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The Ohio State University,
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INFLUENCE OF INJURY, NERVES AND WOUND EPIDERMIS ON CELL CYCLE EVENTS IN AMPHIBIAN LIMB REGENERATION

DISSERTATION

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

By

Robert Merrill Loyd, B.S., M.S.

******

The Ohio State University
1978

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FIELDS OF STUDY

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Studies in Regeneration, Professor Roy A. Tassava


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CHAPTER I
INTRODUCTION

The phenomenon of regeneration has fascinated man since early times. Broadly defined, it may be interpreted as the complete restoration of a lost or missing part. Aristotle spoke of the subject over two thousand years ago. Not until around the middle of the 18th century, however, did it first begin to attract general attention. Trembley (1744) first described experiments with a fresh water hydra that was found to produce two polyps if cut into two pieces. After Trembley's work became generally known, others began describing experiments of their own on regeneration in other life forms (Reamur, 1742; Bonnett, 1745). The most significant among these, for modern workers, is Spallanzani (1768) who, for the first time discovered that a number of higher animals, especially the vertebrates were capable of regeneration. He observed that a tadpole will regenerate its tail and that a salamander will regenerate its tail, legs, and even its upper and lower jaw. These experiments led others to more precise experiments with animals that regenerate and, importantly, made the study of regeneration more widely known and of
general interest.

These early studies laid the ground work upon which later investigators built more rigorous and far reaching studies of regeneration. At the turn of the twentieth century, Morgan (1901) published a wide ranging book on regeneration throughout the animal kingdom. He gave us a more precise definition of the kinds of regeneration found in the various phyla. Of these distinctions, two terms stand out as being important. Morgan (1901) observed that some types of regeneration involved simply a hypertrophy of existing tissues following injury. This type of regeneration he called morphollaxis. A second type of regeneration involved proliferation at the cut surface following removal of the old part and preceding development of the new part. This type of regeneration he called epimorphosis. This latter kind of regeneration will be the main focus of this study.

True epimorphic regeneration is found in several species of larval anurans, which lose this ability as they metamorphose, but is most vividly exemplified in larval and adult urodèles. These organisms have been the subjects of intensive study since the time of Morgan. Several comprehensive texts have been written in an attempt to explain this process of regrowth (Hay, 1966; Thornton, 1968; Schmidt, 1968; and Goss, 1969).
Of those urodele amphibians that show regeneration, the study of two groups predominates. Included in these groups are the larvae of certain species of *Ambystoma*, and the adult newt *Notophthalmus (=Triturus) viridescens*. The various stages of regeneration in these species have been carefully studied and are basically similar in both larval and adult forms, but vary with age. This study will be concerned only with the adult newt and the process of regeneration will be discussed fully in the text.
CHAPTER II
LITERATURE REVIEW

Events of Normal Limb Regeneration.

Although the process of limb regeneration is known to occur to some extent in a variety of anuran and urodele amphibians (Scadding, 1977), it has been studied most frequently in the adult newt and in larval Ambystoma. The present study deals exclusively with limb regeneration as it is seen in the adult newt, Notophthalmus viridescens. The process of limb regeneration in adult newts is initiated immediately upon amputation of the extremity, which sets in motion a series of complex and interrelated events that culminate in the formation of a new limb that is a morphological and functional replica of the one removed (Thornton, 1968). Hemastasis is achieved within minutes after amputation, the clotted blood serving to seal off the wound from the environment (Schmidt, 1968). Next the cells of the skin at the edge of the wound release their desmosomal contacts and migrate over the injured mesodermal tissues (Schmidt, 1968). The free edges of this migrating epithelium meet within 12 to 24 hours to form a thin covering of epidermal cells over the amputation surface.
(Schmidt, 1968; Singer and Salpeter, 1961). This wound epidermis, which lacks glandular tissue and the pigmentation of normal skin epidermis, is of considerable importance to limb regeneration and will be discussed in more detail below. Initially only 1-2 cells thick, the wound epidermis thickens during the ensuing days to reach a maximum of 10-15 cells thick by two weeks in the adult newt (Singer and Salpeter, 1961). The wound epidermis is not underlain by a basement membrane or a thick fibrous dermis (Schmidt, 1968; Singer and Salpeter, 1961). Thus there is direct contact between the wound epidermis and the mesodermal tissues of the stump.

Following formation of the wound epidermis, a period of "demolition" begins (Thornton, 1968). The distal end of the amputated limb becomes edematous and many phagocytes move into the area between the wound epidermis and the stump tissues. These phagocytes are apparently involved in the removal of tissue debris, especially dead and degenerating cells, resulting from the amputation (Thornton, 1968). By 2 days post-amputation, histolysis of muscle and bone begins in the area of the cut. Muscle loses its striations and some pycnotic nuclei can be seen in the various tissues near the wound. These bits of tissue debris are phagocytized and removed. The histolytic events were first described as seen with the light microscope (Thornton, 1938).
and later with the electron microscope (Hay, 1958). The act of amputation naturally interrupts the normal flow of blood. Thus, the stump tissues become somewhat hypoxic. This lack of oxygen forces a shift to anaerobic metabolism, the result of which is the formation of large quantities of lactic acid (Schmidt, 1960). Proteolytic enzymes become much more active in the regenerating limb than in the unamputated normal limb and histolysis of tissues occurs (Orechowitch and Bromley, 1934; Schmidt, 1966, 1968). An enzyme involved in the breakdown of phagocytized material, acid phosphotase, becomes especially active during this period, the first week after amputation (Schmidt, 1968; Weiss and Rosenbaum, 1967).

As the breakdown of tissue continues, large mononucleate cells begin to appear near the distal tip of the regenerating limb. The origin of these cells has been the subject of much speculation and investigation over the past years (Butler, 1935; Chalkley, 1954; Hay, 1968; Hay and Fischman, 1961; Thornton, 1968), but it is now shown conclusively that they originate from the mesodermal tissues of the amputated stump that include bone, muscle, nerve sheath, and connective tissue (Butler, 1935, Chalkley, 1954; Steen, 1968). These cells are embryonic or "blast-like" in appearance, lacking any specific tissue morphology and thus are referred to as blastema cells. An increasing population of these cells eventually forms the blastema or
young regenerate (Thornton, 1968). While these cells dedifferentiate morphologically and are thus not recognizable as to any specific cell type (Hay, 1968; Norman Schmidt, 1967), experiments have shown that upon dedifferentiation, they return to their original cell type (Steen, 1968). In experiments with the axolotl, Steen (1968) transplanted labeled cells into limbs that were then allowed to regenerate. These cells were then identified in the new regenerate according to the label, either $^{3}$H-thymidine, triploidy or both. Cartilage cells redifferentiated into cartilage or connective tissue and dedifferentiated muscle cells appeared to redifferentiate into muscle cells. Since muscle is a complex tissue type, however, the answer to this question is not unequivocal. Hence, metaplasia is not seen to occur in amphibian limb regeneration (Steen, 1968, Namenwirth, 1974; Eggert, 1966).

Mesodermal tissue cells of unamputated limbs are in the $G_0$ state of the cell cycle and, although low levels of RNA and protein synthesis are seen, no DNA synthesis occurs. However, the large mononucleate cells which appear in amputated limbs are seen to be active and viable and, beginning on day four after amputation, they initiate DNA replication as evidenced by autoradiographic studies (Hay and Fischman, 1961) and also, within 2 days, incorporate precursors of RNA (Bodemer, 1962; Kelly and Tassava, 1973).
and protein (Bodemer and Everett, 1959). Beginning on day six following amputation, these dedifferentiated cells begin to undergo division (Chalkley, 1954) as seen by an increase in the mitotic index. The levels of both DNA synthesis and mitosis increase during the next several weeks of regeneration. The cells continue to proliferate and accumulate between the remaining tissues of the stump and the overlying wound epidermis and this results in the appearance of the regeneration blastema.

The early appearance of this outgrowth or blastema, at 2 weeks post amputation, marks the end of the regression phase and the beginning of the progressive phase of regeneration (Thornton, 1968). The proliferating cells continue to accumulate to form a mound and then an elongated cone, extending out from the plane of amputation (Chalkley, 1954). Soon after, this cone begins to flatten distally to form the paddle or palette stage. At this time, chondrogenesis of the long bones is initiated and then those of the wrist and hand in a proximal to distal gradient. Nerves and blood vessels grow into the developing blastema, and new motor end plates are established between muscle and nerves (Lentz, 1969). The new structure becomes continuous with the stump tissues (Stocum, 1975). The wound epidermis gradually disappears as it becomes normal skin, complete with skin glands and dermis (Singer and Salpeter, 1961). In the remaining time, the
regenerate develops the morphogenic appearance of the normal limb complete with digits and becomes functionally and structurally complete, often an exact replica of the amputated limb (Thornton, 1968; Goss, 1969).

