hematoxylin and eosin (Figs. 6A, 65X and 6B, 65X). Figure 7D shows a degenerating nerve bundle 14 days post-amputation/denervation (200X) grainy particulate matter (G) and numerous light-staining cells (arrows) completely filled the bundle. Note no intact nerve fibers were present, confirming completeness of denervation (compare Figs. 7C and D). Samuel's nerve stain.
Figure 7.
could be found growing back into any of the 14-day limb stumps examined (4 cases). Therefore, reinnervation of denervated limb stumps still had not occurred by 14 days post-amputation/denervation.

Day 21 Post-Amputation/Denervation

By 3 weeks post-amputation/denervation, a striking difference between the innervated and denervated limb stumps was apparent. The most obvious histological feature of the control regenerating limb was the accumulation of dedifferentiated cells distally to form a blastema. In denervated limbs, even though a few dedifferentiated cells could be seen beneath the wound epidermis, no outgrowth beyond the amputation surface occurred (Fig. 8B).

Figure 12 is a histogram comparing the changes in the total number of cells seen in distal regions of control, denervated, and redenervated limb stumps throughout a 5-week period. It can be seen that while there was a 5-fold increase in the number of cells in the distal tips of control limbs, between weeks 2 and 3, only a slight increase in the number of cells in denervated limb tips occurred. This increase in blastema cells in control limbs is the expected result of cells traversing the cell cycle and undergoing the sustained mitotic activity needed to establish a blastema. Abundant mitotic figures were observed in the blastema of these regenerating limbs. The fact that the increase in the number of cells in denervated limbs did not parallel control limbs was expected and is consistent with the fact that dedifferentiated cells in denervated limbs undergo very little mitotic activity in the absence of nerves.
A second notable difference between control and denervated 21-day limbs were the changes that occurred in the wound epidermis. While the wound epidermis of 21-day control limbs remained dermis free and thickened (10 cell layers), the wound epidermis on 21-day denervated limb stumps was greatly reduced in thickness, 4 to 5 cell layers (Fig. 8B). The shape of the cells in the wound epidermis also differed. Wound epidermal cells of 21-day denervated limbs were more flattened and closely packed. In only 1 of the 4 denervated limbs examined at 21 days had skin glands reformed across the amputation surface. Therefore, even though these denervated limb stumps appeared to be "healed up" morphologically, as described in previous results, histologically, this healing response (i.e., connective tissue and dermis) was not evident. Thus, in 3 of the 4 denervated limbs examined on day 21, intimate contact still persisted between mesodermal tissues and the wound epidermis.

Examination of the nerve bundles of 4 denervated limb stumps 21 days post-amputation/denervation showed a few, light staining cells in the nerve bundles, and a number of regenerated nerve fibers could be seen coarsing through all nerve bundles examined in each limb (Fig. 8D). More regenerated nerve fibers were present in proximal bundles (humerus levels) than in bundles found in the distal regions of the limb (radius and ulna). These results confirm those of the morphological test for reinnervation (see results of reamputation of denervated limb stumps), which showed that threshold reinnervation of adult newt limb stumps occurred around 21 days post-amputation/denervation. Since individual nerve fiber counts were not done in this study, the extent of reinnervation described in denervated
Figure 8. Micrographs of a longitudinal section through a forelimb regenerate at 21 days post-amputation (Fig. 8A) and a completely denervated limb stump (Fig. 8B) at 21 days post-amputation/denervation. In Fig. 8A, a midbud blastema (B) is present beneath a thickened wound epidermis (WE). The arrows and the proximal skin glands (S) indicate the level of amputation. Typical histological features of a denervated limb at 21 days post-amputation denervation are illustrated in Fig. 8B. Note the absence of a blastema. Dedifferentiated (DE) cells remain present in the distal limb stump. A very thin wound epidermis (WE), free of any underlying skin glands (S), covers the distal limb tip. Hematoxylin and eosin, (65X). Figure 8D shows a nerve bundle at the distal humerus level in a completely denervated limb stump 21 days post-amputation/denervation (50X). Light-staining cells (arrows) are present between a number of dark-staining nerve fibers (n) that have regenerated back into the limb. Note that the full complement of nerves have not returned to the denervated limb stump by 21 days (compare Fig. 8C, control nerve bundle 50X). Samuel's nerve stain.
Figure 8.
limb stumps represents only an estimation.

**Day 28 Post-Amputation/Denervation**

Regeneration of control innervated limbs continued after 3 weeks and had reached the paddle stage of regeneration by 4 weeks post-amputation/denervation. All 4 limbs examined still had a thickened wound epidermis covering the amputation surface. There was a definite increase in the number of blastema cells between weeks 3 and 4 in these regenerating limbs (compare Figs. 8A and 9C). This increase in blastema cells was expected since it is during this time period (weeks 3 and 4) that regenerating limbs of the adult newt initiate a rapid growth phase (reviewed by Singer, 1973).

The histological changes seen in denervated limb stumps 3 weeks post-amputation/denervation became pronounced by the fourth week. Blastemas were still absent from the 4 denervated limb stumps examined. These results are consistent with the "stumped" appearance of denervated limbs 28 days post-amputation described previously (see Results: Regeneration of Once-denervated Limbs).

Several interesting features were revealed upon histological examination of the 4 non-regenerating denervated limb stumps fixed for histology at 4 weeks post-amputation denervation. The wound epidermis was reduced in thickness (4 to 5 cell layers), (Figs. 9A and 9B), and now closely resembled the lateral skin epidermis of the stump. However, an area of dermis-free wound epidermis could be found in all limbs examined (4 cases) (Fig. 9B). There was still no apparent physical barrier between the wound epidermis and underlying tissues.
Blastema-like cells were still present in the distal tip of 4-week denervated limb stumps and had increased in number since day 21 (compare Figs. 8B, 9A, and 9B) (Fig. 12). More of these dedifferentiated cells had accumulated distally at 4 weeks and were densely packed in the small area found between the wound epidermis and the cut edges of the bone (Fig. 9A and 9B). Both the presence of blastema-like cells in denervated limb stumps at 4 weeks and their increase in number were unexpected results since these denervated limbs appeared to be "static" morphologically and showed no signs of regeneration at 4 weeks.

In addition to blastema-like cells, two new cell types also appeared in the distal tip of 4-week denervated limb stumps. In 3 of the 4 limbs examined, cells with elongated nuclei tended to layer just beneath the wound epidermis (Fig. 9A and 9B) and a small amount of newly differentiated cartilage was associated with the cut ends of the bones (Figs. 9A and 9B). Of the 3 limbs observed to have cartilage and layered cells, only one limb had a large amount of cartilage associated with the cut bone (Fig. 9B) while in the majority of these denervated limbs (2/3) only a small nodule of cartilage and a few layered cells were found in the distal tip (Fig. 9A). The one limb without cartilage or layered cells still closely resembled 21-day denervated limb stumps.

Examination of nerve bundles from 4 of the 28-day denervated limb stumps showed a definite increase in the number of regenerated nerve fibers since day 21 post-amputation/denervation (Fig. 9C). These results were expected since earlier results had shown that reamputation of denervated limb stumps on day 21 and 28 post-amputation/denervation resulted in a higher percentage of limbs regenerating on day 28 versus
Figure 9. Micrographs of a longitudinal section through two non-regenerating, denervated limb stumps at 28 days post-amputation/denervation. Figure 9A illustrates the most typical histological features of a 28-day denervated limb stump. A small nodule of cartilage (arrow) caps the radius (R), a few layered cells are present immediately beneath the ulna (U), and an increased number of dedifferentiated cells (DE) are still present in the distal limb tip. A small area of dermis-free wound epidermis (WE) still covers the amputation surface even though a few skin glands (S) are present beneath a part of the distal epidermis. Hematoxylin and eosin (65X). The denervated limb stump in Figure 9B is the only 28-day limb observed to have a large amount of cartilage (C) and layered cells (L) present in the distal tip. Hematoxylin and eosin (65X). Note blastemas are still absent from 28-day denervated limb stumps (compare Fig. 9A and 9B with 9C). Figure 9C illustrates a normally regenerating control limb at the paddle stage of regeneration. This limb is the same amputation age (28 days) as the denervated limbs in Figures 9A and 9B. Note the thickened wound epidermis (WE) covering the blastema (B). The level of amputation is indicated by the arrows. Hematoxylin and eosin (55X). Figure 9E shows at high magnification (88X) a nerve bundle from the proximal radius and ulna level in a denervated limb stump at 28 days post-amputation/denervation. Regenerated nerve fibers (n) stain dark and are numerous (arrows). Compare with control nerves (N) Fig. 9D (50X). Samuel's nerve stain.
day 21 (Table 1). However, complete reinnervation of the denervated limb stump still had not occurred by 4 weeks post-amputation/denervation.

**Day 35 Post-Amputation/Denervation**

By 5 weeks post-amputation/denervation, all control limbs had essentially completed regeneration. Differentiation of the regenerate had advanced to the digit stage of regeneration in all 4 right limbs examined histologically (Fig. 10D). The skeletal elements were present but were still cartilaginous (Fig. 10D) because ossification of these skeletal elements (carpals, phalanges, radius, and ulna) does not occur until the limb has grown to its original size, several months later (Hay, 1966). A thickened wound epidermis (10 cell layers) still covered the regenerate even though differentiation and morphogenesis were underway.

Denervated limb stumps examined histologically 5 weeks post-amputation still closely resembled 28 day denervated limb stumps, in that blastemas were still absent. However, certain histological features had become even more pronounced since 4 weeks post-amputation/denervation. Blastema-like cells were still present in all 22 limbs examined and the number of these cells had increased since day 28 (Fig. 10A; Fig. 12). More of these dedifferentiated cells had accumulated distally by 5 weeks, however this small amount of accumulation was still insufficient for blastema formation. Therefore denervated limbs 5 weeks post-amputation/denervation still remained stumped.

Both the presence and the number of blastema-like cells remaining in denervated limb stumps for 5 weeks was especially interesting since in other amputated non-regenerating limbs, (i.e., post-metamorphic frogs and
mammals), after a minimal amount of dedifferentiative activity, dedifferen-
tiated cells rather quickly redifferentiate to repair damaged stump
tissues (tissue regeneration) (reviewed by Carlson, 1974). These present
results indicate that complete redifferentiation of dedifferentiated cells
did not occur by 5 weeks post-amputation in denervated adult newt limb
stumps.

The wound epidermis was still reduced in thickness 35 days post-
amputation/denervation and only occasionally had skin glands reformed
across the entire amputation surface (3/22 limbs) (Tables 5 and 7).
No apparent connective tissue barrier existed between the wound epidermis
and the underlying tissue as evidenced by Mallory's connective tissue
stain. Therefore, even though the wound epidermis appeared histologically
non-functional (i.e., resembling skin epidermis), its contact with under-
lying stump tissues still existed.

