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RELATION OF NERVES AND WOUND EPIDERMIS TO CELL CYCLE

PARAMETERS OF BLASTEMA CELLS OF THE MEXICAN AXOLOTL, AMBYSTOMA MEXICANUM

DISSERTATION

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

By
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The Ohio State University
1976

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ACKNOWLEDGMENTS

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CHAPTER I
INTRODUCTION

The regenerative process of urodele and anuran limbs has been of interest to scientists since the early studies by Trembley in the 1700's (reviewed by Tardent, 1963). Regeneration is a phenomenon whereby injured parts are renewed through a series of cellular and morphogenetic interactions. Morgan (1901) divides the regenerative response into two broad categories, epimorphic and morphallactic regeneration. In the latter, the organism repairs the injury by reorganizing the remaining parts of the body whereas in the former, the organism repairs the injury by adding to the remaining part of the body. Newts and axolotls exhibit epimorphic regeneration whereas planarians and hydras exhibit morphallactic regeneration (Spiegel and Dudley, 1973; Burnett, 1961). This dissertation will deal solely with the process of epimorphic regeneration as seen to occur in amputated limbs of frog tadpoles, adult newts and various species of Ambystoma. The experimental studies are restricted to axolotls, Ambystoma mexicanum.

Amphibian limb regeneration has been divided into three main phases by earlier workers in the field (Schotte and Butler, 1944) as follows:
(a) **Dedifferentiation.** The cells of the old mesodermal tissues of the stump immediately proximal to the amputation surface dedifferentiate resulting in a mass of undifferentiated mesenchymal cells which are referred to collectively as a regeneration blastema or a blastema.

(b) **Regeneration Blastema.** The blastema grows and is transformed into the limb rudiment.

(c) **Limb Rudiment.** There is growth and visible morphogenesis of the limb rudiment culminating with differentiation and the restoration of the lost part.

These phases will be discussed in greater detail in the following sections.

**Normal Limb Regeneration**

The regenerative process is immediately initiated following the amputation of the limb. There are four major events which take place immediately following injury and during wound closure. Although they are interrelated, they may be distinguished as, (a) detachment from the underlying basement lamella of epidermal cells bordering the wound, (b) mobilization of epidermal cells proceeding in the form of a wave from the wound border to more distal areas, (c) active migration of the epidermal sheet towards the center of the wound, and, (d) simultaneous arrest of movement when closure is effected (Lash, 1955). Within 24 hours, the amputation surface is covered by a wound epithelium or epidermis (W.E.) consisting of one or two layers of cells (Singer and Salpeter, 1961).

The wound epidermis has been shown to be actively synthesizing proteins shortly after the initiation of regeneration. Using the technique of autoradiography, Bodemer and Everett (1959) demonstrated
protein synthetic activity in the wound epidermis by its intense localization of methionine-$\text{S}^{35}$ throughout the first few days of the wound reaction while mesodermal tissues at the amputation surface showed only slight localization. Several workers (Weiss and Rosenbaum, 1967; Schmidt and Weary, 1963) have detected high acid phosphatase activity in wound epidermis of regenerating larval *Ambystoma* and newt limbs. Acid phosphatase is an enzyme associated with lysosomes and, since the wound epidermis has high acid phosphatase activity, it has been speculated that the wound epidermis may be a phagocytic agent during the early phase of regeneration (Weiss and Rosenbaum, 1967; Schmidt and Weary, 1963). Observations of the transfer of material from inside the blastema to the outside via the epidermis led Singer and Salpeter (1961) to refer to the wound epidermis as a "graveyard" for phagocytes and cellular debris of the wound area, which suggests once again that the wound epidermis assumes a phagocytic role during regeneration.

Other roles for the wound epidermis have been suggested. Through a series of ingenious experiments, Thornton (1957;1960) concluded that the epidermal cap (synonymous to the thickened distal-most portion of the epithelium which covers the blastema and is not underlain by a dermis) was the causative agent directing the accumulation of dedifferentiated mesenchymal cells to form a blastema. Upon continuous removal of the epidermal cap of *Ambystoma* limbs, no blastema was formed. By shifting the epidermal cap to an eccentric position, an eccentric blastema was formed underneath the eccentric
epidermal cap. Since nerves are known to innervate the wound epidermis (Singer, 1949), they may provide a pathway of migration for blastema cells (Singer, 1959), thus experiments where the wound epidermis was shifted to an eccentric position do not completely prove or disprove the role of the epidermal cap in directing an accumulation of mesenchymal cells to form a blastema, as shifting the wound epidermis could very well shift the nerves innervating the epidermis also. In order to resolve this dilemma, a third experimental design was employed. Aneurogenic salamander limbs, which regenerate without the presence of nerves, would eliminate the uncertainty in the interpretation of the earlier epidermal cap shifting experiment, because the nerves which may have been acting as a pathway of migration for blastema cells would be eliminated. When the epidermal caps of aneurogenic limbs were shifted to an eccentric position, an eccentric blastema nevertheless formed underneath the shifted cap, thus the epidermal cap does in fact seem to direct the accumulation of mesenchymal cells which form the blastems (Thornton and Steen, 1962). Recent findings by Tassava and Mescher (1975) imply that the wound epidermis does not merely direct an accumulation of mesenchymal cells, but that cells which are close to the wound epidermis remain in the cell cycle and continue to undergo mitosis resulting in a greater number of cells near the wound epidermis as opposed to a less dense number of cells farther away from the wound epidermis. Therefore, what appeared to be an accumulation may be the result of an intimate
interaction between the wound epidermis and mesenchymal cells, an interaction which maintains the latter in the cell cycle.

By twenty-four hours following amputation of the forelimb, a wound epidermis has migrated over the amputation surface. The underlying stump tissues thus are covered by a dermis-free wound epidermis which many experiments have shown is necessary for regeneration. As early as the fourth or fifth day post-amputation, muscle and connective tissue of the limb stump begin synthesizing DNA and proteins. Muscle, cartilage, and other connective tissue of the limb stump begin to break down by the 4th day post-amputation liberating mononucleated muscle, cartilage and other connective tissue cells by a process termed dedifferentiation. Dedifferentiation continues through day 12 in the adult newt. These liberated mesenchymal cells tend to aggregate underneath the wound epidermis and proliferate to become the blastema (Thornton, 1938; Hay and Fischman, 1961; Bodemer and Everett, 1959; Mescher and Tassava, 1975; Tassava et al, 1974).

The blastema cells (10-15μ in diameter) are morphologically indistinguishable from one another as seen with the electron or light microscope and it is impossible to determine the tissue type from which they originated (Steen, 1968; Bryant et al, 1971; Popiela, 1972). These cells have large nuclei and nucleoli, consistent with data indicating rRNA and mRNA synthesis (Bantle and Tassava, 1974). The blastema is an overall conglomerate of mononucleated mesenchymal cells derived from differentiated stump tissues (Hay, 1966).
Cell division begins on the fifth and sixth day post-amputation respectively in regenerating axolotl and newt limbs. The number of mitotic cells is low on day 5 or 6, but increases rapidly in the next few days (Mescher and Tassava, 1975; Tassava et al, 1974; Hearson, 1966; Chalkley, 1954; Litwiller, 1939; Kelly and Tassava, 1973). There appears to be a distal predominance of mitotic activity in the axolotl blastema (Hearson, 1966; Faber, 1960). Hearson divided axolotl blastemas into four zones and determined the mitotic index of each zone. The results showed that a significant distal predominance of mitoses was maintained throughout regeneration until the paddle stage, at which time there was less difference between the distal and proximal zones. Faber (1960) also obtained evidence of a distal predominance of mitoses in axolotl blastemas by an indirect method of carbon marking. The newt blastema does not show the early predominance of mitoses as seen in the axolotl (Litwiller, 1939; Chalkley, 1954). Litwiller (1939) described a gradual distal shift in mitotic proliferation from the base or stump towards the distal portion of the newt blastema as regeneration progressed. By late cone stage, the distal half of the blastemas showed the greatest mitotic activity. Similarly, Chalkley (1954) reported a gradual distal shift from the base proximally to the distal half of the blastema in cell density and mitotic activity and, by the paddle stage, the mitotic activity was greater distally. Thus axolotl blastemas seem to have a greater distal mitotic activity during the early stages of regeneration while newts tend to
show a gradual distal shift in mitotic activity only during the later stages of regeneration. The reason for such a difference is not clear at this time.

Histogenesis of the blastema begins between the second and third week post-amputation. The blastema undergoes a series of morphologically discernable stages which can be related to their nerve-dependency or nerve-independency. Blastema formation and the continued development of mound and early cone stage blastemas require nerves. However, the late cone, paddle and digit stages are nerve-independent. Once the blastema attains nerve-independency, the limb rudiment will continue morphogenesis and differentiation into a small limb even without nerves (Singer and Cravan, 1948; Powell, 1969; Schotte and Butler, 1944).

The Regenerative Response of Denervated Limbs

The regenerative process is drastically affected by denervation when the blastema is nerve-dependent. One of the first morphological features affected by denervation is the wound epidermis. Within four days post-denervatlon of early mound stage blastemas, the wound epidermis begins to show a decrease in the number of cell layers as compared to innervated limbs (Bryant et al, 1971). Electron microscopic studies of the W.E. of denervated and innervated limbs reveal a subcellular difference. The epithelial cells of the W.E. in the denervated limbs do not possess the typical cisternal type of endoplasmic reticulum that is normally seen on innervated limbs
(Singer and Salpeter, 1961; Popiela, 1972). This may imply that the epithelial cells of the W.E. in denervated limbs may be more modest in their protein synthetic capabilities.

Denervation at the time of amputation prevents the formation of a blastema and somehow suppresses mitotic activity of the dedifferentiated mesenchymal cells. However, DNA synthesis is not immediately affected by denervation as determined by autoradiographic techniques (Mescher and Tassava, 1975; Kelly and Tassava, 1973; Tassava et al., 1974; Singer, 1952). The rate of RNA, protein and DNA synthesis in denervated nerve-dependent blastemas (10-13 days post-amputation) drops to 50-60% of the control levels by 48 hours post-denervation (Singer and Caston, 1972). Denervation of older nerve-independent blastemas (18 days post-amputation) showed a decline in RNA, protein and DNA synthesis similar to younger nerve-dependent blastemas (Dresden, 1969). Innervated limbs tend to show an increase in ribosomal and high molecular weight RNA species throughout regeneration, on the other hand, mRNA and rRNA incorporation into polysomes declines after denervation (Morzlock and Stocum, 1971; Bantle and Tassava, 1974). Thus, macromolecular synthesis in young nerve-dependent as well as older nerve-independent blastemas is adversely affected by denervation.

