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Garling, David John

THE RELATIONSHIP OF AMPUTATION, NERVES, AND WOUND EPIDERMIS TO ELECTROPHORETIC AND FLUOROGRAPHIC PROTEIN PATTERNS IN THE FORELIMB OF ADULT NEWTS

The Ohio State University

Ph.D. 1981

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THE RELATIONSHIP OF
AMPUTATION, NERVES, AND WOUND EPIDERMIS
TO ELECTROPHORETIC AND FLUOROGRAPHIC PROTEIN PATTERNS
IN THE FORELIMB OF ADULT NEWTS

DISSERTATION

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

By
David John Garling, B.A.

*****

The Ohio State University
1981

Reading Committee:
Dr. Roy A. Tassava
Dr. Thomas J. Byers
Dr. Lee F. Johnson

Approved By:

Dr. Roy A. Tassava
Adviser
Developmental Biology
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VITA

September 29, 1950 . . . Born - Ashland, Ohio

1972 . . . . . . . . . . . . B.A., Indiana University, Bloomington, Indiana

1975-1979 . . . . . National Institutes of Health Trainee and Teaching Associate, Molecular, Cellular, and Developmental Biology Program, The Ohio State University, Columbus, Ohio

FIELDS OF STUDY

Major Field: Molecular, Cellular and Developmental Biology

Studies in Regeneration, Professor Roy A. Tassava

Studies in Molecular Embryology, Professor Tim Hunt
PUBLICATIONS


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I. INTRODUCTION

The Process of Regeneration

The phenomenon of regeneration has held a long standing fascination for biologists. Spallanzani (1768) is given credit for the first major descriptive and experimental study of anuran and urodele limb regeneration (reviewed by Schmidt, 1968). Since then, much progress has been made in describing the regenerative process and in identifying important parameters that affect it; however, the exact mechanisms by which regeneration is initiated and regulated remain to be elucidated.

The work on limb regeneration in salamanders can perhaps best be summarized by referring to the major problem areas that have emerged. The three most comprehensive areas relate to the roles of injury, nerves, and wound epidermis in regeneration. A brief account of regeneration should precede a discussion of these three major factors.

Limb regeneration in salamanders can be divided into four phases: wound healing, dedifferentiation, proliferation, and redifferentiation. The wound-healing phase follows amputation and involves blood clotting and migration of epidermis from the surrounding skin over the amputation
surface. Ultimately, this wound epidermis becomes several cell layers thick but throughout regeneration remains free of underlying dermis.

Healing and dedifferentiation overlap during the first week of regeneration. Removal of cellular debris occurs concurrently with the dedifferentiation of cells close to the amputation surface. Bone, cartilage, and connective tissue matrices break down and muscle undergoes sarcolysis. Cells of these various tissues progressively lose their differentiated characteristics; and, when fully dedifferentiated, are identical in appearance to each other as seen by either light or electron microscopy (Hay, 1958; Hay, 1959; Hay and Fischman, 1961).

Autoradiographic studies indicate that significant synthesis of DNA, RNA, and protein begins as early as 4 days post-amputation and increases concomittently with dedifferentiation (Hay and Fischman, 1961; Bodemer and Everett, 1959; Kelly and Tassava, 1973). In newts, the mitotic index first increases on day 6, but dedifferentiation continues until about day 14 post-amputation (Mescher and Tassava, 1975). The proliferative phase extends from day 7 to day 35 and overlaps dedifferentiation and redifferentiation. As mesodermal cells dedifferentiate and proliferate, they accumulate under the wound epidermis to form the blastema. During the blastema proliferation phase, the wound epidermis is 10 to 15 cell layers thick.
The origin and fate of blastemal cells was an area of controversy for many years. An extensive histological study by Chalkley (1959) provided strong evidence that blastema cells were derived from the differentiated cells immediately adjacent to the amputation surface and not from reserve cells. An autoradiographic study by Hay and Fischman (1961) employing tritiated thymidine further substantiated Chalkley's findings. In a now classic study, Steen (1968), using cells labeled with $^3$H-thymidine and triploidy showed conclusively that cells of muscle, connective tissue, and cartilage dedifferentiate and contribute to the blastema. Steen's results further suggest that cells retain their original tissue identity throughout blastema proliferation.

A mound-shaped blastema, visible to the unaided eye, appears by day 20 or 22. During the next few days, the mound blastema increases in size and becomes cone-shaped. Redifferentiation takes place from days 25 to 35. As new cartilage, muscle, and connective tissue are formed, the external shape of the blastema progresses through palette and digit stages. A dermal layer reforms under the wound epidermis as skin redifferentiates.