The most obvious difference between 4 and 5 week denervated limb
stumps was the amount of tissue regeneration that occurred in 5-week
denervated limbs. In addition to blastema-like cells, an abundance of
newly differentiated cartilage and layered cells with elongated nuclei
were also very prominent in the distal tip of denervated limbs at 5
weeks.

In 20 of the 22 denervated limbs examined histologically, layered
cells were very densely packed in the area immediately proximal to the
epidermis (Fig. 10B). Newly differentiated cartilage was associated with
the cut ends of the radius and ulna in 19 of 22 denervated limbs examined
(Figs. 10A and 10C). Layered cells were also present in these 19 denervated
limb stumps and were closely associated with the cartilage (10B and 10C).
Only 2 denervated limb stumps had neither cartilage or layered cells (Fig. 11A). In only 1 case (1/22) did layered cells appear alone. These results indicate that both layered cells and cartilage almost always appear simultaneously in denervated limb stumps. The presence of these new tissue types (i.e., blastema-like cells, layered cells, and newly differentiated cartilage) in 4 and 5 week denervated limb stumps must be emphasized since none of these cell types had been previously described, in the histology of long-term denervated newt limb stumps.

Overall, denervated newt limbs had undergone a number of histological changes since the first week post-amputation/denervation and the results are reported in Table 5 and will be highlighted and summarized below. Initially, denervated limb stumps had a thickened wound epidermis that was 13 cell layers thick on day 7 post-amputation/denervation, but 5 weeks later the number of cell layers had been reduced by more than one-half (5 cell layers thick). The wound epidermis had also become more compact. There had been a 6-fold increase in the average number of cells found in the distal tip of denervated limb stumps, (the small area between the wound epidermis and the cut edges of the radius and ulna), since day 7 (Fig. 12). For example, on the average only 107 dedifferentiated cells were present in the distal region of 7-day denervated limb stumps for 3 limbs examined, but by 5 weeks the number of cells had increased to 644 (Fig. 12). However, cell types other than dedifferentiated cells contributed to this total by 5 weeks in denervated limb stumps.

The amount of distal accumulation that occurred by 5 weeks in denervated limbs was another obvious histological feature. By measuring the distance from the cut edges of the radius and ulna to the wound
epidermis 7, 14, 21, 28, and 35 days post-amputation/denervation the degree of distal accumulation of dedifferentiated cells could be determined. Full grids were used to measure the distance between the wound epidermis and radius and ulna. There was no distance between the wound epidermis and the cut edges of the radius and ulna in day 7 denervated and control limbs. The bones were still in contact with the wound epidermis. However, by day 35 this distance increased to three-fourths of a grid for denervated limbs and approximately 4 full grid lengths for the control blastema. This dramatic increase in control limbs was expected and was due to the distal accumulation of blastema cells to form the regenerate. That any distal accumulation of dedifferentiated cells occurred in denervated limbs through time was unexpected since prior to this study it was not known that dedifferentiated cells remained in denervated limb stumps for 5 weeks post-amputation/denervation.

Cell density changes were also determined and a comparison between denervated limbs and innervated limbs was made for day 7 and day 35 post-amputation/denervation. Table 6 shows that the cell density in the distal tip of denervated limbs did increase during the five-week period. On the average 35-day once-denervated limbs had 25 cells/grid area vs. 21 cells per grid area for control blastemas on the same day. It must be emphasized that cell densities for 35-day denervated limbs included all three types (cartilage layered cells and blastema cells) and that even though there was an increase in cell density, this increase was not great enough to account for the obvious size differences that existed between the stumped denervated limb and the control blastema.
Finally, as described previously two new cell types, layered cells and cartilage became more pronounced in denervated newt limb stumps during the fifth week. An analysis was done which categorized these denervated limbs according to the amount of tissue regeneration present in each limb. The summary of this analysis is reported in Table 7. It was typical for a large number of layered cells and only an intermediate amount of cartilage to be present in 35-day denervated limb stumps.

When the percentage of cells found in each tissue type (cartilage, layered cells, and dedifferentiated cells) was examined for 35-day denervated limbs, dedifferentiated cells made up the predominant cell type (42%) with cartilage cells following (33%) while layered cells made up only 25% (Table 8).

Examination of nerve stained sections from all 4 denervated limb stumps 5 weeks post-amputation/denervation revealed that complete reinnervation of the limb stump had occurred by 5 weeks (compare Figs. 10E and 10F). These results confirm those of Experiment 1 where reamputation/denervation resulted in 100% of the limbs regenerating with no delay when compared to control limbs.

Autoradiographical Analysis of Once-Denervated Limbs

Results by Mescher and Tassava (1975) and Loyd (1978), have shown that cells in limbs that do not regenerate (i.e., denervated limbs, inserted limbs, and skin flapped limbs) nevertheless enter the cell cycle, and in some instances undergo a few mitotic divisions. However, it is not known what happens to the dedifferentiated cells in these inhibited denervated limb stumps through time.
Figure 10. Figures 10A, 10B, and 10C are micrographs of a longitudinal section through the forelimb of a denervated stump at 35 days post-amputation/denervation illustrating the most typical histological features seen. Densely packed layered cells (L) are present immediately underneath the thin wound epidermis (WE). Differentiated cartilage (C) is very pronounced in the distal stump, closely associated with the radius (R) and ulna (U). A number of dedifferentiated cells (DE) remain in the distal stump. A portion of the low power micrograph in 10A (65X) can be seen in higher power in 10C (160X). The section in 10B (325X), which has more layered cells, is in the same limb but cut through the distal tip of the ulna. The arrow in 10C point between the thin wound epidermis (WE) and the layered cells. The thin wound epidermis covering the limb tip resembles the epidermis of the limb skin. Hematoxylin and eosin. Figure 10D is a micrograph of a longitudinal section (58X) through a control regenerating limb at the 3-digit stage of regeneration. A few dedifferentiated cells (DE) can be seen in the distal limb tip. The skeletal elements, digits (D), are present. A thickened wound epidermis (WE) still covers the limb tip. The amputation level and the radius and ulna are out of the plane of the micrograph. A high magnification of a completely reinnervated nerve bundle at the radius and ulna level of a 35-day denervated limb stump is illustrated in Figure 10D (250X). Regenerated nerve fibers (n) completely fill the bundle and are smooth in character. Compare with control nerves (N) in Fig. 10E (50X). Samuel's nerve stain.
Figure 10.
### Table 5

A Comparison of Mean Cell Counts and Other Parameters in 7- and 35-Day Once Denervated, Redenervated, and Control Limbs

<table>
<thead>
<tr>
<th>Days Post-Amputation</th>
<th>Experiment</th>
<th>Number of Limbs</th>
<th>Distance from Amputation Surface to Wound Epidermis</th>
<th>Wound Epidermis Thickness</th>
<th>Number of Cell Layers in Wound Epidermis</th>
<th>Average Number of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>control</td>
<td>3</td>
<td>0</td>
<td>.75</td>
<td>12</td>
<td>174</td>
</tr>
<tr>
<td>7</td>
<td>once-den</td>
<td>3</td>
<td>0</td>
<td>.83</td>
<td>13</td>
<td>107</td>
</tr>
<tr>
<td>35</td>
<td>once-den</td>
<td>4</td>
<td>.76</td>
<td>.27</td>
<td>5</td>
<td>644</td>
</tr>
<tr>
<td>35</td>
<td>re-den</td>
<td>4</td>
<td>1</td>
<td>.25</td>
<td>5</td>
<td>604</td>
</tr>
<tr>
<td>35</td>
<td>control</td>
<td>1</td>
<td>4.3</td>
<td>.75</td>
<td>10</td>
<td>3,520</td>
</tr>
<tr>
<td>21</td>
<td>control</td>
<td>1</td>
<td>3</td>
<td>.75</td>
<td>10</td>
<td>1,337</td>
</tr>
</tbody>
</table>

*Distance measured in grid units.

*Individual grid units were used as a measure for determining the thickness of the wound epidermis.

*Areas I and II combined.
In the present study it was of interest to determine the fate of dedifferentiated cells that initially synthesized DNA on day 7. That is, whether they are maintained in the denervated limb stump through time (5-week period), or are eventually lost or removed from the denervated limb stump.

Examination of autoradiographs from 3 amputated, denervated limbs labelled on day 7 (see Experiment 8 Series I) showed that the labelling index of 6% for denervated limb stumps paralleled that of control innervated limbs. These results were expected since it had been shown by Mescher and Tassava (1975) that denervated limb stumps of the adult newt show DNA labelling indices which increase like those of control innervated limbs during the first week after amputation. Therefore, 6% of the total dedifferentiated cells found in the area of dedifferentiation in denervated and innervated limb stumps were synthesizing DNA on day 7. Autoradiographs on days 14, 21, and 35 of the same series described above, showed that labelled dedifferentiated cells from day 7 remained in the denervated limb stump throughout the 5-week period.

As reported earlier the total number of cells found in the distal limb tip of denervated limbs increased throughout the 5-week period (Fig. 12). In addition new tissue types had appeared by day 35 post-amputation/denervation. It was of interest then, to examine the distribution of label with regard to cell type (cartilage, layered cells, and blastema cells) in these amputated/denervated labelled limbs, 35 days later. When autoradiographs from 5 denervated limbs were examined on day 35, it was found that a very low percentage of layered cells and newly differentiated cartilage cells had silver grains above their nuclei.
The preponderance of silver grains were found over the nuclei of blastema-like cells. Therefore, dedifferentiated cells labelled on day 7 remained present in the distal tip of 35-day denervated limb stumps as blastema-like cells and very rarely differentiated into cartilage or layered cells. These results must be emphasized since the presence of dedifferentiated cells in long-term denervated limb stumps 35 days post-amputation/denervation had not been previously reported. The fact that few labelled cartilage and layered cells were found in 35-day denervated limbs could also mean that some of the dedifferentiated cells labelled on day 7 underwent a number of mitotic divisions during the 5-week period such that the number of silver grains found above their nuclei was completely diluted. These cells then differentiated into cartilage and layered cells with little or no silver grains above their nuclei.

It was of interest then, to also note if any dilution of the number of silver grains found above labelled nuclei of dedifferentiated cells present in 7-day innervated and denervated limbs occurred during the 5-week period. A comparison of autoradiographs from innervated and denervated limbs on day 7 with autoradiographs from both innervated and denervated limbs on day 35 showed that labelled cells on day 35 in both groups had fewer silver grains above their nuclei. Therefore, dilution of the number of silver grains above labelled cells did occur during the 5-week period in both innervated and denervated limbs. These results were expected in innervated limbs since it is known that in innervated limbs, dedifferentiated cells undergo proliferation to establish a regeneration blastema.
The most notable difference could be seen when autoradiographs from day 7 innervated limbs were compared with autoradiographs from day 35 innervated limbs. The results are reported in Figures 14 and 15. In control limbs on day 7 the majority of cells synthesizing DNA were heavily labelled (having 50 or more silver grains above the nucleus). However, 35 days later, the majority of the cells had only background labelling (4 silver grains) above their nuclei. These results show that there had been essentially complete dilution of silver grains in innervated limbs by day 35 post amputation. The extent of mitotic activity that occurred in these control innervated limbs through time was also revealed.