The decline in DNA synthesis in the previously mentioned studies of denervated limbs does not seem to be due to decreases in activity of enzymes related to DNA synthesis. Dresden and Moses (1973) were unable to demonstrate a significant difference in DNA polymerase
activity of denervated versus innervated limbs. Manson et al, (1976) observed the activity of aspartate carbamyl transferase (ACTase), and thymidine and uridine kinases but failed to find a significant difference in the activity of these nucleotide synthetic enzymes in denervated versus innervated limbs. Since, the decline in DNA synthesis of denervated limbs does not appear to be controlled by enzymes related to DNA synthesis, there must be other cellular related nerve-dependent processes.

The influence of nerves on regeneration has been known for many years, although the mechanism of action is unknown. The neurotrophic phenomenon has been characterized as follows: (a) all neurons are trophic; motor, sensory and central neurons can supply the nervous factor necessary for regeneration, (b) neither central connection nor reflex circuitry is required for the effect, (c) the neurotrophic phenomenon is independent of polarization of the neuron; it is always centrifugal in direction, emanating from the cell body and flowing thence into the neuronal processes, and (d) there must be a threshold number of nerve fibers at the amputation surface for limb regeneration to occur (Singer, 1952; Singer, 1974).

The neurotrophic or trophic factor is thought to be chemical in nature, however the establishment of the trophic factor as a chemical agent has foiled workers in the field of regeneration for many years. Extracts of nervous tissue do not enhance a visible growth response, although there is an increase in protein synthesis in denervated limbs following brain extract treatment (Singer, 1974; Deck, 1971).
Recently, Singer et al (1976) showed that the loss in protein synthesis of 9-12 day nerve-dependent forelimb regenerates following a 48 hour denervation period can be recovered by infusing into them an extract of frog soluble brain protein. The synthesis of basic proteins shows a greater response to the active brain principle than does that of acidic protein. The active agent of the nervous tissue is heat labile and trypsin sensitive, suggesting that it is likely a proteinaceous substance. The substance is apparently specific to nervous tissue since extracts of liver and spleen do not evoke recovery of protein synthetic activity in denervated blastemas. Additional experiments led Singer et al (1976) to suggest that the nervous agent is likely a basic protein. These results do not specify which if any of the proteins typical for the normal regenerate are synthesized under the control of the nervous agent. Dearlove and Stocum (1974) observed the disappearance after denervation of three electrophoretic bands of proteins and concluded that these represented nerve-dependent proteins of the regenerate. However, it was pointed out by Singer et al (1976), that it remains to be seen if the same proteins that are lost following denervation are recovered upon infusing the active brain extract.

The mechanism of action of the neurotrophic factor, as well as the development of a suitable assay system which will result in a positive regenerative response that can be measured and quantitated quickly, remain to be resolved in the future.
The unamputated limb of salamanders consists of differentiated muscle, bone, or cartilage, and various connective tissue types, as previously mentioned, which are in the G₀ state. There are essentially no cells synthesizing DNA, i.e. in the S phase of the cell cycle, in unamputated limbs (Mescher and Tassava, 1975; Hay and Fischman, 1961). Mescher and Tassava (1975) also showed by microspectrophotometry that there are no cells in the G₂ phase of the cell cycle in unamputated limbs. As early as four days post-amputation, some mesodermal stump tissue cells begin to incorporate ³H-thymidine. Thus, these cells are in the S phase of the first cell cycle. However, mitotic activity is not observed in the mesodermal stump tissue until 6 days post-amputation. There is thus an interval of approximately 48 hours between the onset of DNA synthesis in freshly amputated limbs and the initial onset of mitosis. The 48 hour differential represents a rough approximation of the G₂ + S phases of the initial cell cycle following the amputation of the limb. The actual cell cycle time of the first cell cycle is not known since it is difficult to say exactly when the dedifferentiating cells of the stump enter G₁. Grillo (1972) measured the cell cycle parameters of 18 day newt blastema cells (18 days post-amputation), and the mean $G₁ = \frac{1}{2}M$ (presynthetic or postmitotic phase plus $\frac{1}{2}$ mitosis), S (DNA synthetic phase), $G₂ + \frac{1}{2}M$ (postsynthetic or premitotic phase plus $\frac{1}{2}$ mitosis) and CCT (cell cycle time) were 3.0, 37.5, 4.5, and 45.0 hours duration respectively. The
cell cycle parameters of the first cell cycle following amputation of the limb will probably be worked out in the very near future as well as other stages of regeneration.

Focus of the Dissertation

In an unamputated limb, differentiated muscle, cartilage, bone and various connective tissues are in the non-dividing $G_0$ phase (or state) with regard to the cell cycle (Mescher and Tassava, 1975). Upon amputation, the specialized, differentiated mesodermal stump tissues begin to dedifferentiate by losing their specialized cellular morphology (Hay and Fischman, 1961; Thornton, 1968) and enter the $G_1$ phase ($G_1$) of the cell cycle. In terms of a normal cell cycle, this $G_1$ population of cells proceeds asynchronously through the cycle to a point where $^3$H-thymidine is specifically incorporated into newly synthesized DNA, thus termed the $S$ phase ($S$). Following the $S$ phase, an interval is observed prior to when the $S$ phase cells enter mitosis ($M$), when $^3$H-thymidine is no longer incorporated into DNA and the cells contain a tetraploid (4N) amount of DNA; this gap between DNA synthesis and mitosis is termed the $G_2$ phase ($G_2$) of the cell cycle (Mitchison, 1971; Van't, Hof, 1974; Byron, 1974). Finally, the $G_2$ population of cells eventually enter and complete mitosis giving rise to two daughter cells which repeat the cycle several times resulting in an accumulation of blastema cells. The blastema cells continue
cellular proliferation and finally undergo complex morphogenetic interactions ending with differentiation and the restoration of the amputated limb.

Injury, nerves and the wound epidermis are prerequisites to regeneration and if any of these is absent, regeneration does not occur (reviewed by Singer, 1974; Carlson, 1974; Thornton, 1968). Tassava and Mescher (1975) recently proposed a model which suggests that injury, nerves and the wound epidermis influence the different phases of the cell cycle. Their model hypothesizes that: (1) injury promotes dedifferentiation of differentiated stump tissues in the vicinity of the amputation surface, (2) nerves are necessary for some G₂ event(s) prior to mitosis, and (3) the wound epidermis serves to maintain postmitotic cells in the cell cycle for further rounds of DNA replication and mitosis. This model is strongly supported by data from the initiatory phase or preblastemal stage of regeneration, thus the role of nerves and the wound epidermis is less certain during the later stages of regeneration. Direct measurements of the cell cycle phases under experimental conditions, i.e. after denervation or ultraviolet light inactivation of the wound epidermis are lacking completely. It is important to note that cells proceed into dedifferentiation and into the cell cycle in an asynchronous manner.

The purpose of this study was to examine the role of nerves and the wound epidermis during later stages of regeneration by comparing the cell cycle parameters, ³H-thymidine labelling, and mitotic indices of control and denervated blastema cells. Experiments were designed
to test the applicability of the cell cycle hypothesis of Tassava and Meacher (1975) to blastema stages of regeneration. Specifically, experiments were designed to answer the following questions: (1) Does denervation affect the cell cycle time of blastema cells that are traversing the cycle? (2) If so, which phase(s) of the cycle is affected? (3) Is there a difference in blastema cells that are located either distally or proximally to the amputation surface in terms of their cell cycle parameters, labelling and mitotic indices? (4) Is there a difference in the cell cycle times of blastema cells of nerve-dependent versus nerve-independent stages? (5) Is there a different denervation response in regard to cell cycle parameters when nerve-dependent and nerve-independent blastemas are compared? Answers to questions such as these were thought to be helpful in understanding the influence of nerves and the wound epidermis on the cell cycle during later stages of regeneration.
CHAPTER II
METHODS AND MATERIALS

Larvae of the Mexican axolotl, *Ambystoma mexicanum*, kindly
provided by Dr. R. R. Humphrey, Indiana University and Dr. R. A.
Tassava, The Ohio State University, were grown to an average weight
of 1.59 ± 0.11 gm and snout-tail tip length of 6.3 ± 0.56 cm. Larvae
were maintained at room temperature in conditioned water and fed
strips of beef liver thrice weekly. During experiments, larvae were
transfered to an incubator where the temperature was maintained at a
constant 21.5 ± 1°C. The larvae were also acclimated to experimental
conditions for 3-4 days prior to the start of an experiment.

Operations

Prior to operations, larvae were anesthetized in an aqueous
solution of 0.1% MS222 (ethyl m-aminobenzoate sulfonate, Eastman
Chemical Co.) and placed on moistened gauze. All operations were
performed during the afternoon under a dissecting microscope while
the larvae were anesthetized. The operations were specifically
scheduled for the afternoon in order to avoid complications due to
any possible circadian rhythm that may exist (Litwiller, 1939).

Both forelimbs of each larva were amputated through the distal
third of the humerus and allowed to regenerate. Denervations were
accomplished by transecting the 3rd, 4th and 5th brachial spinal
nerve stain (Samuel, 1953) was also used to determine if nerves were present in the limbs at the end of the experiment.

Each larva was labelled by intraperitoneal injection of 1.5 μCi of $^3$H-thymidine ($^3$H-methyl thymidine, sp. act. 71.6 Ci/mM, New England Nuclear or 60.0 Ci/mM, Schwarz) in 0.05 ml sterile water through the tail musculature (Kelly and Tassava, 1973). A 2 hour incorporation period was used in DNA labelling studies after which the larvae were killed and fixed. Larvae that were used in cell cycle studies were given a single injection of $^3$H-thymidine and fixed at 8 hour intervals through 80 hours.

**Histological and Autoradiographic Techniques**

All limbs were fixed in Carnoy's fixative (1:3, glacial acetic acid: absolute ethyl alcohol). Limbs were histologically prepared and short ribbons, containing 5 sections each, were distributed on 4 slides such that each slide contained representative regions of the limb (Kelly and Tassava, 1973). A single slide of each limb was randomly chosen for Feulgen staining. The Feulgen-stained slides were allowed to dry at room temperature. Following drying, the slides were dipped into a 1:3 dilution of Kodak NTB-2 Nuclear Track Emulsion and distilled water (46° C) and hung perpendicularly in a rack until dry (45 minutes to 1 hour). The emulsion-coated slides were stored at 4° C for two weeks in light-proof plastic slides boxes containing
small packages of dessicant. At the end of the exposure period, the autoradiographs were developed for 3 minutes in D-19 developer, briefly rinsed in distilled water, fixed in Kodak fixing bath for 5 minutes, and washed in running tap water for 10 minutes. Finally the slides were counter stained with fastgreen (10-15 seconds), dehydrated, cleared and coverslips were mounted with Piccolyte mounting medium.

Determination of the Transition Period From Nerve-Dependency to Nerve-Independency

Forelimbs of anesthetized larvae were amputated through the distal third of the humerus and the protruding cartilage trimmed back effectuating a smooth amputation surface. Regeneration proceeded in a normal manner. The right forelimbs served as controls while the left were experimentals. The left forelimbs were denervated by transecting the 3rd, 4th and 5th brachial spinal nerves at 8, 9, 10, 11, 12, and 13 days post-amputation. Since nerves reinnervate the limb at 7 days following denervation (Kelly and Tassava, 1973), it was necessary to redenervate every 6 days in order to maintain the denervated state of the limbs.