The Role of Nerves in Limb Regeneration.

Todd showed as early as 1823 that nerves are essential for salamander limb regeneration to occur. Since that time many workers have amplified and extended these findings. The most comprehensive studies of how nerves influence regeneration were done by Singer in the 1940's, (see Singer, 1952 for review). The forelimbs of the adult newt are normally innervated by the 3rd, 4th and 5th spinal nerves that have both sensory and motor components. Transection of these three nerves results in a failure of the amputated limb to regenerate. Singer concluded that the ability of the urodele limb to regenerate depends upon a critical "threshold" number of nerves at the amputation surface. If this threshold number of nerves is removed, such as by a denervation, a removal of the nerves, then regeneration fails (Singer, 1952). A study by Singer, et al (1967) showed that limbs of *Xenopus laevis* will regenerate even though these limbs have a subthreshold number of nerve fibers. The axonal diameters of the nerves in these animals is larger, however. Thus the threshold concept was
modified slightly to indicate that a minimum cross section­
al area of nerve fibers must be present (Singer, 1968), but
the basic concept is the same. Either sensory or motor
nerves can support regeneration, and the polarity of the
neuron does not influence the nerve effect.

The precise mechanism by which nerves influence re­
generation is still unknown. The major stumbling block in
analysing the effect of the nerve has been the lack of a
suitable bioassay that clearly demonstrates a nerve effect.
However, the most compelling evidence to date indicates
that the nature of the nerve influence is one of some
trophic material which eminates from the cut ends of nerve
fibers following amputation (Singer, 1952; Singer, Maier
and McNutt, 1974). Many attempts have been made to eluci­
date the nature of the trophic factor present in the nerve
which could substitute for the nerve in a denervated limb.
One of the most logical compounds would be a neurotrans­
mmitter such as acetylcholine. The results of injecting
such neurotransmitters into a denervated newt, however,
have no effect on restoring growth (Singer, et al 1960).
Homogenate infusion of whole blastema (Deck and Futch,
1969) or of nerves, brain, or liver (Singer, 1974) also
fail to promote regeneration when infused into a de­
nervated limb.
The failure to restore regenerative ability to a denervated limb by the infusion of extract and homogenates coupled with intense curiosity about the role of nerves in regeneration, led investigators to a more biochemical approach to the problem. Immediately following denervation of a young blastema, decreases in incorporation of precursors to RNA, DNA and protein occur (Lebowitz and Singer, 1970; Singer and Caston, 1972; Dresden, 1969). Thus, a possible bioassay may be the restoration of the level of the incorporation of one or all of these precursors. Singer has shown that some degree of restoration of $^{14}$C-leucine incorporation into protein is seen following infusion of nerve homogenates, synaptosomal concentrates and soluble portions of brain extracts into denervated blastemas (Lebowitz and Singer, 1970; Singer, 1974). The activity of this infused material is found to be heat labile and is also destroyed by trypsin and pronase, indicating that the active substance is a protein (Singer, 1974). More recently Singer and his co-workers have produced a partially purified protein from frog brain that stimulates DNA synthesis in denervated forelimbs of adult newts (Jabaily and Singer, 1977).
The Role of The Wound Epidermis.

As mentioned earlier, a wound epidermis migrates over the cut surface of the amputated limb during the first day following amputation in the adult newt. The absolute necessity of this wound epidermis for regeneration of the urodele limb has long been known (Thornton, 1968), but no precise and definitive role has yet been elucidated. Considerable evidence suggests that contact between a dermis-free epidermis and mesodermal tissues of the stump is necessary for blastemal formation. Early investigators blocked regeneration by suturing full thickness skin over the amputation surface (Tornier, 1906; Godlewski, 1928). Thornton (1954) repeatedly removed the wound epidermis and found that regeneration was inhibited until a new wound epidermis formed. When he shifted the bulk of the wound epidermis to an eccentric location in the limb, regeneration occurred in the direction of the newly positioned wound epidermis (see Thornton, 1968 for review). Mescher (1976) blocked regeneration by placing flaps of whole skin over the amputation surface. In many such cases, the skin flap was incomplete and upon histological examination, more cells were seen in the areas of free wound epidermis that formed at the edge of the flap than in areas where no wound epidermis existed. These cells also demonstrated a greater degree of DNA synthesis as seen by $^3$H-thymidine.
incorporation, and a higher mitotic index than those cells under full thickness skin on the opposite side of the limb.

Goss (1956) inhibited the wound epidermis from forming by inserting amputated forelimbs of adult newts into the coelomic cavity. Precedent for such experiments was established by earlier investigators such as Dent (1954) and Butler (1955) who observed lack of regeneration in limbs that were inserted into tail musculature and did not form a wound epidermis. The forelimbs that Goss inserted into the body cavity failed to show regeneration for as long as they remained inserted. The intracoelomic fluid apparently precludes the formation of a normal wound epidermis, and these limbs subsequently fail to regenerate. Goss further demonstrated in these experiments that if the inserted limbs were exteriorized as long as 7 weeks following insertion, a normal wound epidermis formed over the amputation surface and regeneration proceeded normally. In a second paper Goss (1956b) showed that if, following amputation, insertion was delayed sufficiently long (24 hrs or more) for a wound epidermis to form, these limbs did in fact regenerate inside the body cavity.

These several experiments serve to verify the essential presence of the wound epidermis for regeneration to occur but do not define the nature of the influence. Some workers, especially Rose (1948) and Rose, Quastler, and
Rose (1955), initially thought that the wound epidermis served to provide cells to the blastema and hence gave rise to the regenerate. Following detailed studies of the origin of blastema cells (Chalkley, 1954, Hay and Fischman, 1961; Riddeford, 1960), this view is no longer tenable.

Phagocytic and histolytic activity has also been ascribed to the wound epidermis (Singer and Salpeter, 1961). Taban (1955) observed that a blood clot in the wound epidermis of a salamander was dissolved, and he also observed debris material within the epidermal cells. Thornton (1968) also described "tongues" of wound epidermis engulfing scattered debris from deep within the stump. Little (pers. comm.) has recently observed phagocytes within the wound epidermis, especially that of denervated blastemas.

The Role of Injury.

Both the importance of injury and the role it plays in amphibian limb regeneration are less well defined than for nerves and the wound epidermis. However, there is ample evidence that some type of injury is necessary to initiate the process of regeneration (Thornton, 1968). The effects of injury become most obvious immediately after amputation. There is rapid blood clotting and the tissues become inflamed. The stump becomes edematous and phagocytes move into the area to clean up debris (Schmidt, 1968). Of more
consequence, however, is the effect the injury has on the cells of the remaining stump that will give rise to the new regenerate. That the role of injury is more than a passive one in this regard is suggested by a number of experiments. Experiments of both Orechowitsch and Bromley (1934) and Polezaiev and Faworina (1935) suggested that injury was important. These workers noted that regeneration does not occur when grafts of whole skin are placed over the amputation surface and allowed to heal. If the graft is then carefully removed without inducing new injury regeneration fails to occur. If, however, the stump is reinjured when the skin is peeled off, regeneration occurs normally (Polezaiev and Faworina, 1935).

Goss (1956a) amputated limbs of adult newts and inserted the cut end of the remaining stump into the coelomic cavity. These limbs failed to form a wound epidermis and did not regenerate for as long as they remained inserted. Histological observation of these limbs, however, showed that the cells of the remaining stump did undergo dedifferentiation, allowing that the wound epidermis is not essential to this initial stage of regeneration.

Butler and Schotte (1941) showed that amputated de-nervated limbs of larval *Ambystoma* not only dedifferentiate but are eventually resorbed, a process known as regression. Thornton and Kraemer (1951) made an important observation
with regard to injury (fracture of skeleton) in unamputated larval Ambystoma limbs. They found that if injured limbs of Ambystoma were denervated, and remained so for extended periods, the entire limbs regressed. However, when these injured denervated limbs were permitted to become rein­
nervated, the process of regression stopped and, in fact, these limbs regenerated if the process of regression had progressed to the point of resorption of the digits (Thornton and Kraemer, 1951). Thornton (1953) did a histological study of these injured, denervated, unamputated limbs and found many dedifferentiated cells both distally and proximally to the fracture soon after the injury. Injured, innervated limbs healed within 12 days of the fracture, but in denervated limbs a "secondary dedif­
ferentiation" occurred spreading out in both directions from the injury. According to Thornton (1953) this "un­
checked dedifferentiation" resulted in the regression of the limb for as long as it remained denervated.