When the same comparison described above for 35-day control limbs was made between autoradiographs from day 7 denervated limbs, and autoradiographs from day 35 denervated limbs, only partial dilution of silver grains occurred (compare Figs. 14 and 16). The majority of labelled dedifferentiated cells in day 7 denervated limbs were heavily labelled having 50 or more silver grains above their nuclei. Labelled cells were still heavy to moderately labelled on days 14 and 21 post-amputation/denervation. By day 35 the majority of labelled cells were still moderately labelled having as many as 25 silver grains above their nuclei. The few labelled cartilage and layered cells present in the distal tip were lightly labelled. These results suggest that cell division occurred in denervated limb stumps during the 5-week period and it became important to quantitate the number of cell divisions labelled cells underwent during the 5-week period.

To determine the number of cell divisions the following method was used. Since cell division results in silver grain dilution in labelled
cells, the average number of silver grains over the heaviest labelled nucleus found in 7-day innervated limbs was determined while the average number of silver grains was counted for those cells lightly labelled on day 35 in control limbs. On the average, the heaviest labelled nucleus had 100 silver grains and lightly labelled nuclei on the average had 4 to 6 silver grains. It was then determined that a nucleus having 100 silver grains would have to undergo at least 4 cell divisions in order to dilute its silver grain count to 4. As shown in Figure 15, the majority of labelled nuclei in 35-day control limbs fell in the 4 silver grains per nucleus category. No heavily labelled nuclei were seen in control limb stumps on day 35. Therefore, since day 7 labelled dedifferentiated cells in control limbs underwent at least 4 rounds of cell division.

Using the same method, the number of cell divisions that occurred in denervated limb stumps during the 5 week period was also determined. Figure 16 shows that unlike control limbs, labelled nuclei in denervated limb stumps on day 35 could be found in all categories, with the majority of labelled nuclei falling in the 25 to 13 silver grain per nucleus range. Therefore, on the average labelled dedifferentiated cells in denervated limbs underwent only 2 or 3 cell divisions during the 5-week period.

Since all dedifferentiated cells were not synthesizing DNA during the incorporation period on day 7, every cell did not label. The results described above, therefore represent only 6% (labelling index on day 7), of the total number of dedifferentiated cells found in both innervated and denervated limb stumps.
To determine the extent of cellular activity (i.e., cells synthesizing DNA) in 35-day denervated limb stumps 4 limbs were given a pulse of \(^3\)H-thymidine on day 35. Using the distal tip of the limb as the sample area the labelling index was determined.

Examination of autoradiographs from these limbs revealed that a number of labelled cells were present in the limb tip. These results were unexpected since morphologically, denervated limbs appeared stumped and were considered to be static or inactive. Labelled nuclei were found among all three cell types located in the limb tip (cartilage, dedifferentiated cells, and layered cells). It was interesting to find that no cell type had a preponderance of label. Labelled dedifferentiated cells appeared as frequently as labelled cartilage or layered cells. Therefore, cells from all 3 tissue types were still cycling on day 35 post-amputation/denervation. However, the labelling index in the distal tip of the 4 denervated limbs examined was only 3%. Such a low labelling index suggests that a large percentage of cells, especially dedifferentiated cells, since they represent the dominant cell type in 35-day denervated limbs, are not cycling and that the cellular activity in these limbs had stabilized by day 35. The fact that mitotic figures were never seen in 35-day denervated limb stumps also supports this view. Another striking observation after the examination of the autoradiographs from the denervated limbs described above was that wound epidermal cells labelled as intensely as cells in the proximal skin epithelium. Hay and Fischman (1961) reported that in normal regenerating limbs, prior to 28 days post-amputation, the blastema formation period, only occasionally could a labelled cell be found in the wound epidermis.
actively synthesizing DNA; however, the greatest amount of tritiated thymidine incorporation still occurred in the proximal skin epidermis. Thus the epidermis covering 35-day denervated limb stumps, not only resembled skin epidermis histologically, but also in the pattern of tritiated thymidine incorporation.

Histological Analysis of Redenervated Limbs

It has already been established that tissue regeneration occurs in long-term denervated limb stumps by day 35. However, it is not known if tissue regeneration in denervated limb stumps is dependent upon nerve in growth.

One approach taken to resolve this problem was to prolong the denervation state of amputated adult newt forelimbs by performing two denervations. The first denervation was done one day post-amputation and the second denervation followed 14 days later.

A histological comparison between redenervated limbs of the same amputation age as once-denervated limbs fixed 28, 35, and 49 days post-amputation was made (Tables 5, 6, and 7). Results from the 28-day comparison will be reported first.

Like 28-day once-denervated limbs, 28-day redenervated limbs had a wound epidermis reduced in thickness having only 5 cell layers, and was still free of dermis and skin glands. A number of dedifferentiated cells were present in the distal limb stump and examination of Mallory stained sections revealed that a small amount of tissue regeneration had also occurred in 28-day redenervated limbs (Fig. 11B).
The total number of cells found in the distal tip of 28-day redenervated limbs were less than one-half the number found in 28-day once-denervated limb tips (Fig. 12). Only a small amount of distal accumulation of these cells had occurred. When the same comparison of total cells located in the distal tip was made between 28-day redenervated limbs and 14-day once-denervated limbs (same denervation age of 14 days), the total number of cells was comparable. These results show that the cell division seen in 14-day once-denervated limbs is nerve independent.

A small amount of newly differentiated cartilage capped the radius and ulna in 2 of the 4 limbs examined. Occasionally a few layered cells could be found in the distal tip of these limbs. Even though the amount of cartilage found in 28-day redenervated limbs was the same as that found in 28-day once-denervated limbs, layered cells were fewer in the redenervated limbs. It is interesting to note that tissue regeneration was never seen in any 14-day once-denervated limb stumps (limb stumps of the same denervation age as 28-day redenervated limbs).

Four nerve stained sections of 28-day redenervated limbs were examined and the results show that nerve fibers were not present in any of these redenervated limbs. When redenervated limb stumps were tested for sensitivity of the limb stump, no movement of the stump was observed, suggesting that nerves were still absent.

A total of 15 redenervated limb stumps were examined histologically on day 35. Five of these 15 redenervated limbs were from Experiment 9 the study done to analyze tissue regeneration in redenervated limbs; the remaining 10 were from Experiment 10, the autoradiographical analysis of 35-day redenervated limb stumps. Limbs from both groups were included
when cell counts and density determinations were made for day 35. However, these two groups were analyzed separately when categorized according to the amount of tissue regeneration present in the limb stumps (Tables 7 and 8).

Redenervated limbs examined 35 days post-amputation had a number of interesting histological features. The wound epidermis that covered the amputation surface in all 15 limbs was reduced in thickness (5 cell layers) but was not as compact as the wound epidermis in 35-day once-denervated limbs (Fig. 11C). In only one limb had the skin glands reformed over the entire amputation surface. Therefore, like most of the 35-day once-denervated limbs, no apparent barrier existed between the wound epidermis and the underlying stump tissues.

Blastema formation was never observed in any of the 35-day redenervated limbs examined. However, all 15 limb stumps had a number of dedifferentiated cells present in the distal limb stump. Like 35-day once-denervated limbs, very few of these dedifferentiated cells had accumulated distally by day 35 (Figs. 11C and 11D). However, the total number of cells in the distal tip of 35-day redenervated limbs did increase slightly since day 28 post-amputation (Fig. 12). As shown in Fig. 12, the number of cells in the distal tip of 35-day redenervated limb stumps was considerably less than the number of cells in the distal tip of 35-day once-denervated limbs (372 vs. 524). When the same comparison was made between 35-day redenervated limbs and once-denervated limbs of the same denervation age, 21 days, the average number of cells in the distal limb tip was roughly the same (372 vs. 369; Fig. 13).
Therefore, these results suggest that the low level of mitotic activity seen in 21-day once-denervated limbs is nerve independent, since redenervation resulted in the same low level of mitotic activity.

The density of cells in the distal tip of 35-day redenervated limbs remained relatively stable throughout the 5-week period. These results are seen in Table 6 where the mean number of cells/grid area in the distal tip of control once-denervated and redenervated limb stumps are recorded. Results in this table also show that the density of cells in the distal tip of 35-day once-denervated limbs was much greater than the density in 35-day redenervated limbs. On the average, there were 25 cells per grid area in once-denervated limbs on day 35 while 35-day redenervated limbs had only 19 cells per grid area in the distal tip.

In addition to dedifferentiated cells in the distal limb stump, 10 of the 15 redenervated limbs examined had newly differentiated cartilage and layered cells in the distal limb tip. The amount of tissue regeneration was scored in each of these 10 limb stumps and the results can be seen in Table 7.

In the first group of 35-day redenervated limbs examined histologically, less than one-half (2/5) showed signs of tissue regeneration (Table 7). There had also been no increase over the amount seen in 28-day redenervated limbs (Table 7). Only a small nodule of cartilage was found associated with the radius and ulna (Fig. 11C). Layered cells were absent in both of these limbs. There were present, however, 2 to 3 rows of cells that extended from the radius to the ulna which formed a type of cellular connection between the two bones (Fig. 11C). This cellular connection was not observed in 28-day redenervated limbs, the
second group of 10, 35-day redenervated limbs, nor 35-day once-denervated limbs (compare Figs. 11C, 11D, and 10A). Data in Table 7 and 8 also show that tissue regeneration in the two redenervated limbs in this group was not as prominent as that found in 35-day once-denervated limbs. Ninety-six percent of the total cells found in the distal limb stump was dedifferentiated cells. Cartilage cells made up only 4% of the total cells in the distal tip.

In the second group of 10 35-day redenervated limbs examined histologically, 80% of the limbs showed signs of tissue regeneration (Table 7). The amount of cartilage in the distal tip of these redenervated limbs was comparable to the amount seen in 35-day once-denervated limbs. Layered cells were present in all 8 limbs in this group; however, the number of layered cells was fewer than the number found in 35-day once-denervated limbs (Table 7). Dedifferentiated cells made up 48% of the total cells found in the distal tip of these 35-day redenervated limbs while 34% of the cells were cartilage and layered cells made up only 18% (Table 8).