The blastemas were categorized in stages according to the morphology that each regenerate had attained initially at 8 through 13 days of regeneration. The limbs were observed every alternate day throughout the two week experimental period. At 1 or 2 weeks post-denervation, the forelimbs were harvested and histologically prepared and the final state of the blastemas was examined macroscopically as well as
microscopically at 430X for signs of regression (drastic resorption of the blastema which was present at the initial time of denervation), stationary (the blastemas remained static following denervation), or regeneration (the blastema continued development following denervation).

**Cell Cycle Studies**

Forelimbs of 80 larvae were amputated and allowed to regenerate for 8 days. At 8 days post-amputation, the left forelimbs of all larvae were denervated. The larvae were divided into two groups of 40 larvae each and maintained separately. One group of 40 larvae was injected i.p. through the tail musculature with 1.5 μCi of 3H-thymidine at 9 days post-amputation (1 day post-denervation) and the other group of 40 was similarly injected on day 10 (2 days post-denervation). At two hours post-injection, limbs of three larvae were fixed in Carnoy's fixative. Additionally, limbs of three larvae were fixed at each 8 hour interval post-injection thereafter through 80 hours. Limbs were histologically prepared, serially sectioned longitudinally at 10 microns and short ribbons containing 5 sections each were distributed on 4 slides such that each slide contained representative regions of the limb and blastema. A single slide of each limb was randomly chosen for Feulgen staining of mitotic figures and autoradiography (Kelly and Tassava, 1973).

Each blastema was sampled microscopically at 430X in the middle and on the two sides for labelled mitotic figures using the humerus as a landmark. Background counts were less than 4 grains thus, any
mitotic figure which had 4 or more silver grains (Figs. 4 and 5) was considered labelled. A total of 50-60 mitotic figures per blastema was sampled for labelling. The means of 3 blastemas at each sample time were plotted through the experimental period of 80 hours. In order to determine the influence of the apical wound epidermis on the cell cycle parameters of blastema cells, 9 and 10 day blastemas of control and denervated limbs were divided into distal and proximal halves, i.e. near or distant to the wound epidermis. Each half was examined for labelled mitotic figures, as described above. A total of 25-30 mitotic figures were examined for labelling per region for each blastema. The mean percent labelled mitoses of three distal and three proximal blastema halves were plotted at 8 hour intervals throughout the experimental period. The cell cycle parameters were determined from the plot of percent labelled mitoses as will be described below.

Cell cycle parameters were determined for 9 and 10 day control blastema cells as well as 9 and 10 day blastema cells which were denervated for 24 and 48 hours respectively prior to pulse labelling the larvae with \(^3\)H-thymidine. Cell cycle parameters were also determined on distal and proximal blastema cells of 9 and 10 day control and denervated blastemas. According to the methods of Quastler and Sherman (1959) and Takahashi (1966) the following cell cycle parameters were determined from percent labelled mitoses curves (Fig. 6): (1) Cell Cycle Time (CCT) = time from the first to the third 50% intercept, (2) S phase = time of the first to second 50% intercept,
(3) $G_2 + \frac{1}{2}M = 0$ time to first 50% intercept, (4) $G_1 + \frac{1}{2}M = CCT - (G_2 + \frac{1}{2}M + S$ phase), and (5) Mitosis $(M) = (\text{Mitotic Index}) \times (CCT)$. Once $M$ is determined, the $G_1$ and $G_2$ values can be calculated by subtracting $\frac{1}{2}M$ from the $G_1 + \frac{1}{2}M$ or $G_2 + \frac{1}{2}M$ values.

**Mitotic and Labelling Index Studies**

Forelimbs were amputated and allowed to regenerate for 8 days at which time mound stage blastemas were present (Fig. 1). The left limbs were denervated as previously described, by transecting the 3rd, 4th and 5th brachial spinal nerves which innervate the forelimb. From 3-6 control and denervated blastemas were fixed daily, beginning 8 days post-amputation through day fifteen.

Limbs were histologically prepared and Feulgen stained. A total of 600-700 cells per blastema (excluding erythrocytes) were sampled for mitotic index determinations. Cells with chromosome configurations between late prophase and late telophase were scored as mitotic figures. This mitotic index (MI) was representative of the entire blastema. In order to determine the influence of the apical epidermal cap on the mitotic index of cells located either near or distant to the W.E., the slides that were used to determine the MI of the entire blastemas were resampled distally and proximally for mitotic figures. A total of 300-400 cells were sampled in each blastemal half. The mitotic index was calculated and plotted for each day throughout the experimental period (8-15 days post-amputation).
Portions of the data were statistically analyzed using the Student's t-test when possible.

Labelling indices were determined on 3-4 innervated and denervated blastemas at 9, 10, 11, 13 and 15 days post-amputation. Limbs were amputated and allowed to regenerate for 8 days at which time the left limbs were denervated. Following denervation, axolotls were replaced in an incubator at 21.5° C ± 1. As previously described, each larva was i.p. injected with 1.5 μCi of ³H-thymidine and fore-limbs were harvested and fixed 2 hours post-labelling. Following fixation, the limbs were histologically prepared and processed for autoradiography by standard procedures outline above.

A total of 700-800 cells per blastema were examined microscopically at 430X. Nuclei with 5 or more silver grains were considered labelled. The labelling index (LI) was calculated as follows:

\[ LI = \left( \frac{\text{# cells labelled}}{\text{total # cells examined}} \right) \times 100. \]

Labelling indices were also calculated for distal and proximal blastema regions. A total of 300-400 cells per region were sampled.
CHAPTER III
RESULTS

Determination of the Transition Period From Nerve-Dependency to Nerve-Independency

Experiments have shown that denervation of nerve-dependent blastemas results in regression and a concomitant decrease in mitotic activity while nerve-independent blastemas after denervation show no signs of regression, but a similar less drastic decrease in mitotic activity was observed following denervation (Schotte and Butler, 1944; Butler and Schotte, 1949; Singer and Craven, 1948; Powell, 1969). These studies show that denervation affects early blastemas (ND) to a greater extent than older blastemas (NI). Since the present studies were designed to determine the influence of nerves on the mitotic and labelling indices and the cell cycle parameters of nerve-dependent (8 day denervated) blastemas, it was important to determine when blastemas were nerve-dependent, transitional or nerve-independent.

Blastemas that were denervated 8, 9, 10, 11, 12, or 13 days post-amputation and observed either 1 or 2 weeks later responded quite differently to nerve withdrawal, depending on the stage of regeneration. Upon histological observation, the blastemas were divided into the following categories: (1) regressing, (2) stationary and (3) regenerating. Table 1 shows that 8 and 9 day blastemas were
## Table 1.

**DETERMINATION OF THE TRANSITION PERIOD FROM NERVE-DEPENDENCY TO NERVE-INDEPENDENCY**

<table>
<thead>
<tr>
<th>Age of Regenerate At Denervation</th>
<th>Morphological Stage</th>
<th>Number of Cases</th>
<th><strong>FINAL STATE OF BLASTEMA AT 1 OR 2 WEEKS POST-DENERVATION</strong></th>
<th>Nerve Requirement&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Regressed</td>
<td>Stationary</td>
</tr>
<tr>
<td>8</td>
<td>mound&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>early cone</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>mid-cone</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>late cone</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>early paddle</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>mid-paddle</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup>ND, nerve-dependent; NI, nerve-independent; TP, transition period.

<sup>b</sup>The final stage of the blastema was observed at 1 week post-deneration for this group only and all others were observed at 2 weeks post-deneration.
Figure 1. A light photomicrograph of a longitudinal section through a mound stage blastema at 8 days post-amputation. This is a nerve-dependent blastema which will regress if denervated as shown in Figure 7. Note the distal accumulation of blastema cells (B), wound epidermis (W.E), distal end of the amputated humerus (H) and differentiated stump muscle (M). Harris Hematoxylin and Eosin. X 80.
mound and early cone stages respectively (Fig. 1) and in all cases, these blastemas regressed following denervation, at both days. Unlike the 8 and 9 day blastemas, where 100% of the cases regressed, 1/3 of the 10 and 11 day blastemas were stationary following denervation. Early and late paddle stages (Fig. 3) were present at 12 and 13 days of regeneration respectively. These blastemas continued to develop into small limbs after denervation and did not show any signs of regression, however there was a single stationary blastema in the 13 day group. Since none of these blastemas regressed, they were considered nerve-independent (NI). Considering the entire population of regenerates, the transition period (TP) from nerve-dependency to nerve-independency was the time interval when denervation of the blastemas resulted in regression and stationary activities, i.e. blastemas of 10 and 11 days post-amputation. Some of these blastemas were thus in the process of attaining some degree of cellular stability as well as neural independency since the blastema cells were not removed from the population as compared to denervated nerve-dependent, regressing blastemas (day 9 or younger) (Fig. 7) (Schotte and Butler, 1944; Butler and Schotte, 1949; Singer and Craven, 1948). Thus individual axolotl blastemas undergo a transition from ND to NI some time between 10 and 11 days post-amputation.