In addition to these experiments, other studies have shown the importance of injury in limb regeneration. It has been demonstrated that insertion of small pieces of tissue, especially kidney and lung, under the skin of a newt will elicit formation of a new limb referred to as a supernumerary limb (Carlson, 1967; Bodemer, 1959). Histological analysis of these implants show that wound healing
and phagocytosis occur the first few days after implanta-
tion. Dedifferentiated cells of the host appear shortly
(Carlson, 1974). Thus, these initial events in super-
umarery limb formation are similar to those seen in normal
regeneration and suggest the importance of injury to this
process. It appears from these experiments, that injury
alone is sufficient to initiate dedifferentiation in the
presence or absence of an amputation, the presence or
absence of a wound epidermis, and the presence or absence of
nerves. It is these dedifferentiated cells that are
critical to the formation of a blastema and the regeneration
of the limb.

Regeneration and the Cell Cycle.

Extensive reports have shown the necessity of nerves,
the wound epidermis, and an injury for regeneration to
occur. Only recently, however, have investigators linked
the roles of these elements to the cell cycle. The
cells of the uninjured, unamputated limb of the adult newt
are essentially all in the differentiated or $G_0$ state
(Mescher and Tassava, 1975; Hay and Fischman, 1961). Fol-
lowing amputation, the cells of the stump must leave this
differentiated state and enter the cell cycle at $G_1$,
replicate DNA in $S$, proceed through $G_2$ and divide ($M$ phase)
in order to form a population of cells, the blastema, that
will form the new regenerate. Kelly and Tassava (1973), using $^3$H-uridine autoradiography, showed that RNA synthesis occurred at near normal levels in denervated, amputated limbs of *Ambystoma mexicanum* larvae. These same studies, however, showed that mitotic indices remained near zero in denervated limbs, but normal levels were reached in the contralateral innervated control limb. Kelly and Tassava (1973) concluded from these studies that a denervated limb did not undergo the early events of regeneration as did the control innervated limb. Even though RNA synthesis increases in both limbs on the third and fourth day after amputation, Kelly and Tassava (1973) suggested that the RNA synthesis in the denervated limb was involved in a different developmental pathway than that in the regenerating control limb. Additional studies with larvae by Tassava, *et al* (1974) showed that DNA synthesis is initiated in denervated limbs at the same time as in innervated limbs, but again there is no mitosis in the denervated limb. Mescher and Tassava (1975) reported that incorporation of $^3$H-thymidine into DNA begins on day 4 following amputation in both a denervated-amputated and an innervated-amputated forelimb of the adult newt. Cell division begins about 48 hours later in the innervated limb, but very few, if any, mitotic figures are seen in the denervated limb. These results suggest that nerves are not necessary for cells of an amputated limb to dedifferentiate and enter the
cell cycle nor to replicate DNA in S. There is additional evidence which supports this view. Microspectrophotometric data indicate that there is no $G_2$ population of cells in an unamputated limb. Thus, cells that dedifferentiate must presumably enter the cell cycle at $G_1$ and replicate DNA before they divide. Additionally, Manson (1974) has shown that activities of enzymes involved in the production of DNA precursors rise in both innervated and denervated limbs after amputation.

That the wound epidermis is not involved in the initial dedifferentiation of cells is suggested by several reports, some of which have been described previously. Goss (1956a) reported dedifferentiation in amputated coelom inserted limbs that lack a wound epidermis. Polezaiev and Faworina (1935) noted the importance of injury to dedifferentiation in limbs with skin flaps. Mescher (1976) placed flaps of whole skin over the amputation surface of adult newt forelimbs and found that after the first week dedifferentiation, labeling indices and mitotic indices were similar in both skin flap limbs and normal regenerating limbs.

These results prompted Tassava and Mescher (1975) to propose a model for regeneration in terms of the cell cycle. According to this model, the injury of amputation causes cells to dedifferentiate and enter the cell cycle at $G_1$. 
The cells then replicate DNA during S and proceed through G2 and divide (M). In the absence of nerves, however, the cells become blocked and are unable to traverse G2. Thus nerves appear to be necessary for some G2 event. Foret (1977) has shown in a recent abstract that nerves stimulate mitosis in cultured blastemas, a finding that agrees with the role of nerves suggested by Tassava and Mescher (1975). In this model, the wound epidermis is assigned the function of keeping the cells in the cell cycle so that further rounds of DNA replication and mitosis occur. In a limb without a wound epidermis (i.e. with a skin graft) cells presumably fail to continue cycling. This model is consistent with the preponderance of literature and provides an hypothesis on which to base further experiments regarding each of the necessary factors in regeneration.
CHAPTER III

FOCUS OF THE DISSERTATION

The proposed Tassava-Mescher cell cycle model (1975) provides a convenient hypothesis on which to base experimental analysis of the precise roles of injury, nerves, and the wound epidermis in amphibian limb regeneration. Although the model itself is consistent with the preponderance of literature, several unanswered questions remain and, as with any good hypothesis, new questions can be raised. This study was designed, with the cell cycle model in mind, to look more closely at the early events of limb regeneration and their relationship to nerves, wound epidermis and injury.

The absolute necessity of a wound epidermis for regeneration has been discussed previously. Earlier attempts to define a precise role for this important structure (see Singer and Salpeter, 1961) have not been successful, though several investigators attempted to elucidate the function of the wound epidermis by preventing its formation and then observing what occurred. These experiments, described earlier, have involved suturing full thickness skin over the amputation surface (Tornier, 1906;
Godlewski, 1928), removing the wound epidermis after it forms (Thornton, 1957) and placing flaps of whole skin over the amputation surface (Mescher, 1976). These experiments have provided much useful data, but have failed to satisfactorily describe the function of the wound epidermis.

The experiments by Goss (1956a, b) demonstrated that amputated limbs inserted into the coelomic cavity fail to form a wound epidermis and fail to regenerate. These reports, however, were essentially morphological and histological studies that did not consider cellular events. Mescher (1976) was the first to report a detailed study of cellular events in limbs that had skin flaps. These limbs lacked a wound epidermis and instead had full thickness skin, including dermis, over the amputation surface. Mescher (1976) reported labeling and mitotic indices at one and two weeks after amputation in these limbs. He found them to be similar at one week but by two weeks both the labeling and mitotic indices were lower in limbs that had skin flaps than in normally regenerating limbs. Many of these limbs formed wound epidermis under the skin flap and regenerated. In limbs that did not regenerate because of skin flaps, dermis separated the mesodermal tissues from the epidermis, and this potentially provides a complicating factor in interpreting Mescher's (1976) results, for the dermis could be actively inhibiting regeneration. The inserted limb experiment of Goss provides an additional
means of observing cell cycle events in amputated limbs that completely lack a wound epidermis and also lack full thickness dermis. Experiments were specifically designed to repeat Goss' findings (1956a, b) but then to extend his work by observing cell cycle events in these nonregenerating inserted limbs. The specific questions that were asked were: (1) to what extent, if any, do mesodermal stump cells enter the cell cycle in these nonregenerating limbs? (2) What specific cell cycle events are affected by the wound epidermis? (3) If the cells in these inserted limbs begin cycling in the absence of the wound epidermis, at what point do they stop and what is their fate? (4) How do cell cycle events in inserted limbs compare with those in limbs that have skin flaps? It was hoped that more definitive answers to the role of the wound epidermis could be found using a different system. To further ascertain the role of injury in regeneration, an additional experiment was designed to eliminate both nerves and the wound epidermis.

Another focal point in these studies is the role injury plays in the regenerative process. The work discussed earlier, as well as the Tassava-Mescher model, suggests that injury is important in initiating the process of regeneration. Thronton's (1953) injury experiments with unamputated larvae showed that injury alone could induce widespread dedifferentiation and regression in the
absence of nerves. Thus, specific experiments were designed to observe the effect of injury alone in newts. The specific questions asked were: (1) What affect does injury alone, without amputation, have on cellular events? and (2) What cell cycle events occur in an injured, denervated newt limb?

Another major focus of this dissertation furthered the analysis of the skin-flap studies initiated by Mescher (1976). His initial studies showed only what occurred in limbs with skin flaps at two points, one and two weeks after the operation. The results failed to indicate the possible fate of cells in limbs that did not regenerate. Experiments were thus designed to determine what events occur in limbs that have skin flaps for extended periods of time and to ascertain the kind and extent of injury necessary to induce regeneration in limbs which have been quiescent for extended periods of time because of skin flaps placed over the amputation surface.
CHAPTER IV
MATERIALS AND METHODS

Adult newts (Notophthalmus viridescens) collected in southern Ohio were used exclusively for these experiments. Newts were maintained throughout the course of the experiment in aerated tap water in plastic crispers at 21°C ± 1.5°C. Following all operations, animals were placed in a solution of aqua-aid (Methylene Blue and acriflavin), a fungicide, for 24 hours, then returned to aerated tap water. Prior to all operations, newts were anesthetized in MS-222 (M-ethyl amino benzoate methane sulfonate 1:1000, Eastman).