Examination of the parameters listed in Tables 7 and 8 describing the relative amount of tissue regeneration present in the distal stump and the percentage of cells found in the various tissue types, show that the 8 redenervated limbs showing tissue regeneration in this group resembled 35-day once-denervated limbs more closely than did the first group of 5 35-day redenervated limbs described above. Because of the similarity between the two groups, it became important to examine and compare nerve stained sections from 21-day once-denervated limbs. Nerve stained sections of 4 limbs from each group of redenervated limbs were examined and
the results show no significant difference between the two groups in the
number of nerve fibers that had regenerated back into the limb stumps.
Nerve bundles from both groups of redenervated limbs very closely resem­
bled those found in 21-day once-denervated limbs. Therefore, these
results still did not resolve the apparent histological differences seen
between the two groups of 35-day redenervated limbs described in this
experiment.

Four redenervated limbs were also fixed 49 days post-amputation.
The histology of these day 49 redenervated limbs was not different from
the histology of the second group of 35-day redenervated limbs. There
had been a slight increase in the number of cells since day 35 (Fig. 12)
found in the distal tip of these redenervated limbs. However, when
compared with once-denervated limbs of the same denervation age of 35
days (Fig. 13), 49-day redenervated limbs had fewer cells accumulated in
the distal tip.

Tissue regeneration occurred in 3 of the 4 limbs examined. However,
there was no improvement over the amount seen in 35-day redenervated limbs
(second group).

Examination of nerve stains revealed that reinnervation of the limb
stump had occurred by day 49 (35 days post-amputation) in redenervated
limbs.

Because tissue regeneration occurred in redenervated limb stumps,
especially in 28 day redenervated limbs, the results of this study
therefore suggest that nerves are not needed for tissue regeneration.
However, as shown in Tables 5 and 7, the absence of nerves does affect
both the number of cells found in redenervated limbs and the amount of
tissue regeneration that occurs when compared with the amounts seen in once-denervated limb stumps.

**Autoradiographical Analysis of Redenervated Limbs**

Four of the labelled animals in Series I of Experiment 10 did not survive the length of the experiment, therefore, an autoradiographical analysis of 28-day redenervated limbs was not made. The following results include only the autoradiographical analysis of 7, 35-day redenervated limbs from Series I and 3, redenervated limbs from Series II.

Labelled dedifferentiated cells were still present in the distal limb stump of all 7 redenervated limbs fixed on day 35. Dedifferentiated cells, layered cells, and cartilage had silver grains above their nuclei, but one of the cell types labelled more frequently than the other. Labelling was also found among wound epidermal cells and cells in the proximal skin epidermis. Silver grain dilution did occur during the 5-week period, but not as extensive as it occurred in 35-day once-denervated limbs. Therefore, cells in 35-day redenervated limbs were still heavy to moderately labelled.

To determine if any cells were still cycling in 35-day redenervated limbs, 3 limbs were injected with $^3$H-thymidine on day 35 (Series II). The mean labelling index in these redenervated limbs was 3%. As reported in earlier results 35-day once-denervated limbs also had a labelling index of 3%. Labelling was distributed among all three cell types (cartilage, layered cells, and dedifferentiated cells). However, there were more dedifferentiated cells labelled than any other cell type. These results suggest that dedifferentiated cells are the predominant
cell type in the cell cycle on day 35 in redenervated limbs.
Figure 11. Figure 11A is a micrograph of a longitudinal section (160X) through the forelimb stump at 35-days post-amputation/denervation illustrating less typical histological features seen after long-term denervation. No layered cells or differentiated cartilage were present in this limb. Instead, dedifferentiated cells (DE) are present throughout the distal limb tip. A thin wound epidermis free of skin glands (S) covers the limb stump. Hematoxylin and eosin. Figure 11B is a longitudinal section of a 28-day redenervated limb stump. Histological features closely resemble those of the day 35 denervated limb stump (Fig. 11A). Dedifferentiated cells (DE) are present throughout the limb tip. Layered cells are absent, however, a small amount of cartilage is associated with the ulna (U) (arrow). Layered cells were present in an adjacent section of this limb. The wound epidermis is free of any underlying skin glands and is not as compact as the thin epidermis covering the limb tip of 35-day denervated limbs (compare Fig. 10A and 11A). Hematoxylin and eosin (70X). Figures 11C and 11D are micrographs of longitudinal sections of two 35-day redenervated limbs illustrating the histological differences observed between the two groups. In the redenervated limb Fig. 11C (75X) dedifferentiated cells are prominent in the distal tip. A small amount of cartilage (arrow) is associated with the ulna (U). Layered cells were absent, however, rows of dedifferentiated cells with rounded nuclei are layered between the radius (R) and ulna (U). The wound epidermis (WE) was still not compact and closely resembled the WE of 28-day redenervated limbs (Fig. 11B). Hematoxylin and eosin. Figure 11D shows a 35-day redenervated limb stump with histological features that very closely resembles a typical 35-day once-denervated limb rather than the redenervated limb in Fig. 11C. The wound epidermis (WE) is compact, newly differentiated cartilage (C), layered cells (arrows), and dedifferentiated cells (DE) are all prominent in the distal limb tip. Hematoxylin and eosin (75X).
Figure 12. Comparison of the average number of cells in the distal limb stumps of innervated control, once-denervated and redenervated limbs, 7, 14, 21, 28, 35, and 49 days post-amputation. Areas I and II were combined for determining the mean number of cells/area in both control and denervated limbs for 7 and 14 days post-amputation while Area II only (distal limb tip) was sampled in control, denervated and redenervated limbs 21, 28, 35, and 49 days post-amputation. Regenerating control limbs were not sampled after 5 weeks post-amputation/denervation. Each point represents the mean number of cells/area from 3 limbs.
Figure 12. AVERAGE NUMBER OF CELLS

- CONTROL
- DENERVATED
- RE-DENERVATED

DAYS POST-AMPUTATION

- 7
- 14
- 21
- 28
- 35
- 49
Figure 13. Comparison of the average number of cells found in the distal limb stumps of redenervated limbs 14, 21, and 35 days post-amputation/denervation with once-denervated limbs of the same denervation ages (14, 21, and 35 days). Control limbs were sampled on days 7, 14, 21, and 35 post-amputation. Each point represents the mean number of cells/area from 3 limbs.
Figure 13. DAYS POST-DENERVATION
Figure 14. Histogram showing the average number of labelled cells found in the distal limb stump of 7-day control and denervated limbs. Labelled cells were categorized according to the silver grain concentration above their nucleus. A total of 3 limbs were sampled for day 7 control and denervated limbs. Since no differences in number of labelled cells were observed between the two groups the histograms were combined.
Figure 15. Histogram showing average number of labelled cells in the distal limb stump (blastema) of 35-day control limbs. Labelled cells were categorized according to the average number of silver grains/nucleus. A total of 3 limbs were sampled.
Figure 16. Histogram showing average number of labelled cells in the distal limb stump of 35-day denervated limbs. Labelled cells were categorized according to the average number of silver grains/nucleus.
Table 6

A Comparison of the Cell Density in the Distal Region of Once-Denervated, Redenervated and Control Limbs

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Days Post-Amputation</th>
<th>Number of Limbs</th>
<th>Cell Densities (cells/grid area)</th>
<th>Mean Cell Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>control-innervated</td>
<td>7</td>
<td>3</td>
<td>(18)(19)(16)</td>
<td>18</td>
</tr>
<tr>
<td>once-den</td>
<td>7</td>
<td>3</td>
<td>(17.3)(20)(18)</td>
<td>18</td>
</tr>
<tr>
<td>control (early bud)^b</td>
<td>21</td>
<td>2</td>
<td>(23)(19)</td>
<td>21</td>
</tr>
<tr>
<td>control (digit)^b</td>
<td>35</td>
<td>2</td>
<td>(22)(20)</td>
<td>21</td>
</tr>
<tr>
<td>once-den</td>
<td>35</td>
<td>3</td>
<td>(21)(25)(29)</td>
<td>25</td>
</tr>
<tr>
<td>re-den</td>
<td>35</td>
<td>2</td>
<td>(18)(20)</td>
<td>19</td>
</tr>
</tbody>
</table>

^aCell densities included all 3 cell types in denervated limbs but on the average the density of layered cells was higher (#/grid area) than that of cartilage (#/grid area) or dedifferentiated cells (#/grid area).

^bThese densities were determined over the blastema.
TABLE 7

A COMPARISON OF THE AMOUNT OF TISSUE REGENERATION IN ONCE-DENERVATED AND REDENERVATED NEWT LIMBS 4 AND 5 WEEKS POST-AMPUTATION

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Days Post-Amputation</th>
<th>Number of Limbs</th>
<th>Number of Limbs Showing Tissue Regeneration</th>
<th>Relative Amount of Tissue Regeneration</th>
<th>Number of Limbs with Skin Glands Healed Over the Amputation Surface</th>
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<tbody>
<tr>
<td>once-den</td>
<td>28</td>
<td>4</td>
<td>3</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>re-den</td>
<td>28</td>
<td>4</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>once-den</td>
<td>35</td>
<td>22</td>
<td>20</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>re-den(^a)</td>
<td>35</td>
<td>5</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>re-den(^a)</td>
<td>35</td>
<td>10</td>
<td>8</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>re-den</td>
<td>49</td>
<td>4</td>
<td>3</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)Two histologically different groups of redenervated limbs.

\(^b\), smallest amount of cartilage or number of layered cells observed; ++, average amount of cartilage or number of layered cells observed; +++ , largest amount of cartilage or number of layered cells observed.
TABLE 8
A COMPARISON OF THE PERCENTAGE OF CELLS FOUND IN THE VARIOUS TISSUE TYPES IN THE DISTAL LIMB TIP OF ONCE-DENERVATED AND REDENERVATED LIMBS 35 DAYS POST-AMPUTATION

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Days Post-Amputation</th>
<th>Number of Limbs Counted</th>
<th>% Cartilage Cells</th>
<th>% Dedifferentiated Cells</th>
<th>% Layered Cells</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>once-den</td>
<td>35</td>
<td>2</td>
<td>33</td>
<td>42</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>re-den°</td>
<td>35</td>
<td>2</td>
<td>34</td>
<td>48</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>re-den°</td>
<td>35</td>
<td>2</td>
<td>4</td>
<td>96</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

aAreas I and II were included.

bAll limbs were chosen at random but represented the typical histological features of once-denervated and redenervated limbs 35 days post-amputation.

cTwo histologically different groups of redenervated limbs were analyzed.
CHAPTER VI
DISCUSSION

Regeneration of amputated adult newt forelimbs never occurred if the limb stump was completely denervated at the time of amputation. Amputated-denervated limb stumps of adult newts furthermore never initiated regeneration after nerves returned to the distal limb stump. These observations support the generally accepted view that denervation prevents regeneration of amputated, adult newt forelimbs even after reinnervation (Singer, 1952; Thornton, 1968; Carlson, 1974). While it is still not known why denervated adult newt limbs remain inhibited from regeneration once nerves return to the limb stump, results from my study provide some insight into the identification of the factors responsible.