Figure 7 shows a typical nerve-dependent regressing blastema that was denervated on day 8 and fixed 1 week later. Note that the blastema has been essentially resorbed back to the amputation surface and the dermis is beginning to encroach over the amputation surface.
Figure 2. A light photomicrograph of a late cone stage blastema 11 days post-amputation. This blastema falls within the transition period (TP) from nerve-dependency to nerve-independency (see Table 1). Denervation of this blastema would not result in regression. Cartilage has not yet begun to differentiate although the blastema cells are "condensing" preparatory to differentiation in regions of greater density (arrows). The amputation surface is along the bottom edge of the figure (W.E., wound epidermis). Feulgen stain. X 80.
Figure 3. Longitudinal section through a 15 day nerve-independent, paddle stage blastema. This blastema is considerably larger than 8 day blastemas (compare with Figure 1). Feulgen stain. X 30.
Figure 4. A light photomicrograph of mitotic figures from a longitudinal section through a 9 day blastema. Autoradiography was done on sections from this blastema after a 2 hour $^3$H-thymidine incorporation period (note the labelled nuclei, large arrows). Note the mitotic figures are not labelled indicating that G2 is greater in length than 2 hours (Figure 14). Three mitotic figures are shown in this view (small arrows). Feulgen stain. X 600.
Figure 5. An autoradiograph showing labelled mitotic figures (arrows). Notice the silver grains along the chromosome arms. The high density of the condensed chromosomes tends to obliterate the silver grains. Also notice the high labelling index of the blastema cells which is greater than that seen after a 2 hour incorporation period. Feulgen stain. X 1750.
Figure 6. A method of analyzing the cell cycle parameters from a plot of the percent labelled mitoses post-labelling. This method involves pulse labelling a population of cells with $^3$H-thymidine and scoring the percent labelled mitoses versus time post-labelling (Quastler and Sherman, 1959; Takahashi, 1966; Mitchison, 1971). It should be noted that only those cells in the S phase of the cell cycle will incorporate the DNA precursor as well as those cells entering S during the pulse when the precursor is available, and thus will be labelled. If label is available for only a short duration, a block of cells will be labelled which can be followed through the cycle by harvesting blastemas at various time intervals post-labelling and observing the percent labelled mitoses. The following cell cycle parameters are determined from a plot of percent labelled mitoses versus time post-labelling: (a) $G_2 + \frac{1}{2} M = 0$ time to 1st 50% intercept, (b) S phase = time from the 1st to 2nd 50% intercept, (c) CCT, cell cycle time = time from the 1st to 3rd 50% intercept, (d) $G_1 + \frac{1}{2} M = \text{CCT} - (G_2 + \frac{1}{2} M + S)$ and (e) $G_1 + M + G_2 = \text{time from the 2nd to 3rd 50% intercept.}$
Figure 7. A light photomicrograph of longitudinal section through a regressing nerve-dependent blastema 7 days post-denervation. At the time of denervation, an eight day mound stage blastema was present (Figure 1). Note the great number of glandular cells in the wound epidermis and especially in the distal-most tip (compare this Figure with Figures 1, 2, and 3). The dermis (D) tends to encroach and prevent contact between the wound epidermis (W.E.). The few remaining blastema cells are aggregated in the vicinity of the dermis-free wound epidermis (cells between arrows). Dermis-like material (arrow) can be seen permeating inward towards the blastema cells. (H, humerus). Feulgen stain. X 80.
thus decreasing contact between the wound epidermis and underlying dedifferentiated mesodermal cells. Few mesenchymal cells are located between the amputation surface and the wound epidermis and those that are present can be seen concentrated in the vicinity of the dermis-free wound epidermis (compare Figs. 1 and 2 with Fig. 7). This regressing blastema was comparable in age to a 15 day paddle stage blastema (Fig. 3), i.e. both are 15 days post-amputation, however the latter has many more blastema cells than the former. These results were basically similar to those of Butler and Schotte (1949) for denervated larval Ambystoma maculatum limbs.

The wound epidermis of control and denervated limbs is quite similar during the first 3 days post-denervation, but at 4-5 days, some of the denervated blastemas begin to show a decrease in the relative ration of glandular to cuboidal cells in the wound epidermis. The decrease in cuboidal cells of the wound epidermis of denervated limbs is most pronounced at 5 days post-denervation and closely resembles the case shown in Figure 7. A comparison of Figure 7 with Figures 1, 2 and 3 shows that the wound epidermis of innervated regenerating limbs tends to have more cuboidal cells than glandular cells. The wound epidermis of denervated limbs resembles the skin epidermis that covers the limb stump (Fig. 3).

Denervated, early paddle stage blastemas do not exhibit the typical regression phenomenon observed in denervated nerve-dependent blastemas. By 2 weeks post-denervation (26 days post-amputation), the 12 day, early paddle stage blastemas were well differentiated and a
small 2 digit regenerate was present (Fig. 8). As can be seen morphogenesis occurred quite normally in the absence of nerves even though there was a delay as compared to the 26 day control regenerate (Fig. 9). Figure 9 shows the contralateral control limb which has regenerated for 26 days. Note the 4 digits present indicating that regeneration is almost complete, since axolotls have 4 digits on their forelimbs. Control blastemas reach 2, 3, and 4 digit stages by 18, 20 and 23-24 days post-amputation respectively. In regard to morphologically stages, there appears to be a considerable delay between 12 day control and denervated blastemas at the end of a 2 week denervation period. Normal 12 day blastemas reached the 2 digit stage at 18 days post-amputation, but the 12 day denervated blastemas reach the 2 digit stage on day 26 post-amputation, thus denervation seems to delay morphogenesis by about one week.

**Mitotic and Labelling Index Studies**

Limbs that were denervated on day 8 and harvested daily throughout the following 7 days eventually showed a decline in the number of cells undergoing mitosis and synthesizing DNA. As can be seen in Figure 10, a significant (P<0.01, t-test) difference was not observed between the mitotic indices of 8 day denervated and control blastemas until 4 days post-denervation. The 12 day control and contralateral 8 day denervated blastemas had mitotic indices of 4.26 and 2.23 respectively (Table 2). In the latter, the MI continued to decline to 1/10 the 12 day value by day 15, while the controls declined only
Figure 8. A light photomicrograph of a longitudinal section through a denervated nerve-independent regenerate. At 12 days post-amputation, a paddle stage blastema was present. This limb was denervated on day 12 and fixed 2 weeks later. Limbs were re-denervated at 6 day intervals in order to maintain the denervated state of the limbs. Note that morphogenesis occurred in the absence of nerves (D, digit; R/U, radius or ulna). Feulgen stain. X 70.
Figure 9. A photomicrograph of a section through a 26 day innervated regenerate. This regenerate is comparable in age to the regenerate in Figure 8 which was denervated on day 12. Note that there are 4 distinct digits (D) as well as carpal elements (CE) and radius and ulna (R and U). Feulgen stain. X 70.
Figure 10. Mitotic index of control and denervated blastemas through 7 days post-denervation. All left limbs were denervated on day 8. The right innervated limbs served as controls. The mitotic index of the denervated blastemas was significantly (P<0.01, t-Test) lower than the controls at 4 days post-denervation (12 days post-amputation). (Con, control innervated blastemas; den (day 8), denervated all the left blastemas on day 8). At 15 days post-amputation, denervated blastemas had regressed tremendously (Figure 7) while contralateral innervated blastemas were paddle stage (Figure 3). Vertical lines represent the standard error of the mean of 3-6 blastemas sampled at each point. (n=number of blastemas sampled at each point).
TABLE 2.

COMPARISON OF MITOTIC INDICES IN CONTROL AND DENERVATED LIMBS

<table>
<thead>
<tr>
<th>Day</th>
<th>Mitotic Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>8c</td>
<td>2.02±0.62d</td>
</tr>
<tr>
<td>9</td>
<td>1.94±0.45</td>
</tr>
<tr>
<td>10</td>
<td>2.43±0.31</td>
</tr>
<tr>
<td>11</td>
<td>3.55±0.82</td>
</tr>
<tr>
<td>12</td>
<td>4.26±0.43</td>
</tr>
<tr>
<td>13</td>
<td>3.46±0.46</td>
</tr>
<tr>
<td>14</td>
<td>3.54±0.27</td>
</tr>
<tr>
<td>15</td>
<td>3.62±0.21</td>
</tr>
</tbody>
</table>

*Left limbs of all larvae were denervated on day 8 and were either fixed or allowed to regenerate several days post-denervation (1-7 days post-denervation).*

*Data are represented as the mean±SE of 3-6 limbs.*
slightly. The 4 day delay prior to a significant decrease in the MI of the denervated blastemas may be due to a slow decay or slow utilization of "existing trophic factor" and once exhausted, the levels of mitoses begin to decline. This idea is reasonably consistent with the Tassava and Mescher hypothesis (1975) concerning the role of nerves in relation to the cell cycle. This view will be elaborated upon in the discussion.

The MI of nerve-dependent, transitional and nerve-independent blastemas ranged from 1.94-2.02, 2.43-3.55 and 3.46-4.26 respectively (Table 2). The MI was highest for 11 day (late cone and paddle stage) blastemas. Blastemas denervated on day 8 peaked beyond the controls only twice, on days 9 and 11 (Fig. 11 and Table 2) and steadily declined thereafter to less than 0.3%. During the same time, the MI of control blastemas rose to a peak at day 12 and remained between 3-4% through day 15.

As stated earlier, nerve-dependent blastemas show drastic signs of regression within a week following denervation (Fig. 7). Although cells are clearly being removed from the blastema as regression occurs a few cells nevertheless remain in the cell cycle as is evident by a low level of mitosis and DNA synthesis (Fig. 11) one week following denervation.

The labelling index (LI) for control 9, 10, 11, 13 and 15 day blastemas ranged from 51.4-81.0% (Fig. 12) while 8 day denervated blastemas during the same days post-amputation ranged from 32-77%. Just as denervation significantly lowers the level of mitosis in the
denervates, the number of cells synthesizing DNA is affected also. A significant difference in LI between innervates and denervates was observed at 5 days post-denervation (13 days post-amputation) (Fig. 11), however a 4 day post-denervation point was not included in these studies. The decrease in the LI of the controls at day 15 is probably due to the differentiation of cartilage cells and other mesodermal cells as seen in figures 2 and 3. The condensation of cells along the axis of late cone blastemas represents the first sign of differentiation.

Control blastemas from 8 through 14 days post-amputation did not show a significant difference in the mitotic index of cells located distally and proximally (Fig. 13). As a general trend, the cells located proximal to the amputation surface had somewhat lower mitotic indices than those located distally, but this difference was not statistically significant. However, the denervated blastemas showed a significantly lower MI in the proximal areas than the distal areas at days 10 and 14 (P>0.01 and P<0.02 respectively, t-Test).

The labelling index of distal and proximal denervated blastemas was essentially the same at all the days examined (Fig. 12). The control blastemas were similar to the denervated blastemas in the sense that distal and proximal cells had equal labelling indices at all the days observed except day 15, when there was a significant difference between the distal and proximal LI (P<0.01, t-Test).
Figure 11. Labelling index of denervated and control blastemas. Left limbs were denervated (den) on day 8 post-amputation while right innervated limbs served as controls (con). 3-4 larvae were given a single i.p. injection of $^3$H-thymidine (1.5 μCi per larva) on days 9, 10, 11, 13 or 15 post-amputation, and fixed after a 2 hour incorporation period post-labelling. It is important to point out that blastemas used in labelling index studies incorporated $^3$H-thymidine for 2 hours (a 2 hour pulse) and were fixed immediately thereafter while those blastemas used in cell cycle studies were fixed between 2 and 80 hours post-labelling. The labelling indices of denervated blastemas were significantly lower than the controls at days 13 ($P < 0.005$, t-Test) and 15 ($P < 0.02$, t-Test). Each point represents the mean of 3-4 blastemas. (SD = ± X).
Figure 12. Labelling index of distal and proximal halves of control and denervated blastemas. Left limbs (B) were denervated on day 8 while right innervated limbs (A) served as controls. 3-4 larvae were given a single i.p. injection of $^3$H-thymidine (1.5 μCi) on days 9, 10, 11, 13 or 15 post-amputation, and fixed after a 2 hour incorporation period post-labelling. The distal halves of the 15 day control blastemas were significantly greater (P < 0.01) than the proximal halves. Denervated limbs did not show a significant difference in either half at any of the days observed.
### TABLE 3.
**COMPARISON OF LABELLING INDICES IN CONTROL AND DENERVATED LIMBS**

<table>
<thead>
<tr>
<th>Day</th>
<th>Labelling Index (%)&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>9</td>
<td>80.9±3.54&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>71.0±2.50</td>
</tr>
<tr>
<td>11</td>
<td>67.7±0.98</td>
</tr>
<tr>
<td>13</td>
<td>68.3±4.57</td>
</tr>
<tr>
<td>15</td>
<td>51.5±0.37</td>
</tr>
</tbody>
</table>

<sup>e</sup>All larvae were labelled by i.p., injecting 1.5 μCi of ^3^H-thymidine on days 9, 10, 11, 13 and 15 post-amputation and fixed 2 hours post-labelling.