Inserted Limbs.

Following anesthetization, each animal was placed on moist gauze under a dissecting microscope. The left arm was amputated with scissors through the mid-forearm. A small ring of skin approximately 1 mm in width was then removed from the upper arm to facilitate healing of the arm skin to the flank after insertion. A small incision was then made in the flank skin with fine scissors at a level corresponding to an area just proximal to the elbow.
of the amputated left limb. The hole was carried through to open the coelomic cavity to expose the liver. The cut surface of the amputated stump was then inserted into the coelom so that the arm distal to the elbow was within the coelomic cavity. The limb was held in place by means of three sutures of 4-0 silk placed between the proximal edge of the cut ring of skin in the upper arm and the flank skin. Care was taken in placing the sutures so as not to constrict major blood vessels or severely damage internal limb tissues. Sutures generally began to deteriorate and fall off in about one week, at which time the skin of the arm had usually healed to the skin of the flank, securing the limb in position. If the skin of the arm and that of the flank had not grown together, additional sutures were occasionally necessary.

The right limb served as a control. It was amputated at the same time and in the same manner as the experimental left limb and was allowed to regenerate normally.

Additional controls involved delayed insertion. Left and right limbs of animals were amputated as before, but differing periods of time allowed to elapse before the left limb was inserted into the body cavity. This delay allowed for migration of the wound epidermis over the distal amputation surface prior to insertion.

In another series of experiments, a denervation was performed prior to inserting the limb into the coelomic
cavity. One day before the insertion procedure, the 3rd, 4th, and 5th brachial nerves were transected at the level of the scapula of the left limb. The right limb served as a control and was amputated but not denervated. The left limb was inserted the following day as described above.

In all the experiments mentioned above, three animals were selected at appropriate days and given IP injections of 10 μCi (methyl-^3^H) thymidine (ICU Pharmaceuticals: Specific activity 60 Ci/mM) in 0.1 ml of sterile water three hours prior to fixation. The animals in the first experimental series were killed by decapitation on days 0, 3, 6, 9, 11, 14, 18, 21 and 24 and the entire animal was fixed for 24 hours in Carnoy's fixative (3:1 absolute alcohol: glacial acetic acid). In the second experimental series animals were fixed in a similar manner on days 7, 14, and 21. In the control experiments, animals were injected with ^3^H-thymidine on the 28th day after the insertion procedure and fixed after a three hour incorporation period. The whole body fixation procedure prevented disturbances to the inserted limb which may have adhered to internal tissues in the coelomic cavity. The inserted limbs were not removed until they had been fixed. Limbs were then dissected free and placed in 70% ethanol and prepared for paraffin histology.
Injured Limbs.

To further studies on the role of injury in regeneration, experimental animals were divided into two groups. Newts in the first group were anesthetized and placed on moist gauze. Both the left and right forearms were then fractured midway between the elbow and the wrist by squeezing and bending the arm with watchmaker's forceps. Care was taken not to break the skin and create an area of wound epidermis. Then, in order to control for the fact that the skin may have been slightly damaged, a ring of skin was deliberately torn around the fracture area on the right limb. Three animals were then selected, injected with $^3$H-thymidine as above and fixed 3 hours later on each of days 0, 3, 6, 9, 12, 14, 18 and 21.

In the second series of injury experiments, both left and right limbs were carefully fractured as in the first group so as to not damage the skin. Twenty-four hours later the left limb was denervated by transecting the 3rd, 4th and 5th brachial nerves. The skin on both limbs was left in tact in this group of newts. As each animal recovered from the anesthetic, it was observed to be sure no movement occurred in the denervated limb. As before, three animals were selected on days 0, 3, 6, 9, 12, 14, 18 and 21, injected with 10 μCi $^3$H-thymidine and fixed three hours later. After 24 hours in Carnoy's fixative, all limbs were
placed in 70% ethanol in preparation for paraffin histology.

**Skin Flap Limbs.**

For further study of the role of the wound epidermis and the effects of injury in regeneration, flaps of whole skin were placed over the amputation surface of newt forearms in a slightly modified technique of that described by Mescher (1976). Newts were anesthetized and placed on moist gauze. The left limbs were amputated at the wrist. An incision was then carried through the skin on the medial side of the limb from the wrist to the elbow with fine scissors. With watchmaker's forceps, the skin was then gently freed from underlying mesodermal tissues, and peeled back. The exposed stump was then amputated approximately 1 mm distal to the elbow. The remaining flap of skin was then folded over the amputation surface and secured in place by means of a single 4-0 silk suture taken between the end of the flap and the skin overlying the humerus. The right limb was left intact. Animals were then maintained in aerated tap water for a five week period after which any animal that had regenerated its limb was discarded. Only those animals with complete skin flaps and no regeneration were chosen to participate in the experiment. In these newts, the left limb only contained a skin flap. This limb was now reinjured by cutting a
single transverse incision across the limb stump with a sterile razor blade to a depth of 1 mm into the stump tissue. An attempt was made to make these incisions parallel with the plane of the skin flap but they occasionally varied by as much as 20°. The right limb was then amputated at the same level as the skin flap on the experimental left limb to serve as a control. Observations were then made at weekly intervals to see if the limbs were regenerating and if so, at what rate, and how the right and left limbs were similar or different. A variation of this procedure involved putting skin flaps on left and right limbs. At the end of the five week period, one group of animals containing skin flaps on both limbs was selected to observe what cell cycle events could still be detected, by 3H-thymidine incorporation, in the non-regenerating limbs. Three animals were given IP injections of 10 μCi 3H-thymidine and fixed in Carnoy's three hours later. These limbs were then placed in 70% ethanol and prepared for paraffin histology.

**Histological Procedures and Experimental Techniques.**

Following fixation, all limbs were washed in 70% ethanol. They were then decalcified for 24 hours in a 0.5M solution of EDTA (tetra sodium ethylene-diamine-tetra-acetic acid), washed, and prepared for routine histological
procedures and paraffin embedding. Experimental left limbs and control right limbs were always processed together to allow a direct comparison. Limbs were then sectioned longitudinally at 10 microns with a rotary microtome and sections were distributed on each of 4-5 slides to give a representative part of the limb on each slide. These slides were then subjected to various procedures for determination of labeling index, mitotic index, or absence of nerves in the denervation study.

Two slides from each limb were processed for autoradiography (Kelly and Tassava, 1973). After drying, slides were deparaffinized and hydrated and allowed to dry. In the dark, they were then dipped in a 3:1 dilution of Kodak NTB-2 Nuclear Track Emulsion and hung to dry and cool. When dry and cool, the slides were then packed in light tight plastic boxes containing dessicant and stored at 4°C for two weeks. Following this exposure period, they were developed in Kodak D-19 developer for 3 minutes and fixed in Kodak acid fixing bath for 5 minutes. They were then rinsed thoroughly in running tap water. The slides were then stained for two minutes with Harris Hematoxylin containing 2% acetic acid and counterstained with Eosin. Finally, they were dehydrated, cleared and mounted in preparation for sampling of labeling indices.

To determine mitotic indices, at least one slide from each limb was prepared for the Feulgen stain. The
procedure used represented a slight modification of the technique described by Humason (1962). Slides were deparaffinized and hydrated. They were then hydrolized in 5N HCl at 24°C for one hour, placed in Schiff's reagent (see Humason, 1962) for 2 hours, and rinsed thoroughly in running tap water. The slides were counterstained for 10 sec with 0.5% solution of Fast green in 95% ethanol, dehydrated, cleared and mounted.

For some studies, slides were processed for Samuel's nerve stain (1953) to verify histologically that the limbs were denervated.

Labeling and Mitotic Index Determinations.

The same procedure was used to determine both labeling and mitotic indices in the inserted limb experiments. Three areas were selected in each limb. Two of these areas were as close as possible to the distal end of the limb, one was more proximal. This procedure facilitated examination of events at the distal-most end of the limb and those further removed from the amputation surface in limbs that were not regenerating and in normally regenerating controls. In each of the areas, the grid in the counting reticle of the microscope was placed in four contiguous locations and determinations were made. All counts were done at 430x with a binocular microscope. Twelve grid
areas (3 areas, 0.17 mm$^2$ each; 4 grids/area) were counted in each section. Four sections each separated by at least 30 microns, were counted for each limb for a total of 48 grid areas per limb. Any nucleus with 5 or more silver grains was considered labeled. Labeling indices were expressed as a percent of labeled nuclei per total nuclei and mitotic indices as a percent mitotic figures in the total nuclei.