Several approaches were undertaken in this study to determine the effect of denervation and subsequent reinnervation on amputated adult newt limbs. The first approach was a morphological study to test whether amputated, denervated newt limbs could indeed be stimulated to initiate regeneration upon reinnervation without additional injury. The second approach involved the histological examination of denervated newt limbs through time to identify and analyze those changes that occurred in these denervated limb stumps which explain why reinnervation alone does not result in regeneration.

Adult newt limb stumps in the present study were amputated at three different levels, proximal humerus, distal humerus, and radius and ulna.
and completely denervated. Whether the level of amputation was the proximal humerus, distal humerus, or radius/ulna, denervated newt limb stumps never regenerated, even through 10 weeks post-amputation. Control innervated amputated limbs always completed regeneration. Surprisingly, it was not until the 3rd week post-amputation/denervation that morphological differences existed between denervated and control limbs. At this time, besides the absence of a blastema, denervated limbs had reduced limb diameters consistent with previous reports (Singer and Craven, 1948; Singer and Egloff, 1949), and there was also a clear pigment-free area over the distal end of the limb stump. There were no apparent morphological differences at 1 and 2 weeks. Mescher and Tassava (1975) also observed that through 2 weeks post-amputation, denervated and innervated limbs were similar in histological features. Wound epithelium formation occurred, dedifferentiation of stump tissues occurred and H3-thymidine/labeling indices were similar. Thus, like in amputated, innervated limbs during the first 2 weeks, the histological features present in denervated limbs are an expression of an attempt by the limb to initiate regeneration. However, the mitotic activity necessary for blastema formation was absent since nerves were not present, and regeneration did not occur. The view that nerves are indispensable to the essential phase of regeneration that leads to the establishment of a blastema is further emphasized by my results. These observations are consistent with the cell cycle hypothesis of Tassava and Mescher (1975) and with a growing body of literature (Tassava and McCullough, 1978; Garling, 1981), in showing that amputation does initiate many cellular events (i.e., dedifferentiation) but sustained mitotic activity requires
the presence of nerves.

The clear pigment-free area over the distal limb tip of denervated limb stumps, had the morphological appearance of wound epidermis. This clear pigment-free area was observed beginning at 2 weeks post-amputation denervation and persisted throughout the 10-week period. Singer (1954) also observed a central translucent pigment-free region over the end of amputated forelimbs of non-regenerating post-metamorphic frogs. This clear area was observed as early as 2 weeks post-amputation and persisted for many weeks. Singer did not define this clear area histologically, and therefore, it is not known if this pigment-free region is a part of the whole skin that rapidly regrows over the amputation surface of amputated frog limbs and presumably blocks regeneration of these limbs (Rose, 1944a; 1949; Carlson, 1974). It was of interest to note that this pigment-free region was never observed in any amputated, innervated, regenerating newt limbs nor was it observed by Singer (1954) in those amputated frog limbs that showed a positive regenerative response. It is likely that this clear pigment-free region seen in denervated limbs is a morphological expression by denervated limbs that reflects stump healing.

Prior to the paper by Salley and Tassava (1981), the only report in the literature that examined regeneration of amputated adult salamander limbs after reinnervation was that of Schotte (1923). Schotte (1923) denervated limbs of Triton (European newt) which were amputated at different levels. Some of the shortest limb stumps i.e., those amputated nearest the body, showed delayed regeneration. Schotte reasoned that nerves reached the distal tips of the short limbs before inhibitory healing events occurred. In my study, regeneration of denervated limbs
never occurred, whether amputation was near the body or through the
radius/ulna. It is clear, therefore, that adult newt (Notophthalmus)
limb stumps exhibit subtle healing events before regenerating nerve
fibers reach the tips of even the shortest limb stumps. Schotte observed, present in the limb stumps of those non-regenerating limbs in
his study, a "resistant cicatrix" which he believed, was the mechanical
obstacle that blocked regeneration after nerves returned. Both the
results in my study and those of Schotte make it apparent that in denervated adult salamander limbs, there is a critical time period during
which stump tissues will respond to the return of nerves and initiate
regeneration. If nerve fibers return to the limb stump after this
critical time period, as was the case with simultaneously amputated/
denervated newt limbs in my study and with the majority of the denervated
Triton limbs in Schotte's study, inhibitory healing events have already
occurred and even though nerve fibers eventually completely reinnervate
the limb stump, the stimulus for epimorphic regeneration is no longer
present. Stump healing, therefore, occurs and the limbs fail to
regenerate.

It, therefore, became important to establish the exact time of
reinnervation of denervated limb stumps, a time not made clear in the
literature, and secondly, to design a system that would allow nerves to
return to denervated limb stumps before these critical healing events
occur, to (1) test for regeneration of these limbs upon reinnervation,
and (2) further define the time period when these subtle healing events
were occurring in denervated newt limbs.
The timing of reinnervation was established by using the following approaches: (1) reamputation of denervated limb stumps at various times post-amputation/denervation and (2) histological analysis, including nerve staining throughout a 5-week period.

Using reamputation and subsequent observation for regeneration as an indicator for threshold reinnervation, the first approach showed that threshold numbers of nerve fibers return to denervated distal limb stumps by 21 days post-original amputation/denervation. The observed regeneration of 86% of reamputated limbs, without significant delay, clearly argues that threshold reinnervation had occurred. A smaller percentage (43%) of denervated limbs reamputated at 14 days post-original amputation/denervation regenerated, and only after a one-week delay in initiating regeneration, when compared to regenerating control limbs (Table 1). It is likely that during this delay, more nerve fibers regenerated back into the limb stump, thus establishing threshold innervation. These results are consistent with the view that threshold reinnervation occurs in denervated limb stumps during the 3rd week post-amputation/denervation.

The fact that 57% of the denervated limbs did not regenerate when reamputated on day 14, indicates that healing events inhibitory to regeneration were programmed to occur during the next week prior to additional nerve ingrowth. This assumes, as suggested above, that threshold innervation has returned by 3 weeks post-denervation.

Data in Table 1 show that delaying reamputation allowed more nerve fibers to reach the distal tip and effectively increase the percentage of limbs regenerating after reinjury. By 5 weeks post-reamputation, all
reamputated limbs regenerated with no delay when compared to control limbs, clearly showing that reinnervation of the limb stump was complete at this time.

Nerve staining of denervated limbs throughout the 5-week period verified the morphological times established for reinnervation. Nerve fibers were still undergoing degeneration at 7 days post-amputation/denervation and, by 14 days, degeneration was essentially complete. New regenerating nerve fibers could not be seen in the nerve bundles of denervated limbs until after 21 days post-amputation at the radius and ulna level. Approximately one-third of the normal innervation had returned by that time. An increase in the number of regenerating nerve fibers returning to the limb stump continued after day 21 and by day 35, limbs were completely reinnervated. It should be noted that the extent of reinnervation was a gross estimation and actual nerve fiber counts were not done. Since day by day nerve stain analysis of denervated limbs was not done in this study the exact time (day) of reinnervation was not determined. However, it is clear that threshold reinnervation returns to denervated adult newt limb stumps during the 3rd and 4th week post-amputation/denervation.

One way to get at the problem of the timing of reinnervation in relation to the timing of stump healing was to denervate the newt limbs first and then to amputate the denervated limbs at different levels at various times post-denervation. This system provided an effective means for testing the view that if nerves are allowed to reach the amputation surface before stump healing occurs, then regeneration of the limb will result. My results show that both the level of amputation and the length
of delay post-denervation before amputation, were instrumental in
determining the number of denervated newt limbs which regenerated without
additional injury after nerves returned to the limb stump. Delaying
amputation of completely denervated limbs provided the limb with a fresh
injury and a wound epidermis and also gave nerves a headstart back into
the limb stump. Denervated limbs amputated through the proximal humerus
always regenerated, regardless of whether amputation was 7 days post­
denervation or 14 days post-denervation. These results were not
surprising since proximal humerus limb stumps are short and in-growing
nerves reach the amputation surface very quickly, before stump healing
occurs. Denervated limbs amputated on day 7 were delayed in initiating
regeneration by 1 week when compared to innervated control limbs.
Denervated radius and ulna limb stumps failed to regenerate when
amputated 7 days post-denervation. Nerve fibers, even though given a
head start of 7 days, did not reach the amputation surface in sufficient
quantities before the stump healed up. Radius and ulna limb stumps are
twice the length of proximal humerus limb stumps and require more time to
become reinnervated. If these R/U limb stumps were given an additional
7 days (total of 14 days) reinnervation time before amputation, all of
the limbs regenerated and with no delay when compared to control limbs.
These results mean that inhibitory healing events in denervated adult
newt limb stumps occur between 7 and 14 days post-amputation. Examina­
tion of 7 and 14 day denervated limbs histologically showed no signs of
overt tissue healing (i.e., cartilaginous callus, dermal pad or cicatrix)
in the distal limb tip. These events, therefore, occur covertly and are
not detectable histologically. It is likely that cells in denervated
limbs are being programmed to undergo stump healing rather than epimorphic regeneration as early as 14 days post-amputation/denervation. When nerves return to the distal limb stump, some 3 to 4 weeks later, the conditions for regeneration no longer exist, even though denervated limbs still show histological features of control innervated limbs (i.e., presence of dedifferentiated cells and contact between stump tissues and wound epidermis). These results represent an important finding. Additional experiments should be done to further define these healing events and to also determine if these cellular changes can be detected ultrastructurally, or biochemically (changes in DNA, RNA, proteins) during the 2 to 3 week period post-amputation/denervation. It should be noted that Garling (1981) compared proteins present in both innervated and denervated newt limbs for up to 18 days post-amputation and reported no differences in the proteins that were detectable. These results also suggest that stump tissue cells are programmed first to undergo epimorphic regeneration since getting nerves back in sufficient quantities between 1 and 2 weeks did result in epimorphic regeneration. However, it must be noted that amputation of these limbs was delayed in relation to denervation.

While amputated denervated newt limbs remain stumped after reinnervation, larval Ambystoma amputated/denervated limbs always regenerate when nerves return (Petrosky et al., 1980; Olsen, 1981). The different response by amputated larval limbs to denervation and subsequent reinnervation is not well understood. It has been suggested that the cartilaginous skeleton of larvae plays a role in preventing stump healing from occurring in amputated/denervated limbs (reviewed by Carlson, 1974)
while the bone in newts favors stump healing (Winick, 1952).

Results in my study show that cartilage from larval Ambystoma, grafted into amputated/denervated newt limb stumps, was not successful in stimulating regeneration and preventing stump healing from occurring. Because histology was not done on these cartilage graft limbs, the possibilities cannot be ruled out that these limbs failed to regenerate because the graft was lost from the limb stump during the course of the experiment or that the amount of cartilage grafted to the limb was insufficient. On the other hand if the graft remained in the limb stump, it is also possible that cartilage simply has no role in preventing inhibitory stump healing events from occurring in denervated larval Ambystoma limbs.