<sup>f</sup>All left limbs were denervated on day 8 while right innervated limbs served as controls.

<sup>g</sup>Data represents the mean±SE of 3-4 limbs.
Figure 13. Mitotic index of distal and proximal halves of control and 8 day denervated blastemas. Limbs were denervated on day 8 and 3-6 control (A) and denervated (B) limbs were fixed at 8 through 14 days post-amputation. The distal and proximal halves of the control blastemas were not significantly different at any of the days sampled the distal halves of the denervated blastemas were significantly greater than the proximal halves at days 10 (P < 0.01) and 14 (P < 0.02) (con-d, distal half of the control blastema; con-p, proximal half of the control blastemas; den-d, distal half of the denervated blastemas; den-p, proximal half of the denervated blastemas).
Cell Cycle Studies

It has been hypothesized that nerves and the wound epidermis influence the different phases of the cell cycle (Tassava and Mescher, 1975; Mescher and Tassava, 1975) during the initiation of regeneration. Thus it was hoped that an analysis of the cell cycle parameters of blastema cells following denervation would help define the role of nerves and the wound epidermis during later stages of regeneration as well.

The length of the cell cycle parameters of 9 day blastema cells denervated on day 8 (24 hours of denervation at the time of labelling) was increased by about 34% over the controls. This increase was due to an increase in the mean duration of $G_1$ and/or $G_2$ phase(s) of the cell cycle (Fig. 14 and Table 4). Control 9 day blastema cells were found to have a mean $G_1+G_2$, S, M and cell cycle time of 6.94, 32.00, 1.06 and 40.00 hours duration respectively while the mean $G_1+G_2$, S, M and cell cycle time of 9 day (24 hour denervated) blastema cells were 21.78, 30.50, 1.22 and 53.50 hours duration respectively. Thus, denervation does not appear to affect the M or S phase of the cycle, but the $G_1+G_2$ phases were increased by 3 fold.

The above data do not distinguish between an increase in $G_1$ or $G_2$ phase alone, since the initial $G_2+M$ is not affected by a 24 hour denervation period (Fig. 14), but the time interval between the 2nd and 3rd 50% intercepts shows an increase which could be due to $G_1$ as well as $G_2$ in that this time interval is equal to the duration of
Figure 14. Percent labelled mitoses of 9 day control (cong) and denervated (den) blastemas through 80 hours post-labelling. Left limbs were denervated on day 8 post-amputation. The right innervated limbs served as controls. All larvae were labelled on day 9 (0 time) by i.p. injecting 1.5 μCi of $^3$H-thymidine per larva. Of the 1.5 μ Ci of $^3$H-thymidine injected on day 9, 20% of the radioactivity of the injection dose was lose to the water by 4 hours post-labelling and 50% within 24 hours. A plateau was reached at 24 hours and the 50% level of radioactivity of the water was maintained through 72 hours post-labelling. The radioactivity of the plasma at 4 hours post-labelling was 80% less than the activity at 2 hours post-labelling. These data tend to suggest that a single injection of $^3$H-thymidine (1.5 μ Ci per larva) does constitute a pulse label of about 4 hours duration. Also the plots of percent labelled mitoses suggest that cells were not continuously labelled since distinct peaks and troughs were observed. If the injection dose constituted a continuous level, distinct peaks and troughs would not have been observed.
### TABLE 4.
**CELL CYCLE PARAMETERS OF 9 DAY BLASTEMA CELLS**

<table>
<thead>
<tr>
<th>Blastema</th>
<th>Cell Cycle Parameters (hr)</th>
<th>Cell Cycle Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G₁+G₂</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>6.94</td>
<td>32.00</td>
</tr>
<tr>
<td>Denervated (day 8)</td>
<td>21.78</td>
<td>30.50</td>
</tr>
</tbody>
</table>

### TABLE 5.
**CELL CYCLE PARAMETERS OF 10 DAY BLASTEMA CELLS**

<table>
<thead>
<tr>
<th>Blastema</th>
<th>Cell Cycle Parameters (hr)</th>
<th>Cell Cycle Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G₁</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>1.44</td>
<td>38.00</td>
</tr>
<tr>
<td>Denervated (day 8)</td>
<td>3.44</td>
<td>34.00</td>
</tr>
</tbody>
</table>
$G_1+M+G_2$ phases (Fig. 6). An increased M phase can probably be ruled out since M is generally a constant and short duration compared to the more variable $G_1$ and $G_2$ phases (Mitchison, 1971; Young, 1962). The $G_2$ phase which is included in the 2nd and 3rd 50% intercepts is the second $G_2$ phase following the pulse label at 0 time (Fig. 6) and thus could very well be increased over the initial $G_2$ phase that is traditionally determined from the 1st 50% intercept.

In an attempt to determine if nerves were affecting the $G_1$ or $G_2$ phases in the above experiment, a second experiment was designed. Assuming that nerves are required for some $G_2$ event(s) prior to mitosis (Tassava and Mescher, 1975), a longer denervation period may be required before an initial increase in the $G_2$ phase occurs, i.e. cells "use up" trophic factor slowly. For this reason, 8 day blastemas were denervated and cell cycle studies were begun 48 hours post-denervation (10 days post-amputation). Control and denervated 10 day blastema cells were shown to have cell cycle times of 46.50 and 50.00 hours duration respectively. The mean $S$, $G_1$, $G_2$ and M phases of the 10 day control blastema cells were 38.00, 1.44, 5.94 and 1.12 hours in duration respectively (Fig. 17 and Table 5). These data suggest that both $G_1$ and $G_2$ were increased in length by two fold following a 48 hour denervation period, however the $S$ and M phases were essentially unaffected. Even though the mean duration of $G_1$ and $G_2$ is doubled in the denervated blastemas, the total cycle time is increased only about 8% as compared to the controls.
In summary, denervation of nerve-dependent blastemas (mound and early cone stage blastemas; day 8 and 9 post-amputation respectively) result in regression of the blastemas within one week post-denervation, lowered mitotic and labelling indices at 4 and 5 days post-denervation respectively and a 2 fold increase in the G₁ and G₂ phases of the cell cycle following a 48 hour denervation period. Even though the G₁ and G₂ phases are affected by denervation, the M and S phases are relatively constant following 24 and 48 hour denervation periods. The cells which remain in the cycle in 9 and 10 day blastemas (24 and 48 hour denervation periods respectively) continue to cycle within about 50 hours which is slightly longer than control 9 or 10 day blastema cells (which cycle in 40.0 and 46.6 hours respectively). Thus, it can be concluded that denervation of ND blastemas does not appear to greatly extend the overall cell cycle time of blastema cells following a 48 hour denervation period.

Effects of the Wound Epidermis on the Cell Cycle

Several workers (Hearson, 1966; Faber, 1960) have presented evidence of an apical predominance of mitotic activity in mound and cone stage blastemas of axolotls. Recently, Tassava and Mescher (1975) hypothesized that the wound epidermis serves to keep post-mitotic cells in the cell cycle for further rounds of DNA replication and mitoses during the initiation of regeneration. Thus, it is possible that the distal wound epidermis of the blastema or the
"apical epidermal cap" (Thornton, 1968) influences the mitotic activity as well as the cell cycle time of blastema cells located directly underneath the apical epidermal cap as opposed to those cells located farther away.

The influence of the apical epidermal cap on blastema cells located distally or proximally to the amputation surface was examined in terms of the cell cycle. Essentially no differences were found in the cell cycle parameters of 9 day control or denervated blastema cells located either distally or proximally within the blastema. In the controls, the cell cycle times of distal and proximal cells were 39.00 and 39.50 hour duration respectively (Fig. 15 and Table 6). Similarly, the cell cycle times of distal and proximal cells of denervated blastemas were 52.0 and 53.5 hour duration respectively (Fig. 16 and Table 6). Table 7 data (Figs. 18 and 19) show that the distal and proximal location of blastema cells in either 10 day control or denervated (denervated on day 8) blastemas resulted in essentially no effect on the cell cycle time. Blastema cells located either distally or proximally (i.e. close to or distant from the W.E.) in 10 day control blastemas had cell cycle times of 46.00 and 48.50 hour duration respectively while the cell cycle times of distal and proximal cells of contralateral denervated (48 hour denervation period) blastemas were 49.5 and 51.0 hour duration respectively. Thus, it appears that cells of 9 or 10 day control or denervated blastemas
Figure 15. Percent labelled mitoses of distal and proximal halves of 9 day control blastemas through 80 hours post-labelling. Each point represents the mean of 3 blastema halves (S phase, DNA synthetic phase; con-d, distal half of control blastemas; con-p, proximal half of control blastemas).
Figure 16. Percent labelled mitoses of distal and proximal halves of 8 day denervated blastemas. Left limbs were denervated on day 8 while the right innervated limbs served as controls. Each larva was labelled by injecting 1.5 μ Ci of ^3H-thymidine on day 9. Three blastemas were fixed at 8 hour intervals through 80 post-labelling. Each point represents the mean of 3 blastema halves (S phase, DNA synthetic phase; den-p, proximal half of denervated blastemas; den-d, distal half of enervated blastemas).
Figure 17. Percent labelled mitoses of 10 day control (con) and denervated (den) blastemas through 72 hours post-labelling. Left limbs were denervated at 8 days post-amputation and each larva was labelled on day 10 (0 time). The right innervated limbs served as controls. Each point represents the mean of 3 control or denervated blastemas (S phase, DNA synthetic phase).
Figure 18. Percent labelled mitoses of distal and proximal halves of 10 day control blastemas through 72 hours post-labelling. All larvae were labelled on day 10 (0 time) and fixed at 8 hour intervals through 72 hours post-labelling. Each point represents the mean of 3 blastema halves (S phase, DNA synthetic phase; con-d, distal half of control blastemas; con-p, proximal half of control blastemas).
Figure 19. Percent labelled mitoses of distal and proximal halves of 8 day denervated blastemas. Limbs were denervated on day 8 and each larva was labelled on day 10. Blastemas were fixed through 72 hours post-labelling. Each point represents the mean of 3 blastema halves (S phase, DNA synthetic phase, den-p, proximal half of denervated blastemas; den-d, distal half of denervated blastemas).
TABLE 6.