To make determinations in the injury experiments, three areas again were selected. Two of these were in the immediate vicinity of the fracture. Where possible, one on the medial side and one on the lateral side of the injury. In each of these areas, 4 grids were counted as above for a total of 8 grid areas in the vicinity of the fracture. The third area was divided into two sections as far from the break as possible. Two grid areas were counted on the distal most end of the limb and two on the proximal most end. Thus, again, 12 grid areas were counted per section. As before, four sections at least 30 microns apart were counted for each limb. Labeling indices were again expressed as percent of labeled nuclei per total nuclei and mitotic indices as percent of mitotic figures per total nuclei.
Generalized Experimental Design.

Insert Limbs

Experimental left limbs  Control right limbs

I. How does lack of wound epidermis influence cell cycle events? What happens to cells in limbs that do not regenerate?

Insert limbs - no wound epidermis
Inject with $^3$H-t on days 0, 3, 6, 9, 11, 14, 18, 21, and 24
Observe LI, MI, and Cell Density.

Amputated right limbs regenerate normally.
Inject same time periods and compare.

II. Do limbs with a wound epidermis regenerate inside the body cavity?

Allow limbs to heal with wound epidermis, then insert. Label and fix at 4 weeks, Observe LI, MI.

Amputated right limbs regenerate normally.
Label and fix as experimental.

III. How does lack of wound epidermis and nerves influence cell cycle events? What is fate of cells in limbs which do not regenerate?

Amputate, denervate, and insert.
Label and fix on days 0, 7, 14 and 21
Observe LI, MI, and Cell Density.

Amputated right limbs regenerate normally.
Label and fix as experimental.
IV. What is the fate of cells in inserted limbs? Do exteriorized limbs regenerate?

Insert limbs for 5 weeks. Amputate right limbs at time left is removed from coelom.
Remove from body cavity. Compare rate of regeneration.
Observe rate of regeneration.

Injured Limbs
Experimental left limbs Control right limbs

I. How does injury influence cell cycle in absence of wound epidermis?

Injure limbs Injure limbs. Remove ring of skin to control for accidental damage to skin on left limb.
Label and fix on days Label and fix as experimentals.
0, 3, 6, 9, 11, 14, 18, 21
Observe LI, MI.

II. How does injury influence cell cycle in absence of wound epidermis and nerves?

Injure and denervate limbs Injure - no denervation and no skin damage.
Label and fix on days 0, Label and fix as experimentals.
3, 6, 9, 11, 14, 18, 21
Observe LI, MI.
Skin Flaps

Experimental left limbs  Control right limbs

What is the fate of cells in limbs with long term skin flaps?

Place skin flaps on limbs.
Allow to remain for 5 weeks.
Reinjure - Observe rate of regeneration.

Amputate right limbs at time of reinjury to left - Compare rate of regeneration.

Place skin flaps on limbs for 5 weeks.
Label and fix at 5 weeks.
Observe LI and MI.
CHAPTER V

RESULTS

Inserted Limbs.

As previously reported by Goss (1956a) limbs of newts in these experiments, amputated and immediately inserted into the coelomic cavity, consistently failed to regenerate. A total of 24 animals had their left limbs inserted into the coelom. These limbs remained inserted for varying periods of time as described previously. Contralateral control limbs with a single amputation through the mid-forearm regenerated normally. The inserted limb, deprived of a wound epidermis, nonetheless underwent cellular dedifferentiation as seen in Figures 1 and 2, as do control limbs bearing a normal wound epidermis (Figure 3).

In addition to dedifferentiation, the cells of both control and inserted limbs initially enter the cell cycle and replicate DNA as shown by $^3$H-thymidine incorporation. In fact, up to day 14 the labeling index for experimental and control limbs is very much the same (Figure 4). By day 18, however, the labeling index in the control limb is twice that of the inserted limbs. In inserted limbs, the
labeling index appears to level off at the 14 day value. The data in Figure 5 show that dedifferentiated cells from both control and experimental limbs also proceed through G₂ and undergo mitosis, as might be expected because nerves are present (Tassava and Mescher, 1975). As with ³H-thymidine incorporation, the mitotic index increases in both control and experimental limbs through day 14. However, by day 18 the mitotic index in the control regenerates is roughly 4 times that of the inserted limbs but the mitotic index of inserted limbs never rises above the 11 day value and subsequently shows a slight decrease. Thus cells of inserted limbs do dedifferentiate and proceed through the cell cycle to mitosis. Some cells appear to continue to label and divide throughout the 3 weeks of the experiment in the inserted limbs but not to the same extent as in the controls and the inserted limbs do not form a blastema and do not regenerate.

**Inserted Limbs with Wound Epidermis.**

In order to test in these experiments, as Goss has previously shown (1956b) that limbs that have a wound epidermis will regenerate normally inside the body cavity, some limbs were amputated and allowed to heal before being inserted. Although some limbs regenerated in the coelom after only a 24 hour period between amputation and insertion, the environment of the body cavity apparently
sometimes caused the breakdown of the wound epidermis, and these limbs failed to regenerate. This problem was subsequently avoided by waiting 48 hours after amputation before inserting the limb into the body cavity. The right limbs in these control animals were amputated to serve as a comparison with the inserted controls. The inserted limbs that had a wound epidermis underwent typical blastema formation and regenerated as shown in Figure 6. Labeling and mitotic indices examined after limbs with wound epidermis had been inserted for 4 weeks were found to be the same as contralateral limbs regenerating outside the body cavity.

**Denervated, Amputated Inserted Limbs.**

A second series of experiments involved denervating the left forelimb before it was amputated and inserted into the coelom. This procedure allowed for effective elimination of two of the three critical elements in regeneration, nerves and the wound epidermis, and offered the possibility of studying the initial stages of regeneration in the absence of these two factors. The right limb was amputated as before and served as a normally regenerating control. Figure 7 is a graph comparing the labeling indices at days 7, 14, and 21 in limbs that have no nerves and no wound epidermis with the normal limb. Cells enter the cell cycle and proceed to S at all 3 sample
times as evidenced by incorporation of $^3$H-thymidine. The levels of labeling remain very similar in the experimental and control limbs for the first week. By two weeks, however, the labeling index in the control limb is higher than in the experimental and by three weeks the difference is substantially greater ($p < .005$, Student's t-test) (Figure 7).

Figure 7 is a graph of the mitotic indices in limbs from this experiment. Very few mitotic figures are seen in the denervated inserted limbs, but the mitotic index steadily increases in the uninserted, innervated control limbs. Thus the cells seen in S in Figure 7 do not undergo mitosis (Figure 8).

In all of these experiments, the cells participating in the response to injury are those near the distal end. Histolysis of bone, muscle, and connective tissue near the amputation surface provides dedifferentiated cells that label with $^3$H-thymidine and divide (if nerves are present). Observation of cells in stump tissues 2 mm proximal to the vicinity of the injury does not show any labeling or mitosis. Hence, these cells are assumed to be differentiated and in $G_0$. 
Cell Density.

One of the most obvious histological features of a regenerating limb is the accumulation of dedifferentiated cells to form the regeneration blastema. This accumulation is the expected result of cells traversing the cell cycle and dividing. However, as shown by Mescher and Tassava (1975) and Mescher (1976), and present experiments (graphs 4 and 5 and 7 and 8) cells of limbs that do not regenerate, nevertheless enter the cell cycle and in some cases apparently divide during the very early periods after amputation. It is of interest, therefore, to determine what happens to these cells in a limb that will not regenerate. Since the same number of grid areas were counted in each limb instead of the same number of cells, it was possible to examine the change in cell density in selected areas of the limb through time. These results are seen in Figure 9. This histogram represents the mean number of cells/grid area in the distal-most areas of the control and inserted limbs. The number of cells increases with time in both limbs, consistent with the fact that cells of both limb types replicate DNA and divide (Figures 3 and 4). At day 14 and beyond, the difference in cell density between the controls and inserted limbs becomes more pronounced and, after day 18, the density in the inserted limb levels off or drops but that of the control limb
continues to rise.

A similar situation obtains in denervated, inserted limbs (Figure 10). The density of cells in the distal-most areas of the limb stump remains relatively stable and only slightly lower than in control limbs, for the first two weeks. By the third week the density of cells in the control, regenerating right limb rises dramatically compared to the experimental left limb in which the density of cells remains roughly constant.