Winick (1952) proposed that an antagonistic effect exists between the quantities of nerve versus bone in adult newt limbs; the presence of bone only favors stump healing while the presence of nerves only delays stump healing and promotes normal regeneration. According to Winick's theory, removal of both bone and nerves maintains a balance in the limb and allows regeneration to occur. Overloading the balance in either direction will cause either stump healing or regeneration to occur. It stands to reason, therefore, that if adult newt limb stumps are denervated and the limb bones (radius and ulna) are removed, regeneration of the limb should occur once nerves return to the limb stump. Preliminary results from such an experiment proved to be negative. Amputated/denervated adult newt limbs from which the radius and ulna had been removed showed no signs of regeneration after nerves returned to the limb stump (Tassava and Salley, unpublished). It has been noted in similar
experiments (bone removals) that old skin tends to collapse over the limb stump when skeletal support is lacking and thus inhibits regeneration (Carlson, 1974). This observation could also explain the regenerative failure of denervated limbs from which the R/U were removed.

The consequences of denervation at the time of amputation in larval Ambystoma are far more reaching than in the adult newt (Schotte and Butler, 1941; Butler and Schotte, 1941; Petrosky et al., 1980; Olsen, 1981). Stump cells undergo dedifferentiation but blastema formation does not occur. Stump tissues in the larval limb undergo regression in a proximal direction until entirely resorbed. Dedifferentiation essentially occurs unchecked until nerve fibers reinnervate the limb stump. It is possible that regression (the continued dedifferentiation and loss of stump tissue cells), in amputated/denervated larval Ambystoma limbs could be involved in preventing inhibitory stump-healing events from occurring. Thus, when nerves reinnervate the limb stump, dedifferentiated stump cells are able to respond and epimorphic regeneration results.

In the present study, simultaneously amputated and denervated adult newt forelimbs were never observed to undergo any type of regression. It has been demonstrated, however, that the stem regenerates of the adult newt will resorb if denervated at the time of amputation (Schotte and Liversage, 1959). However, regression of these stem regenerates only occurred back to the adult limb tissues. The amount of regression was also related to the age and thus the differentiated state of the regenerate at the time of denervation. Older stem regenerates (70 to 75 days old) did not show any appreciable amount of regression while younger
stem regenerates regressed completely. I designed additional experiments to determine if stem regenerates of the adult newt would manifest some of the same properties displayed by amputated larval Ambystoma limbs after amputation and denervation, especially whether initiation of regeneration would occur after reinnervation. Stem regenerates of the adult newt (70 days old) were amputated and denervated and observed through a 10-week reinnervation period. Thirty percent (6/19) of these limbs were able to regenerate after nerves returned to the limb stump, but blastemal outgrowth was not observed until 52 days post-amputation/denervation. This long delay in initiating regeneration by these limbs was surprising since previous results in this study had shown that complete reinnervation of the adult newt limb stump occurs by 35 days post-amputation/denervation.

All amputated/denervated stem regenerates (19 limbs) in the present study resorbed back to the original level of amputation (adult newt limb stump) by 2 weeks. These results differed from those of Schotte and Liversage (1959) who observed little or no resorption of their amputated, denervated 70-day stem regenerates. It could be that the stem regenerates in my experiments, although 70 days old, were not as differentiated histologically as those in the Schotte and Liversage study and resembled instead their intermediate stem regenerates (45 to 60 days old) which did undergo regression after amputation and denervation (Schotte and Liversage, 1959).

One important observation that emerges from the results of this experiment is that not only do stem regenerates of adult newts show the regression effect, a property thus far confined to limbs of amputated
denervated larval urodeles and anuran tadpoles, but a small percentage (32%) of these limbs also have the capability of initiating regeneration after reinnervation, another phenomenon confined thus far to larval Ambystoma amputated, denervated salamander limbs (Petrosky et al., 1980; Olsen, 1981).

Unfortunately, the potential use of this stem regenerate system for analyzing cellular events (labelling index or mitotic index) that occur in denervated newt limbs upon reinnervation is extremely limited due to the small percentage of limbs that regenerated in this experiment. Both the length of the experiment and the long delay observed before these amputated/denervated limbs initiated regeneration could also pose problems.

The results of the experiments discussed thus far emphasize the fact that reinnervation alone does not result in the regeneration of denervated adult newt limb stumps. In an effort to further define the regeneration limiting factors (i.e., wound epidermis and/or injury), in amputated/denervated newt limb stumps, the histology of these limbs was examined throughout a 5-week period and the effects of various kinds of reinjury on these amputated denervated newt limb stumps at 5 weeks was also tested.

The present study is the first detailed histological study of the effects of long-term denervation on amputated adult newt limb stumps. Previously, reports either described the cellular and histological changes that occurred in denervated newt limb stumps only 2 to 3 weeks post-amputation/denervation (Mescher and Tassava, 1975) or they included general statements describing typical features of adult newt limbs.
blocked from regeneration but without providing experimental evidence or adequate micrographs to support the descriptions (Rose, 1944; Singer, 1952; Singer, 1956).

I amputated and denervated adult newt limb stumps and examined them histologically throughout a 5-week period in hopes of finding or identifying a histological feature common to all denervated newt limbs which overtly signaled that regeneration was blocked. These types of histological features have been previously described in non-regenerating, amputated limbs of hypophysectomized newts (Schotte and Hilfer, 1957), newt limbs with skin grafts (Mescher, 1976), amputated limb stumps of frogs and mammals (Rose, 1944; Polezhaev, 1946; Schotte and Smith, 1959). They include the following: 1) a wound epidermis that becomes very rapidly underlain by dermis, 2) a cartilaginous callus surrounding the cut edges of the bone, 3) a distal fibrocellular scar formed between the end of the bone and the wound epithelium, and 4) fluid-filled cavities between the wound epidermis and the underlying tissues of the stump. The majority of these "histological markers" formed very quickly in these non-regenerating limbs (2 weeks) and were thought to act as a mechanical obstacle for preventing or disrupting the intimate contact between the wound epidermis and underlying stump tissues that is critical to the regeneration process.

The week-by-week study of the histological effects of denervation on amputated adult newt limbs allowed me to make observations on how the absence of nerves affects the early or initiatory phases of regeneration and also on how denervated newt limb stumps respond to the return of nerves (reinnervation).
Histologically, denervated newt limb stumps did not differ from control innervated limbs the first two weeks post-amputation. Both limb stumps underwent dedifferentiation, and a thickened wound epidermis, not underlain by dermis, covered the amputation surface. These results are consistent with those of Mescher and Tassava (1975) which show that through 2 weeks post-amputation, denervated and innervated limbs were similar in histological appearance, wound epidermis formation, and H3-thymidine labelling indices. These results further emphasize that the neural influence is not necessary for wound epidermis formation or for dedifferentiation of stump tissues during the initiatory phase of regeneration.

Beginning at 3 weeks post-amputation/denervation, significant histological differences could be seen between innervated and denervated limb stumps. While dedifferentiated cells in the innervated limbs underwent mitotic proliferation to form a regeneration blastema, cells in denervated limb stumps remained relatively stationary and only showed a slight increase in number. The sustained mitotic proliferation needed for blastema formation never occurred in denervated limbs. These findings are in agreement with earlier results (Mescher and Tassava, 1975; Tassava and Mescher, 1976) which indicate that the absence of nerves have an early and immediate adverse effect on mitosis in denervated newt limbs, such that in their absence, cells block in the cell cycle and do not undergo continued proliferation to form a blastema.

Changes in the appearance of the wound epidermis were also apparent by 3 weeks. The wound epidermal cells were flattened and the wound epidermis was more compact, having less than half the number of cell layers
it had on days 7 and 14 post-amputation/denervation. Neither dermis nor connective tissue could be found beneath the wound epidermis of denervated limbs examined on day 21. Epidermal contact with the underlying dedifferentiated cells still existed. This observation was an important one since it had been generally accepted that between 2 weeks and 3 weeks, dermis reforms beneath the wound epidermis of denervated newt limbs and blocks regeneration even though nerves return (Singer, 1952; Carlson, 1974). Denervated limbs in the present study at 3 weeks had a small population of dedifferentiated cells, still present in the distal limb stump, a wound epidermis and threshold innervation, yet no evidence of blastema formation could be detected histologically. Earlier results in this study established that critical healing events occur in denervated newt limbs already by 2 weeks post-amputation/denervation. It is interesting to note that these stump healing events were not overt and could not be detected histologically even at 3 weeks. These covert events may involve gene programming which is not overtly manifested until 4 weeks, at which time denervated limb stumps showed the first overt histological signs of stump healing. For the first time, newly differentiated cartilage appeared in the distal limb stump, always associated with the cut edges of the radius and ulna. A few cells, with elongated nuclei (layered cells), could also be seen layered beneath the epidermis in 28-day denervated limbs. In addition to cartilage and layered cells, denervated limb stumps at 4 weeks also had dedifferentiated cells still present in the distal limb stump. The wound epidermis had not undergone any further changes since day 21 and maintained the appearance of skin epidermis. Even though the number of nerve fibers that regenerated back
into the limb stump had increased beyond that of day 21, blastemas were still absent. Therefore, only after 4 weeks post-amputation denervation do denervated adult newt limbs show similar histological features (mainly tissue regeneration) of non-regenerating amputated limbs of mammals and post-metamorphic frogs.

The fact that the distal outline of denervated limb stumps never changed histologically to resemble a blastemal outgrowth, verified the stumped morphological appearance that characterized these denervated newt limbs throughout this experiment. Although stumped in appearance, denervated limbs had present in the distal limb tip at 5 weeks a number of cells (cartilage, dedifferentiated cells, and layered cells), densely packed into the small area found between the wound epidermis and the bone. The following question was raised as a result of the above observation: Is the cell number in the distal tips of denervated limb stumps 35 days post-amputation/denervation different from that of a young regeneration blastema? This question was based on the view that perhaps the cells located in the distal limb tip, even though their nuclei were compact and in a layered arrangement, could if given the proper regeneration stimulus, change their morphology as they began cycling and form a regeneration blastema. In other words, possibly the number of cells in the distal tip of denervated newt limb stumps at 5 weeks was not different from the number of blastema cells in an early bud regenerate; only the morphology and density of the cells differed. If this were true, it could possibly mean that considerable cell division occurs in denervated limb stumps but is not manifested in blastema formation. Olsen (1981) raised the same question concerning the compact cells found in the distal
tip of denervated *Ambystoma* larval limbs. Cell densities were not determined for these denervated limbs, however, the morphology of these compact cells was observed to change (cell nuclei became enlarged) as reinnervation of the larval limb stump occurred and these compact cells became cycling blastema cells. My results show that the cell density of denervated adult newt limb stumps, even though it increased slightly throughout the 5-week period, could not account for the obvious size differences that exists between a regeneration blastema and a denervated limb stump at 5 weeks post-amputation/denervation.