CELL CYCLE PARAMETERS OF 9 DAY BLASTEMA CELLS:
Distal Versus Proximal Blastema

<table>
<thead>
<tr>
<th>Blastema</th>
<th>Cell Cycle Parameters (hr)</th>
<th>CCT (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G_1 + G_2$</td>
<td>$S$</td>
</tr>
<tr>
<td>Control</td>
<td>6.08</td>
<td>32.00</td>
</tr>
<tr>
<td>Proximal</td>
<td>5.88</td>
<td>32.50</td>
</tr>
<tr>
<td>Denervated (day 8)</td>
<td>19.40</td>
<td>31.50</td>
</tr>
<tr>
<td>Proximal</td>
<td>20.00</td>
<td>32.00</td>
</tr>
</tbody>
</table>

TABLE 7.

CELL CYCLE PARAMETERS OF 10 DAY BLASTEMA CELLS:
Distal Versus Proximal Blastema

<table>
<thead>
<tr>
<th>Blastema</th>
<th>Cell Cycle Parameters (hr)</th>
<th>CCT (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G_1$</td>
<td>$S$</td>
</tr>
<tr>
<td>Control</td>
<td>1.98</td>
<td>36.00</td>
</tr>
<tr>
<td>Proximal</td>
<td>0.91</td>
<td>39.50</td>
</tr>
<tr>
<td>Denervated (day 8)</td>
<td>4.10</td>
<td>33.00</td>
</tr>
<tr>
<td>Proximal</td>
<td>3.55</td>
<td>36.00</td>
</tr>
</tbody>
</table>
either distally or proximally located within a respective blastema
will cycle within 40-50 hours, however the lengths of the $G_1$ and $G_2$
phases of the blastema cell cycle appear to be increased 2 fold
following a 48 hour denervation period.
The present study was an attempt to determine the influence of nerves and the apical epidermal cap on the mitotic and labelling indices and cell cycle parameters of larval axolotl (*Ambystoma mexicanum*) blastema cells. Nerves have been shown to be essential for normal levels of cell proliferation and macromolecular synthesis throughout salamander limb regeneration (Singer, 1952; Singer, 1974; Thornton, 1968). Following amputation of the limb, mesodermal cells of stump tissues in the vicinity of the amputation surface dedifferentiate and begin synthesizing DNA by 4 days post-amputation. If nerves are present, mitotic proliferation is activated by 6 days post-amputation and blastema formation ensues within the second week (Hay and Fischman, 1961; Thornton, 1938; Mescher and Tassava, 1975). If the limb is denervated at the time of amputation, mitotic proliferation is not initiated nor does a blastema accumulate (Mescher and Tassava, 1975; Tassava *et al.*, 1974; Kelly and Tassava, 1973; Schotte and Butler, 1941; Singer, 1952).

Schotte and Butler (1941; 1949) demonstrated that denervation shortly after amputating the limb prevents regeneration in larval *Ambystoma maculatum* and *Triturus viridescens* (22-44 mm in length),
but if denervation was delayed beyond the "critical period" or transition period (TP), essentially no effect on morphogenesis was observed. The TP for Ambystoma maculatum larvae was between days 7 and 9 and blastemas 7 days old or younger were nerve-dependent (ND) while 10 day and older blastemas were nerve-independent (NI). Limbs that were maintained in the denervated state prior to the TP regressed starting at the level of the blastema and proceeding proximally.

Table 1 shows that the TP for blastemas of axolotl larvae ranging from 57 to 68 mm in length (snout-tail tip) was between 10 and 11 days and blastemas younger than 10 days were ND while 12 day and older blastemas were NI. Figure 7 shows a typical nerve-dependent regressing blastema that was denervated on day 8 (Fig. 1) and observed 1 week later. If denervation was delayed to day 12, when the blastema was NI, the blastema underwent differentiation and morphogenesis and a small two digit regenerate was present (Fig. 8) at 2 weeks post-denervation (26 days post-amputation). The difference between larval Ambystoma maculatum and larger Ambystoma mexicanum (axolotls) in terms of the time blastemas become nerve-independent is that the former regenerates faster than the latter, thus the stages were attained faster. Smaller larvae generally regenerate faster and higher temperatures (up to 26°C) also enhance regeneration rate (Tassava, unpublished).

Denervated regressing blastemas have been shown to contain a greater degree of intercellular debris, which suggest autolysis of cells (Butler and Schotte, 1949), as well as a general connective
tissue appearance (many sparsely spaced cells with a high degree of matrix material in between) (Bryant, et al., 1971; Butler and Schotte, 1949). These observations were confirmed in the present study. Figure 7 shows a regressing blastema that was denervated on day 8 and fixed 1 week later. As can be seen, there were few undifferentiated cells remaining distal to the amputation surface and the overall character of the cells resembles that of connective tissue in that the cells are surrounded by fibrous matrix material similar to the collagenous dermis that lies between the epithelium and the mesodermal tissues of the limb. The increased matrix material may be attributed to cell loss as well as increased matrix synthesis by the remaining blastema cells or some unidentified cell type within the limb stump.

Singer and Craven (1948) showed that denervation of adult newt regenerates prior to day 13 resulted in varying degrees of regression which suggests that these regenerates were ND, but if denervation was delayed beyond day 13, growth in length and morphogenesis occurred nevertheless, thus these regenerates were NI. Their study also showed that the level of mitoses in denervated nerve-independent blastemas decreases only slightly from that of the innervated controls through 10 days post-denervation, however denervated nerve-dependent blastemas showed a precipitous decline in the level of mitosis to the baseline level of unamputated limbs within 10 days. Similarly, the present study showed that denervated ND blastemas exhibited a precipitous decline in the level of mitosis and, at day 7 post-denervation (Fig. 10), the level of mitosis reached baseline levels. Why does the level of mitoses remain higher in denervated NI blastemas
as compared to denervated ND blastemas? It is possible that NI blastema cells may have sufficient stored trophic factor to undergo several further rounds of cell division after denervation, while ND blastema cells have less stored trophic factor and thus undergo only 1 or 2 rounds of mitosis after denervation. The pale or vacuolar cells observed by Bryant et al (1971) and Popiela (1972) in denervated, regressing ND blastemas may represent cells which are blocked in some phase of the cell cycle and are in the process of breakdown (Mescher and Tassava, 1975). Consistent with this idea are the data in figures 10 and 11 which show that while regressing blastemas have no significant mitotic activity 7 days post-denervation, the labelling index with \(^3\text{H}-\text{thymidine}\) is still above that of unamputated limbs. Thus, after denervation of day 8 blastemas, those cells which undergo mitosis may continue to proceed in the cell cycle and replicate DNA but become blocked in \(G_2\). The labelling index would therefore decrease less rapidly than the mitotic index as the data show. It is important to note also that the mitotic index of axolotl blastemas is highest on days 10, 11 and 12 post-amputation, when blastemas are becoming nerve-independent.

This study does not confirm a rapid initial rise in the number of mitoses per mm\(^3\) immediately following denervation of blastemas as seen by Singer and Craven (1948). Rather than a rapid rise in the number of mitoses following denervation, an equal increase was observed in both control and denervated blastemas from day 8 to 11 post-amputation. One source of difference between these studies could be
the choice of sampling technique. Singer and Craven (1948) sampled cross sections of blastemas and expressed their results as the number of mitoses per mm$^3$ of regenerate, whereas in the present study, the number of mitoses was expressed as a percentage of the total blastema cells counted in longitudinal section. Another source of difference between the two studies was that Singer and Craven (1948) often used a single regenerate per sample point for the experimental series. The present study utilized 3-4 blastemas in the denervated studies and 3-6 blastemas in the control groups per sample point.

Autoradiographic and biochemical studies have shown that denervation adversely affects blastema cell macromolecular synthesis. Autoradiographic studies have revealed that denervation does not immediately affect the level of DNA synthesis during the early stages of regeneration. DNA synthesis was initiated in dedifferentiating mesodermal stump tissues by four days post-amputation in innervated as well as denervated limb stumps (Mescher and Tassava, 1975; Hay and Fischman, 1961; Tassava et al, 1974). However, even though denervated and innervated stumps both initiate DNA synthesis, only the innervated stumps continue and maintain high levels of synthetic activity beyond 1 week post-amputation. Mescher and Tassava (1975) showed that by 10 days post-denervation, the labelling index of denervated limbs (LI = 18%) was $\frac{1}{2}$ that of the innervated limbs (LI = 36%). Similarly, axolotl regenerates denervated at the time of amputation initiated DNA synthesis on day 4 as did the control regenerates, but by 1 week post-denervation, the labelling index (22%)
was 1/2 that of the contralateral control limbs (Tassava et al., 1974). These experiments were interpreted to mean that dedifferentiation and the initiation of DNA synthesis were nerve-independent events in that they occurred in the absence of a nerve supply. However, an increase in the labelling index occurred only in innervated limbs because mitosis increased the number of dedifferentiated cells. Mitosis did not occur in denervated limb stumps (Mescher and Tassava, 1975).

One way to explain the constant LI (15–20%) in denervated/amputated limbs at 2 weeks post-denervation, in the absence of mitosis, is that dedifferentiation of the stump tissue continues for an extended time beyond that of the normal limbs. Bodemer and Everett (1959) have shown that dedifferentiation continues well into the second week post-amputation and thus could account for the constant LI in denervated limb stumps. Essentially no cells were found in 8 and 14 day denervated limbs with more than the normal G₂ amount of DNA (4N) (Mescher and Tassava, 1975), thus the constant level of DNA synthesis is not due to continued rounds of DNA synthesis without mitosis (amitosis). Schotte, Butler and Hood (1941) seem to think that the accumulation of a blastema at the amputation surface tends to check the extent of dedifferentiation and go as far as to suggest that a transplanted blastema onto a freshly amputated larval limb will prevent dedifferentiation of the stump. According to this view, since no blastema ever accumulates on denervated limb stumps, dedifferentiation could
very well continue beyond the normal time interval and could account for a constant but low labelling index in those stumps.

Figure 11 shows that nerves are also necessary to maintain normal levels of DNA synthesis during later blastema stages, nerve-dependent as well as nerve-independent. The labelling indices of control 9 day and experimental 9 day (24 hours post-denervation) blastemas were 80.9 and 77.2% respectively. By 1 week post-denervation the labelling indices of control and denervated blastemas declined to 51.4 and 38.0% respectively. This decline observed in the control blastemas at 15 days (Fig. 11) was probably due to the withdrawal of cells from the cell cycle to form condensed masses of cells (Fig. 3) prior to morphological and cytological differentiation. Only slightly fewer mitoses are observed among the inner most cells of these masses. Differentiation and proliferation are considered to be mutually exclusive events and is true in this case also (Cairns, 1966). Chalkley (1954) and Litwiller (1939) have shown that as differentiation progresses in the regenerate, the mitotic proliferation activity decreases.

Biochemical studies have demonstrated that the rate of blastemal protein, RNA and DNA synthesis are adversely influenced by nerves. Dresden (1969) reported that the rate of synthesis of these macromolecules declined after nerve transection in 18 day nerve-independent regenerates. The greatest rate of change was observed within the first 48 hours and was followed by a decrease which reached a plateau at about 60% of the normal innervated level. Singer and Caston (1972)
observed similar results using 10-13 day nerve-dependent blastemas. They observed an outburst in accumulation of newly synthesized protein, RNA and DNA which was followed by a decline which reached a plateau at about 60% of the control, innervated level at 48 hours post-denervation. These results support the view that a certain level of blastema macromolecular metabolism is nerve-dependent.