Another series of experiments with inserted limbs involved observation of regeneration in these limbs when exposed to the environment after having been inserted in the body cavity for 5 weeks. In this experiment, the left limb of a group of 15 animals was inserted and then exteriorized 5 weeks later. The right limb served as a control and was amputated at the time the left limb was exteriorized. Only 8 of the animals survived to participate in the experiment. The results can be seen in Table 1. The exteriorized limbs that had been inserted reach advanced stages of regeneration before the newly amputated limbs. By 3 weeks, the exteriorized limbs had reached cone, paddle, and even in 1 case digit stages, but the amputated limbs were all in mound stages. By 5 weeks, 6 of these limbs had well developed digits, but only one of the amputated limbs produced a digit stage, and this lagged
behind the experimental limbs in extent of development and number of digits.

Injured Limbs.

In an attempt to further analyze the effects of injury in regeneration, I performed experiments to isolate the injury phenomenon in the absence of nerves and the wound epidermis and without an amputation. In the first such series of injury experiments, a cuff of skin was torn around the fracture point to control for possible skin damage and formation of a wound epidermis. This ring can be detected histologically at day 0 and occasionally persists through day 3. By day 6, however, all evidence of the torn skin is gone and the epidermis appears similar and contiguous all along the limb. Histologically, the fractured radius and ulna are obvious (Figure 11). Muscle insertions in the vicinity of the fracture are disturbed but little cellular activity is detected by mitotic figures or $^3$H-thymidine incorporation during the first three days following the fracture (Figures 12 and 13). By day 6, however, the labeling index in the immediate vicinity of the fracture rises to 6% in both the injured limbs and the injured limbs from which the cuff of skin was removed (Figure 12). This level of labeling stays constant until day 18 and then drops off slightly. According to the Student's t-test there is no significant difference in the
labeling index between experimental and control limbs in this experiment. It can be concluded that slight damage to the limbs' skin and thereby possible wound epidermis formation, has no effect on internal cell activities.

The mitotic index (Figure 13) in these unamputated, injured limbs remained very low throughout the 21 day course of the experiment and reached a peak of only 0.50% in both control and experimental limbs on day 14.

The second series of injury experiments involved denervating the left limb and then fracturing it. The right limb was fractured but otherwise remained intact, i.e., received no denervation or cuff of skin. The labeling index in the vicinity of the fracture in both the denervated left limb and the control right limb can be seen in Figure 14. The labeling index in both limbs is very similar, and furthermore, is very similar to that seen in the previous series of injury experiments without a denervation.

Very few mitotic figures were found in the denervated, injured limb and a significant increase in mitotic index was not observed. The mitotic indices in the control right limbs on day 14 were similar to those found in the first series of injury experiments.

Neither labeling nor mitotic indices increased in areas of the limb considerably removed from the fracture.
Skin Flap Studies.

To further the work of Mescher (1976) and to further examine the effects of skin flaps placed over the amputation surface, a series of studies was initiated to examine long term effects of skin flaps and the effects of reinjury to the limb after these grafts of whole skin had remained over the amputation surface for 5 weeks. The results from these reinjury experiments can be seen in Table 2. In these limbs, a ridge formed along the incision within 4 days of reinjury in eight out of nine cases, and mound blastemas were present by one week. The table shows that some of these regenerates reached cone and paddle stages of regeneration by 2 weeks after reinjury and digit stages by 3 weeks. The contralateral control limbs, amputated at the time of the reinjury to the experimental limb, lagged behind (Table 2).

To determine if any cells were still in the cell cycle after having skin flaps for 5 weeks, I injected 3 animals with complete skin flaps on both the right and left limb at 5 weeks with $^3$H-thymidine. The mean labeling index from these limbs at this one point in time was found to be 12.5% and the mitotic index 0.52%.
Figure 1. An autoradiograph of a longitudinal section of a limb that was amputated, immediately inserted into the body cavity, and fixed 18 days later. The $^{3}$H-T labeling period was for 3 hrs prior to fixation. Some of the dedifferentiated cells distal to the muscle (M) and the ulna (u) have incorporated $^{3}$H-thymidine. Some mitotic figures can also be seen in these limbs. The distal most margin of the skin epidermis can be seen (arrows); the amputation surface is devoid of a wound epidermis. Hematoxylin-eosin (H&E). Mag. X 15. A higher magnification of a portion of this limb can be seen in Fig. 2.
Figure 2. An autoradiograph showing a portion of the limb in Figure 1 but under higher magnification (75 X). $^3$H-thymidine labeled cells can be seen (arrows). There is no wound epidermis over the amputation surface. Some internal body tissue (BT) is adhering to the distal end of the limb. H&E.
Figure 3. A photomicrograph of a normally regenerating control (normal control) limb of the same amputation age (18 days) as the limb in Figure 1. Note the well-formed wound epidermis (WE) and normal blastema (B) formation beyond the level of amputation. The ulna (u) is the only bone present in this photomicrograph as the radius is out of the plane of the section.
Figure 4. A comparison of the labeling index in control regenerating limbs (dashed line) and inserted, nonregenerating limbs (solid line) for 24 days after amputation. Each point represents the mean labeling index of three limbs. Where appropriate, the vertical lines at each point indicate standard error of the mean.

P < 0.025 day 18
P < 0.050 day 21
Figure 5. A comparison of the mitotic index in control regenerating limbs (dashed line) and inserted nonregenerating limbs (solid line) for 24 days after amputation. Each point represents the mean labeling index of three limbs. Where appropriate, the vertical lines at each point indicate standard error of the mean.

P < .005 day 18
P < .050 day 21
Figure 6. A photomicrograph of a longitudinal section of the control limb (delayed insertion control) that has been amputated and inserted into the body cavity after a 48 hour delay, a time sufficient to allow a wound epidermis to form over the distal end. The limb is 10 days older in amputation age (a total of 28 days) than the control in Fig. 2 and is further along in regeneration. Mag. X 25.
Figure 7. A comparison of the labeling index in control regenerating limbs (o) and limbs that have been denervated, amputated, and immediately inserted (●) and are not regenerating. Vertical lines at each point indicate standard error of the mean. Each point represents the mean labeling index of three limbs.

It is important to note that sampling times for this experiment were different from previous ones (Figs. 3 & 4) thus, comparisons are valid only between control and experimental values of each experiment and not between controls of the two experiments.
Figure 8. A comparison of the mitotic index in normal regenerating limbs (o) and in limbs which have been denervated, amputated, and inserted into the body cavity (●). Each point represents the mean mitotic index of three limbs. Where appropriate, the vertical lines at each point represent the standard error of the mean. Sampling times for this experiment were different from those not involving a denervation (Figs. 3 & 4). Thus, comparisons are valid only between control and experimental values of each experiment and not between controls of the two experiments.
Figure 9. A histogram showing relative cell density in the distal part of normal regenerating limbs (□) compared to inserted nonregenerating limbs (■) through 24 days after amputation. Each point represents the mean (X) number of cells/area from three limbs of both control and experimental animals.
Figure 10. A histogram showing relative cell density in the distal part of normal regenerating limbs (□) compared to denervated, amputated, and inserted nonregenerating limbs (■). Each point represents a mean (X) number of cells/area from three limbs of both control and experimental animals.
Figure 11. A photomicrograph of a longitudinal section of a limb that has been injured by fracturing both the radius (R) and the ulna (U) midway between the elbow and the wrist. The disjoined ends of the bones can be seen in the picture. Mag. X  .
Figure 12. A comparison of the labeling index in control injured limbs with a wound in the skin (○) and injured limbs that have no such wound (●). Each point represents the mean labeling index of three limbs.
Figure 13. A comparison of the mitotic index in control injured limbs with a skin wound (o) and injured limbs without the wound (●). Each point represents the mean mitotic index of three limbs.
Figure 14. A comparison of the labeling index in denervated injured limbs (●) and limbs that were only injured (○). Each point represents the mean labeling index of three limbs.
Table 1. Enhanced regeneration of limbs which have been exteriorized following a five week insertion in the body cavity compared to amputated controls.*

<table>
<thead>
<tr>
<th>Limb (Exteriorized vs amputated controls)</th>
<th>Week of Observation</th>
<th>Stage of Regeneration Reached</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>Pre-mound Mound Cone Paddle Digit</td>
</tr>
<tr>
<td>Exteriorized</td>
<td>0</td>
<td>8 0 0 0 0</td>
</tr>
<tr>
<td>Amputated</td>
<td>8</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>Exteriorized</td>
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<td>3 3 2 0 0</td>
</tr>
<tr>
<td>Amputated</td>
<td>3</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
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<td>5</td>
<td>0 4 3 1 1</td>
</tr>
</tbody>
</table>

*Control limbs were amputated at the time the inserted limbs were exteriorized.
<table>
<thead>
<tr>
<th>Type of Re-injury</th>
<th>Week of Observation</th>
<th>Stages of regeneration reached</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-mound</td>
<td>Mound</td>
</tr>
<tr>
<td>Transverse incision</td>
<td>1</td>
<td>3</td>
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<tr>
<td>Amputation</td>
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<tr>
<td>Transverse incision</td>
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<td>Amputation</td>
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<tr>
<td>Transverse incision</td>
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<td>Amputation</td>
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<tr>
<td>Transverse incision</td>
<td>4</td>
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<tr>
<td>Amputation</td>
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</table>

*Only 5-week skin graft limbs which showed no external signs of regeneration were used for these re-injury experiments. The transverse incision was made to a depth of 1 mm into the stump. Skin grafts were made only on left limbs at which time right limbs were not amputated. Thus at 5 weeks, right limb controls were amputated for the first time. All control limbs and eight out of nine re-injured skin graft limbs eventually completed regeneration. Newts were maintained at 21 ± 1°C throughout the experiment.*
CHAPTER VI

DISCUSSION

The results of these experiments are consistent with those of earlier investigators (Dent, 1954; Butler, 1955; and Goss, 1956a) in showing that amputated limbs inserted immediately into the body cavity do not regenerate. Since limbs that are allowed to heal with a wound epidermis for 24 or 48 hours before insertion do show typical regeneration, it is apparent that the lack of a wound epidermis is the critical element in preventing regeneration of the inserted limbs.