That a population of dedifferentiated cells still persisted in denervated newt limb stumps at 5 weeks was most interesting. Evidence that dedifferentiated cells remain in the limb stump of other non-regenerating adult newt limb stumps over an extended period of time, has also been provided by Tassava and Loyd (1977) for skin-flap limbs and by Loyd and Tassava (1980) for coelom inserted limbs. My histological results are the first to show that dedifferentiated cells remain present in the distal tip of denervated adult newt limb stumps for as long as 5 weeks post-amputation/denervation. Autoradiographic analysis of these 35-day denervated limbs show that some of these dedifferentiated cells were among the initial population of cells that dedifferentiated during the first week post-amputation/denervation. This mitotic proliferation resulted in the complete dilution of the number of silver grains found above the nucleus. Therefore, the cartilage and layered cells formed as a result of redifferentiation of these dedifferentiated cells, had little or no silver grains above their nuclei. These results also show that the cartilage and layered cells found in
denervated newt limb stumps originated from the initial (7-14 day) dedifferentiated population. It was even more surprising to find that a small percentage (3%) of these dedifferentiated cells in 5-week denervated adult newt limb stumps were able to incorporate H\textsuperscript{3}-thymidine. These findings confirm that some of these dedifferentiated cells are still in the cell cycle, i.e., not in G\textsubscript{0}, as late as 5 weeks post-amputation/denervation. Complete redifferentiation of dedifferentiated cells in denervated newt limb stumps therefore does not occur, at least not by 5 weeks post-amputation/denervation. Similar results have also been found in long-term skin-grafted limbs (Loyd, 1978). Loyd found a small but significant number of labelled cells as well as mitotic figures in 5-week non-regenerating skin-grafted adult newt limbs.

It is still not understood why some cells in denervated adult newt limb stumps remain in a dedifferentiated state for such an extended period of time, even though regeneration of the denervated limb never occurs. Future experiments should be designed to determine which phase of the cell cycle these cells are in. The ultimate fate of the cells should be investigated (i.e., How long will they remain in a dedifferentiated state in denervated newt limb stumps, even though conditions favor redifferentiation?).

I found the most striking histological feature of 5-week denervated newt limb stumps to be the amount of newly differentiated cartilage and layered cells present in distal tips of these limbs. Even though differentiating caps and/or nodules of cartilage have been described in a number of other non-regenerating limbs, for example, long-term skin graft limbs of the adult newt (Mescher, 1976), amputated coelom inserted
limbs of the adult newt (Goss, 1956), and in amputated mammalian and post-metamorphic frog limbs (Rose, 1944; Schotte and Smith, 1959), the present results are the first to describe newly differentiated cartilage and layered cells as a histological feature typical of 5-week denervated adult newt limb stumps. Approximately 80% of the 5-week denervated limb stumps had newly differentiated cartilage present in the limb stump tips. Cartilage cells made up 33% of the total cells in the distal limb tip. Every denervated limb stump examined histologically on day 35 that had newly differentiated cartilage (20/22 limbs) also had an abundance of densely packed layered cells, just beneath the epidermis, in close association with the cartilage. Layered cells, although making up only 25% of the total cells in the distal limb tip, had the highest density when compared to cartilage and dedifferentiated cells. In only one limb stump were layered cells present in the absence of cartilage and only two denervated limbs were observed to have neither cartilage nor layered cells. These results indicate that both layered cells and cartilage almost always appear together in denervated limb stumps. The single case without cartilage or layered cells is perhaps very instructive for it illustrates that these tissues need not be present for regeneration to be inhibited. It further emphasizes that a non-regenerating condition need not appear overtly different from a regenerating limb. Furthermore, the cartilage and layered cells are likely the result, not the cause of regenerative failure upon reinnervation, a point to be elaborated on below.

It should be noted that cartilage and layered cells initially appeared in denervated newt limb stumps 28 days post-amputation/
denervation, but because they were not prominent in all of the denervated limbs examined, it was not known if the presence of these two tissue types was a typical feature of denervated limbs.

It has been suggested that cartilage differentiation or "cartilaginous callus" formation in denervated limbs is tissue-regeneration (Carlson, 1978). The layered densely packed cells observed in the denervated newt limbs in the present study may be a part of this tissue-healing process, variously referred to in the literature as disto-fibrocellular scar, "scarring over," "cap-like scar," and "cicatrix" (reviews by Hay, 1966; Carlson, 1974). However, because these layered cells almost always appear in close association with the newly differentiated cartilage, it cannot be ruled out that these layered cells are a part of periosteum. Periosteal chondrogenesis occurs in normal regenerating adult newt limbs and a cartilaginous cuff or sleeve of cartilage forms around the distal portions of the amputated bones (Carlson, 1978). Cells in the periosteum of these limbs show a close resemblance to the layered cells described in the present study.

The abundance of layered cells and chondrocytes present in denervated limb stumps at 5 weeks suggests that some cellular proliferation occurred in these denervated limbs. Figure 13 shows that there was a 6-fold increase in the number of cells located in the distal stump (compare day 7 and day 35) of these denervated limbs. Since it had been shown by Mescher and Tassava (1975) that very little cell division occurs in denervated limbs during the first 2 weeks post-amputation, it became important to determine when this cellular proliferation occurred, i.e., before or after nerves returned to the limb stump.
Experiments were designed which delayed reinnervation of denervated adult newt limb stumps by performing 2 denervations during the 5-week period. These redenervated limbs were compared with once-denervated limbs of the same amputation age and denervation age. If the increase in number of cells observed in once-denervated limbs was due to cellular proliferation that occurred in the absence of nerves (nerve-independent), then the same number of cells should be present in redenervated limb stumps. On the other hand, if the cellular proliferation that occurred in once-denervated limbs was nerve-independent, then redenervated limbs would have fewer cells. Data from these experiments show that redenervated limbs, as compared to once-denervated limbs on the same days post-amputation (28 and 35 days), had fewer cells in the distal limb stump (Fig. 13). This suggests that a percentage of the cellular proliferation that occurs in once-denervated limbs on days 28 and 35 was nerve-dependent. When redenervated and once-denervated limbs were compared according to the same denervation age, the following results were obtained. Prior to day 35 post-amputation/denervation (14 and 21 days), both once-denervated limbs and redenervated limbs had roughly the same number of cells. Once-denervated limbs on day 35 had more cells than redenervated limbs. It would appear from this data that only a low level of mitotic activity occurs in denervated limb stumps prior to nerve ingrowth but significant mitosis occurs after nerves return (28 to 35 days post-amputation/denervation). It is also during this same time period that cartilage and layered cells appear in the denervated limb stumps. It is likely that the mitotic activity that occurs after nerves return results in cells that differentiate into the cartilage and layered
cells found in the distal tip of denervated limbs.

By prolonging the nerveless state of the denervated newt limb, it was also possible to determine if the absence of nerves affected the tissue regeneration that normally occurs in once-denervated limbs. Examination of sections stained with Mallory's for cartilage and connective tissue from 28-day redenervated limbs confirmed the presence of a small amount of cartilage and layered cells in the distal tip of 2 limbs. These redenervated limbs were only 14 days post-redenervation and nerve fibers had not yet returned to the limb stump. It would therefore appear that a small amount of tissue regeneration can occur in the absence of nerves.

A number of histological differences existed among the redenervated limbs examined 35 days post-amputation/denervation. One group of redenervated limbs very closely resembled 35-day once-denervated limbs in relation to the large amounts of newly differentiated cartilage and layered cells present in the distal limb stump while a second group had a negligible amount of only cartilage present. The fact that these two groups of redenervated limbs were so different was surprising, and raised a question about the completeness of denervation of these limbs. The same denervation technique was used for both groups of limbs and it could not be determined after examining nerve-stained sections from both groups of redenervated limbs whether a partial resection of the nerves occurred in one group, while a complete resection of the nerves occurred in another, since the number of fibers that had regenerated back into these redenervated limb stumps was not different. Even if it is typical of redenervated limbs to show such varied amounts of tissue regeneration 35
days post-amputation/denervation, the data from these experiments would
tend to suggest that tissue regeneration can occur in the absence of
nerves. However, nerves affect the amount of tissue regeneration that
occurs.

I believe it is important to conclusively demonstrate whether or
not the phenomenon of tissue regeneration is nerve-dependent. A repeat
of these experiments should involve at least 3 denervations in an attempt
to prohibit any reinnervation of the limb stump from occurring during the
course of the experiment (5 weeks). This problem is a very important one
to resolve for a number of reasons. First of all, strong tissue regenera­
tive responses occur in non-regenerating limbs of higher vertebrates
(i.e., mammals) and it is thought that these rapid healing events are in
themeselves inhibitory to an overall epimorphic response to regeneration
by these amputated limb stumps. In other words, tissue regeneration is
thought to somehow inhibit epimorphic regeneration (reviewed by Carlson,
1974, 1978). Alternatively, it may also be possible that epimorphic
regeneration has an inhibitory influence upon tissue regeneration, since
epimorphic regeneration is the dominant process that occurs in regenerating
limb stumps. Secondly, in the present study, differentiated carti­
lage and or layered cells could be instrumental in preventing denervated
adult newt limbs from regenerating after reinnervation. It has also been
reported by Loyd (1978) that denervated fractured newt limbs do not heal
and repair their fractures through 3 weeks. This would tend to suggest
that tissue regeneration does not occur in denervated fractured limbs.
Thirdly, even though various reports in the literature state that tissue
regeneration, unlike epimorphic regeneration, does not require nerves
and dedifferentiated cells at the limb tip. A new wound epidermis covered the amputation surface. Stump cell dedifferentiation was presumably initiated by the new amputation and as expected, typical epimorphic regeneration followed in 100% of the cases, confirming histological observations that above threshold reinnervation had occurred.

A single razor incision 1 mm into the limb stump, an intermediate degree of injury, stimulated regeneration in 60% of the cases. These limbs regenerated at a rate comparable to their contralateral controls. The razor incision allowed new wound epidermis to form but over a restricted area. In those limbs which regenerated, the wound epidermis area expanded over the entire amputation surface and regeneration proceeded typically as if the limb was amputated. This is in contrast to the regenerates resulting from razor incisions to skin graft limbs (Tassava and Loyd, 1977), which developed in the plane of the incision. These regenerates were restricted to the incision area, and also regenerated at a faster rate than controls. The layered cells and/or cartilage in denervated limbs are not in themselves inhibitory to regeneration, since the razor incision did not physically remove these cell types. Alternatively, the new injury may have modified these cell types, perhaps by stimulating their dedifferentiation. The different responses of denervated limbs and skin graft limbs to a razor incision cannot at present be resolved. Lack of regeneration in 40% of the razor incision limbs could have been due to an insufficient area of wound epidermis and/or rapid closure of the incision.