As previously shown, the DNA synthetic activity of nerve-dependent and nerve-independent blastemas is affected by denervation (Dresden, 1969; Singer and Caston, 1972). All stages exhibited a decline in the rate of incorporation of $^3$H-thymidine into newly synthesized DNA following denervation. This decline was probably not due to decreased activities of DNA polymerase (Dresden and Moses, 1973), thymidine and uridine kinases and aspartate carbamyl transferase (ACTase) (Manson et al, 1976) which are all related to DNA synthesis. Thus, the subsequent decline in the LI of axolotl blastemas denervated 8 days post-amputation (Fig. 11) is probably the result of a decreased proliferation rate (Fig. 10 and Table 2) which would decrease the number of cells entering the S phase from the $G_1$ phase. Nerves have been shown to produce a trophic factor which is important in the regenerative process (reviewed by Singer, 1974; Singer et al, 1976). Singer and Caston (1972) have suggested that the decline in the incorporation of $^3$H-thymidine into DNA as well as decreased RNA and protein synthesis following denervation are due to an exhaustion of the existing trophic factor. Data in Figure 10 and Table 2 show that the MI is significantly ($P<0.01$, t-Test) decreased at 4 days post-denervation. Singer and
Craven (1948) also observed a precipitous decline in the level of mitoses following denervation of ND blastemas. Common to all three of these studies is the fact that ND blastemas are drastically affected by denervation. These results can be interpreted to mean that as the trophic factor falls below a critical threshold level, the blastema cells cease to undergo mitosis and block in some phase of the cell cycle, subsequently affecting the level of RNA, DNA and protein synthesis of the whole blastema indirectly.

The specific phase in which cells are withdrawn from the cell cycle during regression is not known nor is it known how long these cells remain viable following denervation or the mechanism by which these cells are removed from the population during regression. In any case, that cells are removed from the blastema is evident from observations that (1) the MI and LI decrease tremendously (Figs. 10 and 11), (2) the number of vacuolar and pale cells increase (Bryant et al, 1971) following denervation of nerve-dependent blastemas, and (3) the blastema eventually is completely lost (Fig. 7).

Experiments utilizing microspectrophotometry and $^3$H-thymidine autoradiography could possibly distinguish the specific phase of the cell cycle ($G_1$, $G_2$, or S phase) in which blastema cells block after denervation prior to being removed during regression. Microspectrophotometric data would determine the fraction of cells containing the $G_2$ amount of DNA (4N or tetraploid). The fraction of cells in mitosis could be determined for the same slides used in the microspectrophotometric study by counting the number of mitotic cells per
total number of cells observed. In order to determine the number of cells in the S phase, i.e. synthesizing DNA, the slides used in the microspectrophotometric and mitotic index studies are decoverslipped and processed by standard autoradiographic techniques, after which the numbers of nuclei covered by silver grains per total nuclei observed are recorded. Finally the following determinations can be drawn from such data:

(1) Fraction of total CCT occupied by $G_1 = \frac{\# \text{ 2N cells}}{\text{Total \# cells observed}} \times 100$

(2) Fraction of total CCT occupied by $S = \frac{\# \text{ labelled cells}}{\text{Total \# cells observed}} \times 100$

(3) Fraction of total CCT occupied by $G_2 = \frac{\# \text{ 4N cells}}{\text{Total \# cells observed}} \times 100$

(4) Fraction of total CCT occupied by $M = \frac{\# \text{ mitotic cells}}{\text{Total \# cells observed}} \times 100$

If the fraction of $G_1$, $S$, or $G_2$ cells increase significantly following denervation as compared to the contralateral control blastemas, then it will be possible to specify the particular phase(s) that cells are blocked following denervation prior to being removed during regression. Experiments such as these are certainly warranted.

The role of nerves has been discussed from a biochemical and histological perspective and now the role of nerves in terms of the blastema cell cycle will be examined. Recently Mescher and Tassava (1975) proposed a model which suggests that nerves are necessary for some $G_2$ event(s) prior to mitosis of dedifferentiated cells of the
amputated limb. Initially it was thought that this hypothesis could be tested by actually measuring the different blastema cell cycle phases following the withdrawal of nerves from the limb. The present cell cycle data are generally in agreement with those for regenerating newt limbs. The first cell cycle study in regenerating limbs was that of Grillo's (1972). He showed that 18 day newt blastemas had a mean cell cycle time of 45.0 hours and the mean S phase, $G_2 + \frac{1}{2}M$ and $G_1 + \frac{1}{2}M$ phases were 37.5, 4.5 and 3.0 hours duration respectively. As can be seen from tables 4 and 5, these values were very similar even though the newt blastema was closer to being NI than the 9 or 10 day axolotl blastemas. The only other cell cycle study in the literature in regenerating salamander limbs was that of Wallace and Maden (1976) which was also the most recent. They utilized axolotls which were approximately twice the size of those used in the present study and employed a different technique of histologically preparing the blastemas for microscopic observation. They reported a 54.0 hour cell cycle time for cone stage blastema cells and the S, $G_1$, $G_2$ and M phases were 38.0, 5.0, 10.0 and 1.0 hour duration respectively. It is interesting that the S phases were essentially the same in all three studies, including the present study, and the $G_2$ phase occupied the second largest phase of the cycle. Older salamanders generally require a longer time to regenerate than younger ones, thus the 2 fold increase in the $G_1$ and $G_2$ phases of the older axolotls was not unexpected.

The applicability of the percent labelled mitoses method of analyzing cell cycle parameters to a population of growing cells rests
on the following conditions: (1) asynchrony of cell division and
(2) constancy of the mean cell cycle time (Takahashi, 1966; Mitchison,
1971).

That the above conditions are met by cells of larval axolotl limbs
during regeneration is indicated by the following observations:
(1) There were no precipitous decreases followed by sharp increases in
the mitotic and labelling indices (Figs. 10 and 11) within the normal
cell cycle time of 9 and 10 day blastema cells (40.0 and 46.5 hour
duration respectively). If the blastema cells were synchronized, a
precipitous decrease followed by a sharp increase in the mitotic and
labelling indices at some point within a 40–47 hour interval or every
48 hours would have been observed. (2) Blastema cells seem to have a
rather constant mean cell cycle time. The mean cycle time of 9 and 10
day innervated blastema cells was 40.0 and 46.5 hour duration respec-
tively (Tables 4 and 5). Similarly, the mean cycle time of 9 and 10
day denervated blastema cells (24 and 48 hour denervation period
respectively was 53.5 and 50.0 hour duration respectively (Tables 4
and 5). Thus ND and early NI blastema cells appear to be an asynchro-

nous population of cells and tend to have a rather constant mean cell
cycle time, both of which are essential to the applicability of the
percent labelled mitoses method of cell cycle analysis. Data from
Singer and Craven (1948) and Wallace and Maden (1976) are consistent
with this view.
In regard to the cell cycle, nerve-dependent and nerve-independent blastemas cells probably do not have significantly different cell cycle parameters. Control 9 day labelled blastema cells are ND while 10 day labelled blastema cells are a mixture of both ND and partly NI cells, and thus fall within the transition period from nerve-dependency to nerve-independency (Table 1). Data in tables 4 and 5 from control 9 and 10 day blastemas show that the $G_1 + M + G_2$ phases, i.e. the time interval from the second to the third 50% intercept was 8.0 and 8.5 hours duration respectively while the $S$ phases are 32.0 and 38.0 hours duration respectively. It should be pointed out that the cell cycle parameters of control 9 and 10 day blastema cells are determined within 48 hours post-labelling (from 0 time to the third 50% intercept; Figs. 6, 14 and 17), thus 9 day ND blastema cells are transitional cells at 48 hours post-labelling. Similarly, 10 day transitional blastema cells at the time of labelling (0 time, Fig. 17) are completely NI at 48 hours post-labelling (day 12 post-amputation). This means that the cell cycle parameters of 10 day blastema cells could probably represent the cell cycle parameters of NI blastema cells since 10 day blastemas are partly NI at the start of the cell cycle analysis and are completely NI at the end of the time interval required to analyze the cell cycle parameters. Thus, it is possible to conclude that the cell cycle parameters of NI and ND blastema cells are essentially the same. This view is important when considering the mechanism by which blastemas become nerve-independent. It can be said therefore that
the transition of blastemas from ND to NI does not involve any major changes in the total CCT or in any of the times of the phases (M, G₁, S or G₂).

Figure 14 and table 4 show that the mean S and M phases of 9 day blastema cells of control and 8 day denervated blastemas were essentially the same, but the mean G₁ + G₂ duration was tripled in the denervates. The fact that constant S and M phases were observed was not surprising, since these two phases were found to be the least variable in many mammalian and avian somatic cells in vivo (Cameron, 1964; Young, 1962) as well as in vitro (Cameron and Greulick, 1963). These latter studies also showed however, that the G₁ phase was generally more variable than the G₂ phase. It was not possible to determine if the delay in the second ascending arm of the percent labelled mitoses curve in figure 14 of the denervates was due to an increase in G₁ or G₂ phase or both, since the time from the second to the third 50% intercept (Fig. 6) was equal to the mean duration of G₁ + M + G₂ phases; thus an increase between these two intercepts could theoretically represent an increased G₁ or G₂ phase, but rarely an increased M phase (Mitchison, 1971). Therefore the durations of the G₁ and G₂ phases was combined in this instance and expressed as a single unit, G₁ + G₂. The mean cell cycle time was increased by approximately 34% in the denervates which was due to an increase in the G₁ + G₂ time. The S phase, or DNA synthetic phase, occupies 80% of the total cell cycle time in the controls and 57% in the denervates.

This decrease in the percentage of the cell cycle occupied by the S
phase in the denervates, again was due to an increase in the duration of the $G_1 + G_2$ phases, which automatically increases the duration of the total cell cycle while the $S$ phase remains constant (Table 4). These data suggest that the $G_1$ and/or $G_2$ phase(s) of the cell cycle were influenced by nerves while the $M$ and $S$ phases remained relatively constant following nerve withdrawal.

A comparison of the percent labelled mitoses curves of denervated and control blastemas (Fig. 14) reveals that the withdrawal of nerves did not affect the normal flow of labelled mitoses until 40-48 hours post-labelling or 64-72 hours post-denervation. This is consistent with the observation that a decline in blastema MI was not observed until 3 days post-denervation (Fig. 10). A significantly ($P<0.01$, t-test) lower mitotic index was observed at 4 days post-denervation and beyond in experimental blastemas (8 days plus 4 days post-denervation) compared to control blastemas of similar post-amputation age.