Goss (1956a) described the histological changes in these nonregenerating inserted limbs and found evidence of cellular dedifferentiation similar to that seen in control regenerating limbs for at least the first 10 days, although it did not appear to be quite as extensive in the inserted limbs as in the controls. The experiments presented here extend the findings of Goss (1956a, b) to include a study of cell cycle phenomena by examining labeling and mitotic indices and by observing events in denervated, amputated, and inserted limbs. Of consequence are the findings that the cells of the inserted stumps dedifferentiate, enter the
cell cycle, replicate DNA, and divide. The progeny of these cells do not accumulate, however, and blastema formation and regeneration do not occur. Mescher (1976) found similar results after placing flaps of whole skin over the amputation surface. In those limbs in which the flaps healed and the limbs did not regenerate, dedifferentiation, labeling and some mitosis occurred at a level equal to the controls for the first week following amputation. The data from the present study are consistent with Mescher's (1976) finding, which, taken together, suggest that the wound epidermis is not required for the initial dedifferentiation of cells and their entry into the cell cycle. It is of interest, however, that even though inserted limbs show no regeneration, a substantial number of cells exhibit DNA synthesis throughout the more than three week time course of the experiment. \(^{3}\text{H}\)-thymidine labeling indices of control limbs are higher during the last 2 weeks of the experiment indicating that these cells continue in the cell cycle after they have divided. It should be pointed out here also, that the number of cells increases in the control limb as the blastema forms and thus the absolute number of cells replicating DNA and undergoing mitosis is much higher in the controls than in the inserted limbs.
In this context, it is interesting that the cell density of the inserted limb distal stump increases only marginally, suggesting that little or no accumulation of cells occurs near the amputation surface. Cells that undergo mitosis in inserted limbs thus do not form a blastema nor do they accumulate in the limb stump. Determining the fate of these cells is of critical importance with regard to the role of the wound epidermis and my experiments have uncovered this important problem. There are several alternative explanations for what happens to these cells. It is possible that some of the progeny cells seen to replicate DNA and divide in inserted limbs are removed from the limb, perhaps by phagocytes. Secondly, some daughter cells may be lost to the environment of the coelomic cavity since there is no barrier between the mesodermal tissues and the coelom. It is also conceivable that the cell cycle in these inserted limbs is protracted, by an extended $G_1$, $S$, or even the entire cell cycle. These alternatives would all explain the appearance of labeled and dividing cells in limbs that do not form a blastema or regenerate. I believe it is important, in elucidating the role of the wound epidermis, to ascertain which of these alternatives is responsible for the observed results.

A second series of insertion experiments involved denervating the left limb before it was inserted into the body cavity. These limbs were thus injured but lacked both
nerves and a wound epidermis. The results show a sizeable percentage of cells that still enter the cell cycle and replicate DNA. However, the mitotic index in these denervated inserted limbs is very low. This finding suggests that nerves are essential for cells to traverse the $G_2$ phase of the cell cycle. It is consistent both with the Tassava-Mescher model (1975) and with a number of recent reports that were instrumental in developing the $G_2$ block model for the neural effect in limb regeneration (Kelly and Tassava, 1973; Mescher and Tassava, 1975). Mescher and Tassava (1975) demonstrated that nerves were not necessary for cell division in the newt limb. It was further shown that the wound epidermis also was not essential for dedifferentiation and cellular proliferation during initiation of limb regeneration (Mescher, 1976). My experiments confirm those results by examining the cellular events in a different system than that used by Mescher (1976) and extend them by combining denervation and insertion. As injury is the only critical element necessary for regeneration that is present, then injury alone seems sufficient to initiate the process of regeneration. Even at three weeks, some cells in inserted limbs without nerves or wound epidermis are seen to label with $^3$H-thymidine. The data do not specify whether these are newly dedifferentiated cells or cells in a prolonged $S$ phase. That the $S$ phase is elongated seems unlikely since the density
of silver grains over each nucleus is similar to that seen in controls for the same 3 hour incorporation period of the isotope. The results also indicate that there is no significant change in cell density during the 3 weeks of the experiment. This suggests the fact that no division of cells is occurring in these limbs.

In the first series of inserted limb experiments that did not involve a denervation, both the labeling and mitotic indices were the same in the experimental and control limbs for the first two weeks, showing a significant difference only after that time. In the denervated, amputated, and inserted group, however, there is a substantial difference in the patterns of DNA synthesis and mitosis beginning on day seven and increasing to day 14. It appears that in the presence of nerves, cells of inserted limbs persist in the cell cycle for at least 14 days, are then adversely influenced by the lack of a wound epidermis, and leave, are removed or stop cycling. In the absence of nerves, however, the cells apparently block (presumably in $G_2$, Mescher and Tassava, 1975) no mitosis occurs, no daughter cells are formed, and thus the level of DNA synthesis decreases after the first week. Thus the projected role of the wound epidermis is to keep the dedifferentiated cells of the limb cycling. It would seem, however, that cells can complete one or two rounds of the cell cycle in
the absence of a wound epidermis and in the presence of nerves. The nerves have an early and immediate affect on mitosis such that in their absence, cells are prevented from cycling as they become arrested before reaching M. The wound epidermis on the other hand, would seem to have a more subtle influence, causing cells already cycling to undergo continued rounds of proliferation to eventually form a blastema and hence a regenerate.

The fact that the amputated limb stumps are inserted into the environment of the coelomic cavity is potentially significant with regard to the above interpretation. It is possible that coelomic fluids perpetrate an injury phenomenon that causes continued dedifferentiation and somehow precludes regeneration. It was important therefore, to obtain regeneration within the coelomic cavity. Limbs that were allowed to heal for 2 days prior to insertion into the body cavity regenerated normally, and labeling and mitotic indices were found to be similar to those of normally regenerating controls. It is additionally possible that an injury phenomenon due to the coelomic fluids causes continued dedifferentiation and that this continued dedifferentiation accounts for the labeling indices seen at 3 weeks in inserted limbs.

The fate of cycling cells in innervated inserted limbs that lack a wound epidermis is unclear. Goss (1956a) reported that limbs inserted for 7 weeks subsequently
regenerated if exteriorized. Likewise, in the present study, limbs that were removed from the body cavity at various times after insertion always formed a wound epidermis and regenerated. What is not clear, however, is whether inserted limbs that are exteriorized initiate regeneration anew, beginning with dedifferentiation, or whether cells remain in the dedifferentiated condition and immediately re-initiate cycling upon wound epidermis formation. Several lines of evidence from my data indicate that the latter possibility is correct. As shown in Table 1, limbs that have been inserted into the body cavity for 5 weeks and then removed regenerate more quickly than contralateral control limbs that are amputated at the same time as the experimental limbs are exteriorized. For the benefit of future research in this area, I think it is most logical to hypothesize that the cells in these long-term inserted limbs become arrested in the dedifferentiated condition at some point in the cell cycle, most likely in $G_1$. Although only 8 animals survived the long (10 weeks) and rigorous procedure, all but one showed more advanced stages of regeneration sooner in the exteriorized limb than in the control limb on the same animal, reaching digit stages within 5 weeks. This observation is consistent with the notion that cells of inserted limbs remain dedifferentiated or at least dedifferentiate more quickly than those cells in freshly amputated limbs. Additionally, as
Table 2 indicates, limbs that are reinjured after having skin flaps placed over the amputation surface for 5 weeks showed a particularly fast rate of regeneration when compared to contralateral control limbs amputated at the time of this reinjury. In skin flap studies reported by Tassava and Loyd (1977) regeneration stages were also reached very early after entire or 1/3 skin graft removals. If the cells of inserted nonregenerating limbs remain in the cell cycle and do not redifferentiate, then regeneration of such limbs following exteriorization might be expected to be fairly rapid. It is possible that cells in inserted limbs without a wound epidermis remain stationary in some phase of the cell cycle and require only a signal from a newly formed wound epidermis to begin cycling. However, the possibility that additional dedifferentiation is necessary to obtain regeneration of inserted limbs that are removed from the body cavity has not been ruled out.