Carefully removing the healed tissues covering the amputation surface imparted a minimum of injury to underlying tissues but still allowed new
(Singer, 1956, Singer et al., 1957), the experimental evidence supporting such a view is lacking.

Thus far, it has been established that amputated, denervated adult newt limb stumps at 5 weeks have present the full complement of nerves, a population of dedifferentiated cells, various amounts of cartilage and layered cells, and a wound epidermis healed over the limb tip that is still in intimate contact with the underlying stump tissues. Even though this wound epidermis resembled the proximal skin epidermis in thickness and appearance, it was not known if this epidermis was non-functional. What then inhibits these limb stumps from regenerating? Perhaps one or more of the morphological and histological changes described in this study; 1) lack of a functional wound epidermis, 2) differentiated cartilage, or 3) densely packed layered cells, is/are instrumental in preventing these denervated limbs from regenerating after nerves return to the limb tip.

In an attempt to further define the regeneration limiting factor(s) in denervated newt limb stumps, the effects of various kinds of reinjuries were tested at 5 weeks post-amputation/denervation. Quantitating the amount of injury necessary to initiate regeneration provided a means to investigate the 1) competence of the old wound epidermis for supporting regeneration, 2) the effects of a new injury, and 3) effects of new wound epidermis formation on stimulating regeneration. It was also of interest to compare the reinjury responses of denervated limbs to those of skin graft limbs as reported by Tassava and Loyd (1977). The most extensive type of reinjury, a fresh amputation 1 mm into the limb stump, effectively removed the layered cells, differentiated cartilage,
wound epidermis formation. In these cases, regeneration was elicited in 80% of the cases from the R/U level and in 100% of the cases from the humerus level. This operation resulted in the removal of the epidermis covering the stump and removal of most of the layered cells. Regeneration occurred more rapidly, 2 or 3 stages ahead of contralateral controls. As suggested by Tassava and Loyd (1977), a rapid rate of regeneration would be expected if dedifferentiated cells are already present in the limb stump in reasonable numbers and nerves are back in the limb. Removal of the healed tissues re-established a wound epidermis, the final prerequisite for regeneration. The time normally needed for dedifferentiation was eliminated, and rapid blastema formation resulted.

The presence of dedifferentiated cells in the distal limb tip of denervated limbs in this study was confirmed histologically and autoradiographically, a result which agrees with the observations of Tassava and Loyd (1977) that these dedifferentiated cells participate in rapid blastema formation.

Even though removal of the healed tissues from the amputation surface resulted in a large percentage of limbs regenerating, 20% of these reinjured limbs remained inhibited at the R/U level. During the process of tissue removal, perhaps all of the layered cells were not removed from the distal stump. Were this the case, intimate contact (Singer and Salpeter, 1961) between the new wound epidermis and underlying dedifferentiated cells would have been prevented. The new wound epidermis would be unable to function without potentially cycling cells (Tassava and
Peeling back the flap of healed tissues covering the distal limb tip and immediately returning the flap to its original position exposed the amputation surface only briefly and imparted minimal injury to the underlying mesodermal stump tissues. Nevertheless, regeneration was stimulated in 50% of the reinnervated, denervated limbs. These results suggest that the epidermis healed over the limb tip became functional after minimal injury and supported regeneration of these limbs. Injury could, therefore, stimulate epidermis to become functional wound epidermis. The possibility that new wound epidermis formation occurred cannot be ruled out, however, because these limbs were not examined histologically. Additional studies using this system should be carried out.

Injury of mesodermal stump tissues alone (15 needle penetrations) on day 35 never resulted in regeneration and demonstrated that injury alone could not restore the regeneration capabilities to denervated limb stumps. The small areas of wound epidermis formed after needle penetrations were insufficient to support regeneration.

The results of this study are consistent with the proposed role of the nerves in the Tassava-Mescher model (1975) and emphasize again that, in the adult newt, dedifferentiation and entry of stump tissue cells into the cell cycle are not dependent on nerves. A dermis-free wound epidermis forms initially over denervated amputated newt limb stumps (Mescher and Tassava, 1975; Singer and Inoue, 1964), but the absence of nerves prevents subsequent mitotic proliferation that is necessary for blastema formation. By the time nerves fully reinnervate the limb stump 3
weeks later, certain critical healing events have covertly occurred such that the conditions for regeneration no longer exist and, even though a significant number of dedifferentiated cells are still present at 5 weeks, the limb remains "stumped." What then inhibits amputated denervated limbs from regenerating when nerves return? A functional wound epidermis could be one limiting factor since changes in the wound epidermis did occur in these long-term denervated newt limbs and merely removal of healed tissues covering the amputation surface or a single razor incision, both of which allowed new wound epidermis formation, were successful in stimulating regeneration in these inhibited limbs.

In the present study, of 98 limbs reinjured, 75 showed a positive response to reinjury by regenerating. All of the limbs that regenerated had formed a new wound epidermis and had at the same time received at least a minimal amount of injury. In some instances, new wound epidermis formation alone was insufficient to initiate regeneration. Of those 23 limbs not regenerating after reinjury, 16 formed a new wound epidermis but still failed to regenerate. It is likely that the underlying mesodermal stump tissues in these limbs were not injured sufficiently. The importance of injury in initiating regeneration was also shown by the skin flap experiments of Polezhaev (1933) in which full thickness skin flaps were carefully removed from the distal tip of 8-10 week non-regenerating axolotl limbs. Although new wound epidermis formed over the exposed stump tissues, regeneration failed to occur unless the stump tissues were damaged with a needle. Unfortunately, the techniques used to reinjure denervated limbs in this study do not completely separate the wound epidermis effect from that of injury. According to
Carlson (1974), the methods useful in stimulating limb regeneration in limbs not normally regenerating (i.e., mammals and adult frogs) and limbs in which the normal regenerative capacity has been inhibited (i.e., denervated newt limbs) can be grouped in the following categories: 1) manipulations affecting the epidermal-mesodermal relationships; 2) traumatic stimuli to mesodermal tissues; 3) augmentation of the number of nerves in the limb stump; and 4) attempts to depress the formation of connective tissue which interposes between the wound epidermis and the underlying tissues of the stump. The injuries used in the present experiments to induce regeneration of 5-week denervated newt limbs, fall into categories 1 and 2. Injuries employing categories 3 and 4 were not used. Since the full complement of nerves had returned to the limb stump by 5 weeks, inadequate innervation was therefore not considered a regeneration limiting factor. Even though healed tissues (cartilage and layered cells) were present in the distal stump of these denervated limbs at 5 weeks, and represented a potential barrier to epimorphic regeneration, this tissue regeneration was viewed as the end product of an overall healing response by denervated newt limbs rather than a primary inhibitory factor. Most of the reinjury methods described by Carlson (1974), and the ones used in this study, affect more than one limb component, and any one technique is effective only in the presence of the other factors necessary for normal limb regeneration. For example, trauma induced regeneration in frog limbs does not occur if the limbs are denervated (Singer, et al., 1957) and in the present study, successful epimorphic regeneration of denervated limbs occurred only if a functional wound epidermis formed after the limb had been injured.
sufficiently.

Rapid healing and scarring of stump tissues occurs in both frog and mammalian limbs and it has been suggested that this healing response prevents regeneration of these limbs (reviewed by Carlson, 1974). A number of attempts have been made to reduce or depress scar tissue formation in amputated limbs of both frogs (Schotte and Wilber, 1958) and mammals (Scharf, 1961; 1963). None of these attempts however, were successful in stimulating a convincing degree of epimorphic regeneration. Much more work on the role of scarring in non-regenerating limbs is needed. That subtle covert inhibitory healing events also occur in non-regenerating limb stumps of frogs and mammals as occurred in the denervated adult newt limbs in the present study is also likely and open to further investigations.
CHAPTER VII
SUMMARY AND CONCLUSION

Results of the present study indicate that amputated/denervated limbs of the adult newt never regenerate, regardless of the level of amputation, even after threshold innervation returns to the limb stump some 3 to 5 weeks later. Denervated newt limbs very closely resemble innervated, amputated control limbs both morphologically and histologically during the first 2 weeks post-amputation/denervation. In the absence of nerves, denervated limbs form a thickened wound epidermis and differentiated cells of the limb dedifferentiate, enter the cell cycle and replicate DNA but do not undergo the sustained mitotic activity necessary for blastema formation. Therefore, the denervated limbs remain stumped. These findings are consistent with the proposed role of the nerve in the Tassava-Mescher cell cycle model (1975).

Despite the early resemblance of denervated limbs to innervated limbs, inhibitory healing events occur covertly as early as 2 weeks in the denervated limb stump which block these limbs from regenerating even after nerves fully reinnervate the limb 5 weeks later. These early healing events later manifest themselves overtly, after 4 to 5 weeks post-amputation/denervation as newly differentiated cartilage which caps the cut edges of the bones and as layered cells beneath the epidermis. This tissue regeneration in denervated newt limb stumps is viewed as the result of the denervated limb being denied an opportunity to regenerate.
epimorphically, rather than as the cause of the limb not regenerating after reinnervation, since a few denervated limbs without cartilage or layered cells also did not regenerate. Cells that initially dedifferentiate during the first week in these denervated newt limbs, did not all leave the cell cycle immediately to redifferentiate into stump tissues as they do in other non-regenerating vertebrate systems. Some of these cells remained present and were histologically visible as blastema-like cells 5 weeks later. A small percentage (3%) of these blastema-like cells incorporated H\(^3\)-thymidine on day 35 post-amputation, denoting that some cell cycling activity was still occurring in these limbs even though they were stumped and not regenerating.

It was of special interest to also find that various kinds and degrees of injury could successfully restore the regeneration capacity to 5-week denervated newt limb stumps. Successful reinjuries and the regeneration response they elicited include the following: 1) Reamputation of a 1 mm portion of the distal stump, 100%; 2) removal of healed tissues covering the distal limb tip, 80%; 3) a transverse razor incision, 1 mm into the stump, 60%; and 4) peeling back the epidermis and healed tissues covering the distal limb tip and returning it to its original position, 50%. These results demonstrate that only after mesodermal stump tissues are injured sufficiently and a functional wound epidermis forms over the distal limb stump, do amputated/denervated, reinnervated newt limb stumps undergo epimorphic regeneration. The importance of nerves, injury, and the wound epidermis in initiating regeneration of newt limbs is emphasized.
In conclusion, amputated/denervated adult newt limb stumps do not regenerate because stump cells that initially dedifferentiate divide too infrequently to form a blastema. In the prolonged absence of nerves, these dedifferentiated cells are programmed to undergo stump tissue healing. This programming takes place in denervated adult newt limb stumps between the first and second week post-amputation/denervation, before ingrowing nerve fibers reach the distal limb stump. Even though adequate innervation eventually returns, these healed stump tissues are unable to respond, that is, epimorphic regeneration fails to occur.
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