It was seen from the above study that denervation for 24 hours did in fact increase the duration of the $G_1 + G_2$ phases of the cell cycle. Thus, a second cell cycle study was designed to determine if the $G_2$ phase of the cell cycle was increased following a 48 hour denervation period prior to pulse labelling the larvae. The rationale for allowing a 48 hour denervation period over a 24 hour period was to label a cohort of cells that had begun to respond to nerve withdrawal by increasing their cell cycle time as noted above, since blastema cells may not respond to denervation until their supply of trophic factor is exhausted. However, table 5 shows that a 48
hour denervation period did not greatly increase the cell cycle time beyond the 24 hour denervation period (Table 4). The most obvious differences between the 10 day control and contralateral 8 day plus 48 hour denervated blastemas were the 2 fold increase in the $G_1$ and $G_2$ phases.

It can be concluded that the rate of DNA synthesis by an individual blastema cell is relatively unaltered by denervation during the 5 day experimental period (9-13 days) in that the S phases are relatively close to the control values. These results are in agreement with studies where the activity of DNA polymerase, uridine and thymidine kinases and aspartate carbamyl transferase were shown to be essentially unaffected by denervation (Dresden and Moses, 1971; Manson et al, 1976).

Denervation has been shown to increase the mean duration of the $G_1$ and $G_2$ phases of the cell cycle by 2 fold (Tables 4 and 5), however this does not rule out the possibility that some cells are blocked in the $G_2$ phase of the cell cycle as Mescher and Tassava (1975) have suggested. If cells permanently blocked in the $G_2$ phase of the cell cycle, they would, thus not affect the cell cycle analysis which is based on the percent labelled mitoses observed post-labelling, i.e. those labelled cells that continue to cycle and undergo mitosis. $G_2$ populations of cells have been reported for chicken esophagus (Cameron and Cleffman, 1964), mouse kidney and duodenum (Pederson and Gelfant, 1970), human bone marrow in pernicious anaemia patients (S. N. Wickramasighe et al, 1969), and rat tumors (Janik, 1974; Post and Hoffman, 1969).
Mescher (personal communication) has recently observed an increase in the number of mitoses within 7 hours post-treatment of amputated/denervated newt limb stumps (7 days post-amputation and denervation) with fibroblast growth factor (FGF). These preliminary results suggest that dedifferentiated mesenchymal cells of amputated/denervated limb stumps may be blocked in the G\textsubscript{2} phase of the cell cycle since 7 hours is within the duration of a slightly extended G\textsubscript{2} phase but is not long enough for cells to proceed from G\textsubscript{1} or S to M. These studies also imply that fibroblast growth factor may be the "trophic factor" or that both represent a functionally related class of proteinaceous mitogenic substances.

The role of nerves has been discussed from a histological, biochemical and cell cycle point of view and the emphasis will now turn to the apical epidermal cap and its possible influence on the mitotic and labelling indices and the cell cycle of blastema cells. The apical epidermal "cap" is the distal most portion of the wound epidermis which migrates over the amputation surface and thus is not underlain by a dermis.

The earliest study to describe a difference in the mitotic activity of cells located distally or proximally within the blastema was that of Litwiller (1939) whose data showed a gradual shift in mitotic proliferation from the stump toward the distal half of the blastema in the Japanese newt. Chalkley (1954) observed that the mitotic activity
was greatest in the proximal half of the spotted newt blastema during the early stages of regeneration, but shifted distally during later stages of regeneration.

The above studies with newts tend to suggest that the epidermal cap does not affect the mitotic activity of blastema cells located directly underneath the cap, at least during the early stages of regeneration when the mitotic activity was highest.

Hearson (1966) and Faber (1960) did not confirm the proximal predominance in mitotic activity during the early stages of regeneration whereas Litwiller (1939) and Chalkley (1954) found the opposite in newts. The conflicting results of studies utilizing newts and axolotls may be due to different sampling techniques. In the former, cross sections were sampled at 100-160 μm intervals starting from the distal tip and proceeding proximally towards the amputation surface. In the latter, several longitudinal planes along the axis of the limb were sampled in four zones (Hearson, 1966). In each case, mitotic figures were scored and MI was calculated. Faber (1960) did not use a parameter related to cell proliferation, but followed cells that were indirectly marked with charcoal during an earlier stage of regeneration. The difference between these studies remain to be resolved by further experiments similar to those in newts so that a more direct comparison can be made between their proliferation patterns.

The results of the present study do not agree with those of Hearson (1966) and Faber (1960). No significant difference was found in the mitotic index of innervated blastema cells located distally or
proximally to the amputation surface (Fig. 13) during the four main stages of regeneration. However, limbs denervated showed a significant difference in the mitotic index of distal and proximal halves at 10 and 14 days post-amputation. As compared to the results of Chalkley (1954) and Litwiller (1939), the innervated blastemas of this study should have had a higher proximal MI. In regard to Hearson's (1966) and Faber's (1960) results, the present results were reversed in that the distal half of the blastemas should have been higher than the proximal halves. Why these studies were not corroborated is difficult to say, except that the areas sampled as well as the sampling technique may have resulted in the observed difference.

Labelling index studies again revealed that there were essentially no differences between the distal and proximal blastema halves in either innervated or denervated blastemas (Fig. 12). These results as well as mitotic index studies on newt blastemas tend to suggest that the wound epidermis may be less of a factor in keeping cells in the cell cycle during blastema stages (mound and cone stages) as compared to pre-blastema stages or that cells were affected equally by the W.E. throughout the blastema in regard to labelling and mitotic indices. If the apical wound epidermis was keeping cells in the cell cycle, a higher MI and LI should have been observed in the distal half of the blastemas, however these observations were not seen in the present study. Furthermore, cell cycle analysis of 9 or 10 day denervated or control blastemas reveal that there was essentially no difference in the cell cycle time of cells located distally or proximally (Figs. 18
and 19; Tables 6 and 7). These data imply that cells which remain in the cell cycle following denervation continue to cycle at the usual rate regardless of their location within the blastema.

The labelling index of control blastemas dropped from 68% on day 13 to 51.4% on day 15 (Fig. 11) which suggests that the fraction of cells remaining in the cell cycle was decreasing or that the other phases of the cell cycle were increasing while the S phase remained constant. In any case, fewer cells were in the S phase of the cycle. Figure 12 shows that there were significantly (P<0.01, t-test) fewer labelled cells in the proximal half of the 15 day control blastemas which could be interpreted to mean that cells were dropping out of the cycle in the process of redifferentiating. Early signs of differentiation were observed at the late cone stage (Fig. 12) which started proximally at the level of the amputation surface. Blastemas of 14 and 15 days were not included in these cell cycle studies, however it would be of interest to see if, in fact, the cell cycle time of the proximal differentiating cells were increased. However, it is more likely that these cells, prior to dropping out of the cycle, complete their final cycle within the normal 40-50 hours and enter the $G_1$ phase and, rather than continue in the cycle, they turn-on the biochemical pathways which lead to cytological differentiation into cartilage, muscle and various connective tissue types. Thus it is possible to have a lower LI, as the degree of differentiation increases, as may be the case in these 14 or 15 day blastemas.
The point of view that the proximal differentiating cells discontinue their proliferative activities without increasing their cell cycle time is supported by observations made in growing rat livers (Bucher and Malt, 1971). It was shown that at any one time 4-9 percent of the hepatocytes were synthesizing DNA in preparation for cell division at 3 weeks after birth. By 8 weeks of age, the proportion dropped to 1.5-2.5 per cent, a 66% decrease in the LI. Throughout this period, in spite of diminishing growth rate, the duration of the cell cycle remains relatively constant at 21.0-21.5 hours. There are similarities between these results and those of the present study. Firstly, the LI was higher at a time when the blastema was growing and less differentiated and declined with age as the degree of differentiation increased. Secondly, the cell cycle time remained constant even though differentiation was well underway. Similarly, Janner and Searls (1970) demonstrated that there were changes in the rate of cellular proliferation during differentiation in the mesenchyme of the embryonic chick wing without significant changes in the cell cycle parameters at different stages of development. They determined labelling indices in wings at stage 19, 22 and 24 to be 40-50%, 30-40% and 20-30% respectively. The cell cycle parameters of distal and proximal areas were essentially unchanged at all three stages. It was concluded that the decrease in LI in regard to increasing age was due to a decreased rate of cellular proliferation since the cell cycle parameters were essentially the same throughout the wing.
Thus, differentiation of some cells within a population can and does occur without affecting the overall cell cycle time of the proliferating cells. However, the relationship between the degree of differentiation and the LI seems to be inversely proportional in the chick wing, growing rat liver, and nerve-independent axolotl blastema.

The results of the various experiments conducted in this study are summarized below:

1. Denervation of 8 day blastemas causes an increase in the $G_1$ and $G_2$ phases of the blastema cell cycle (Tables 5 and 6) after both 24 and 48 hour denervation periods. Those blastema cells which continue to cycle do so in about 50 hours, a moderately longer time than control, innervated blastema cells. Through this same post-denervation time period (72 hours-7 days), the mitotic index drops in denervated blastemas and the blastema decreases in size by resorption. These observations suggest that beginning at 72 hours after denervation, some cells of the blastema block completely, fail to cycle, and are removed from the blastema. Those cells which continue to cycle eventually reach mitosis ($M$) and can be observed for label. It was not possible to distinguish if cells were being blocked in the $G_2$ phase of the cell cycle, as might be predicted by the Tassava and Mescher (1975). If cells are completely blocked in any phase of the cell cycle, cell cycle parameters would not be affected since the cell cycle analysis is based on plots of percent labelled mitosis. Thus cells must reach mitosis ($M$) to be recorded.

Experiments utilizing a combination of microspectrophotometry and autoradiography would give some information on the possibility of a $G_2$ blocked population of cells following denervation. Data from such experiments would allow an analysis of the build-up in the fraction of $G_2$ population of cells, assuming that the number of cells blocked in the $G_2$ phase was great enough to be detected beyond the normal $G_2$ population of cells.
As stated earlier, differentiated muscle, cartilage, bone and various connective tissues are in the non-dividing $G_0$ phase (or state) with regard to the cell cycle. Following amputation, these differentiated limb tissues begin to dedifferentiate and synthesize DNA by 4 days post-amputation, but mitosis is not initiated until day 6, this 48 hour interval between the onset of DNA synthesis and the initiation of mitosis suggests a long initial $G_2$. It would be interesting to see whether the initial cell cycle time is longer than subsequent cell cycles, as measured in the present investigation.

(2) The apical wound epidermis either affects cells equally throughout the blastema or affects them only slightly in that there was essentially no difference in the cell cycle time of cells in either the distal or proximal blastema halves (Tables 5 and 6). Nor was there a significant difference in the labelling and mitotic indices of distal or proximal halves except in the 15 day control blastemas (Figs. 12 and 13). The present results do not rule out the possibility that the entire wound epidermis which surrounds the blastema was actively influencing the blastema cells throughout the blastema, thus the cell cycle time, labelling and mitotic indices would be relative equal throughout the blastema as was observed in this study. Thus, a separate and influential role of the apical W.E. "cap" is not supported by this research.
BIBLIOGRAPHY