That a few cells persist in the cell cycle during the five week period that limbs have skin flaps, however, is suggested by the results of the labeling experiment at 5 weeks post skin flap application. Clearly some cells labeled with $^{3}H$-thymidine and some mitotic figures were observed. It is not possible to tell whether these represent newly dedifferentiated cells or cells moving very slowly through the cell cycle. It is also not possible to tell if these are cells that would participate in regeneration
if the skin flap were removed. Additional experiments would be required to test these possibilities. What is apparent from the results of these mentioned studies is that total redifferentiation of cells in skin flap and inserted limbs does not occur. Mescher (1976) found evidence of redifferentiation in skin flap limbs examined after 2 months. He observed cartilage nodules and muscle reformation after this time period. It is possible that the low levels of DNA synthesis and mitosis I observed are indicative of the beginnings of limited myogenesis and chondrogenesis. However, cells that are stationary in some phase of the cell cycle, although remaining dedifferentiated, would presumably not label with radioactive precursors nor divide.

The proposed role of the wound epidermis of keeping cells in the cell cycle, although consistent with Mescher's finding (1976), is not completely adequate, especially in view of the fact that cells are seen to label and divide in limbs lacking a wound epidermis for extended periods. I would like to emphasize that this shows that the role of the wound epidermis is still not clearly understood and additional experiments are necessary. Cells of the wound epidermis actively incorporate radiolabeled precursors of RNA (Bodemer, 1962) and protein (Bodemer and Everett, 1959; Darda and Anton, 1969) but not DNA (Hay and Fischman, 1961).
Chapron (1974) has demonstrated that a radiolabeled glycoprotein precursor introduced to normally regenerating limbs is located first on epithelial cells and then on mesenchymatous cells. These findings suggest that the wound epidermis is active in synthesis of products that may be elaborated into the limb stump and in some way affect blastema formation.

The results of experiments by Mescher and Tassava (1975) and Mescher (1976) and those in this study, especially experiments involving inserted limbs that lack both nerves and wound epidermis, strongly suggest that injury alone cause cells to leave the differentiated $G_0$ state and enter the cell cycle. In order to investigate this problem further, experiments were designed in which the animals sustained injury but in the absence of amputation and thereby in the absence of wound epidermis or wound epidermis and nerves. Thornton and Kraemer (1951) first described such experiments in larval *Ambystoma* and examined fracture healing in the presence or absence of nerves and described gross morphological characteristics. These authors observed that larval axolotl limbs that are injured and denervated, regressed as long as they remained denervated. Once the limbs became reinnervated, the process of regression stopped. Thornton (1953) then went on to discuss the histological changes in these injured
Ambystoma limbs. He observed dedifferentiation in the vicinity of the fracture that he called primary dedifferentiation. In denervated limbs, this dedifferentiation spread in both proximal and distal directions from the injury and was termed secondary dedifferentiation. In the present study, newts were used instead of larvae. The results show that dedifferentiated cells begin to appear in 3-4 days after the fracture of the limb and cells can be observed in S by day 6 as evidenced by $^{3}H$-thymidine incorporation. In these experiments, care was taken not to produce an injury to the skin during the fracture procedure that might cause wound epidermis formation. One of the controls, therefore, consisted of intentional damage to the skin on one limb of each animal. The results indicate that no significant difference in labeling or mitotic indices occurs as a result of this procedure and it can be concluded that slight damage to the skin does not affect the results of these experiments. While the labeling index in the innervated injured limbs reaches a level of 6% and levels off after day 6, the mitotic index remains very low throughout. It reaches a peak of 0.5% on day 14 and then drops again.

The most interesting results in the injury experiments are found in the denervated injured limbs. As has been pointed out previously, both amputated denervated (Butler and Schotte, 1941) and injured denervated (Thornton and
Kraemer, 1951) limbs of _Ambystoma_ larvae regress all the way to the shoulder if they remain denervated. In newt limbs, only the blastema regresses following denervation (Singer and Craven, 1948), and, as shown in these experiments, regression following fracture and denervation does not occur. However, as seen in Figure 13 the injury alone, in the absence of wound epidermis and nerves, is sufficient to cause the cells in the vicinity of the injury to dedifferentiate, enter the cell cycle and proceed to S as indicated by autoradiography. In fact, the labeling index in these limbs reaches the same levels in both denervated and innervated limbs, suggesting that the nerve has no influence on the cell cycle up to this time. This data is consistent with the Tassava-Mescher cell cycle model that provides that the nerves are responsible for some G2 event. Consistent with this view also, is the fact that a very low mitotic index (.08\% on day 14) was observed in the denervated injured limbs.

Of consequence also, is the fact that Thornton (1953) found that fractured innervated limbs of _Ambystoma_ larvae healed within 12 days by formation of new cartilage. This did not appear to be the case in either innervated or denervated newt limbs. No healing was observed during the 21 days of the experiment. This is also in contrast to what is found in some mammalian limbs. Frymoyer and Pope
(1977) found that fractures healed more quickly in the sciatically denervated rat limb. However, in the rat, the sciatic nerve innervated the muscles that originate and insert on the fibula, thus many other nerves were present in the limb. The reason why injured newt limbs showed no healing response during the 3 weeks of the experiment cannot be ascertained from these data except in the case of the denervated limbs. It is possible here that since no cells proceed through M there are insufficient cells to engage in the repair process. It is probable that healing takes longer than 3 weeks and would have eventually been seen in the innervated limb since some cells divide in those limbs, and daughter cells would presumably be available for the healing process.

It should be pointed out that these injured limbs are not engaged in epimorphic regeneration. The labeling and mitotic indices remain low in comparison to those achieved in the blastema of limbs which have been amputated. The reasons for this may be several-fold. The area of actual injury is small and the larger trauma of amputation is absent. Secondly, no wound epidermis is present. Since the proposed role of the wound epidermis is to keep cells cycling, it is not surprising that in its absence no large accumulation of cells occurs. Finally, although nerves are present in at least one group of injured limbs, these
nerves are not injured to any great extent as in an amputated limb where they must undergo growth. There are very few, if any, cut surfaces in the nerve of an injured limb, and this may be an important prerequisite for stimulation of mitosis in epimorphic regeneration.

It is shown by the experiments reported here, however, that injury alone, in the absence of nerves and a wound epidermis, is sufficient to cause differentiated cells of the limb to leave that differentiated state, enter the cell cycle, and replicate DNA. Further, that in the presence of nerves, some limited cell division does occur, but that in the absence of nerves almost no mitotic figures are observed. These findings are consistent with the proposed role of the nerve in the Tassava-Mescher model (1975) and further delineate and emphasize the role of injury in this model.

The information garnered from the inserted limb and skin flap experiments also confirms and emphasizes the role of nerves, injury, and wound epidermis in limb regeneration as suggested by the Tassava-Mescher model (1975). However, these experiments raise several new potentially important questions. It is not clear why some cells of inserted limbs, without a wound epidermis, persist in the cell cycle throughout the 3 weeks of the experiment. Since no accumulation of cells or regeneration occurs, the fate
of these cells seen to be labeling and dividing is not clear. Additionally, there are labeled cells and mitosis occurring in limbs which have had skin flaps for 5 weeks. In regard to the inserted limb experiments, it should be helpful to remove these limbs from the body after 5 weeks, and study cell cycle events through time, beginning on day 0 as the wound epidermis forms. This information coupled with that found in a control amputated limb should help provide the answer to whether or not extensive cellular dedifferentiation is maintained in these limbs and provide some information as to where these cells may be blocked in the cell cycle, if indeed they are. It should also be useful to observe the number of macrophages present in limbs that are inserted for varying periods of time. Since Little (unpublished pers. com.) has found an increase in the number of macrophages in denervated, nonregenerating, regressing limbs of *Ambystoma* larvae, it is important to test whether macrophages are involved in removal of cells in nonregenerating denervated and/or inserted limbs.

Certainly additional experiments designed to examine cellular activities after reinjury of skin flap limbs are important to do. Experiments to determine labeling and mitotic indices through time following reinjury of 5 and 10 week skin flap limbs should provide additional insight
into the fate of cells in these limbs and the role of the wound epidermis. Similar studies, after exteriorizing 5 and 10 week inserted limb stumps, will add valuable comparative data.
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