The times given above for the various stages of regeneration apply to the adult newt, Notophthalmus viridescens, the species used in the present study. Larvae of most salamander species regenerate much more rapidly.
The Role of Injury in Regeneration

The role of injury in regeneration is not well understood; however, it is known that injury alone is sufficient to induce dedifferentiation of stump cells (Mescher, 1976; Loyd, 1978). If the wound epidermis is prevented from forming by inserting the distal end of the amputated limb into the coelomic cavity (Goss, 1956; Loyd, 1978) or by sewing whole skin over the amputation surface (Godlewski, 1928), dedifferentiation still occurs. Mescher (1976) demonstrated that the DNA labeling and mitotic indices in newt limbs with skin flaps increased to the same extent as in control amputated limbs through day 7 post-amputation, but subsequently subsided and regeneration was inhibited.

Loyd (1978) found that cells of innervated limb stumps inserted into the coelomic cavity dedifferentiated, replicated DNA, and divided but did not remain in the cell cycle for additional rounds of proliferation. Loyd noted that dedifferentiated cells did not accumulate in the inserted limb stump; and, hence, no blastema formed. Likewise, if limbs are amputated, denervated, and then inserted into the coelomic cavity, cells of the stump dedifferentiated and replicate DNA, but the mitotic index is very low (Loyd, 1978). Taken together, these studies clearly indicate that injury alone, in the absence of both nerves and wound epidermis, is a sufficient stimulus for cellular dedifferentiation and replication of DNA.
Tassava and Loyd (1977) reinjured skin graft limbs of newts 5 weeks following amputation and skin grafting. They found that the regeneration rate after reinjury was markedly faster than in control limbs. This implied that the de-differentiation induced by injury could be preserved for at least 5 weeks. Injury has not been defined in biochemical terms to date. It may be that injury is mediated through changes in protein synthesis.

The Role of Nerves in Regeneration

The effect of nerves on limb regeneration has received much attention. Todd in 1823 (Singer, 1952) was the first to show that a denervated limb would not regenerate. Denervation of the forelimb is normally performed by severing the 3rd, 4th, and 5th brachial nerves in the shoulder region with sharp watchmaker's forceps.

Many parameters of the neurotrophic effect have been elucidated. These parameters fall into three broad classes: morphological-histological observations, biochemical effects, and attempts to characterize the presumptive neurotrophic substance(s). The morphological observations can be further subdivided into the threshold requirement, the type and connections of the nerves, and the time course of the neurotrophic effect.

Singer performed a long series of studies that culminated in the concept of a nerve threshold requirement. In
adult newts, an average of 9.6 nerve fibers per 100 \, \text{um}^2 \text{ of amputation surface is required for regeneration to occur (Singer, 1946; 1952). Trauma, in addition to that caused by amputation, lowers the threshold requirement (Singer and Mutterperl, 1963). Singer (1954) found that deviating the sciatic nerve to the forelimb of frogs evoked a regenerative response that is normally lost by frogs during metamorphosis. Since the ratio of nerve fibers to amputation surface is higher in the forelimbs of tadpoles than in adult frogs, this study provides further evidence for Singer's threshold concept. Singer (1954) found that either sensory or motor nerves alone could support regeneration. Hence, the direction of impulse conduction was not a factor in the neurotrophic effect.}

Overton (1950) grafted nervous tissue into the dorsal fin of *Ambystoma* larvae and observed a stimulation of mitotic activity in the overlying epidermis. Control grafts of liver, muscle, cartilage, glass, and paraffin to the tail fin did not lead to an increase in mitosis.

Kamrin and Singer (1955) implanted spinal cord pieces with limb segments into the dorsal fin of *Ambystoma* larvae. The limb segments became innervated by nerves growing from the spinal cord pieces and regenerated. This indicated that nerve connections to the spinal cord and brain are not essential for limb regeneration and that nerves act locally in the amputation area.
The neurotrophic effect is also related to the time course of regeneration. In newts, denervation at early times arrests regeneration and the early blastema is resorbed. Slightly older blastemas may resorb or continue to regenerate after denervation. All blastemas of cone or palette stages continue to develop after denervation and obtain normal morphology but are volumetrically smaller than innervated controls (Singer and Craven, 1948). About 50% of the limbs denervated at the early cone stage will regenerate. Limbs denervated at the mid-cone stage or later continue to regenerate (Powell, 1969). These three stages are referred to as nerve-dependent, transition, and nerve-independent respectively. A similar pattern with earlier times applies to young larvae. Limbs of Ambystoma larvae denervated as late as day 6 (early blastema stage) regress all the way to the shoulder region (Schotte and Butler, 1941; 1944). The denervated early blastema of an adult Ambystoma regresses, but the limb itself does not regress.

Ambystoma limbs that have never been innervated do not require nerves for regeneration (Yntema, 1959a; 1959b). These aneurogenic limbs were obtained by parabiosing two Ambystoma embryos and removing the neural tube from one of them. Consequently, the motor and sensory ganglia which normally supply the limb innervation do not develop. If the aneurogenic limb becomes innervated by grafting it to a
normal larva, then it becomes nerve-dependent (Thornton and Thornto, 1970). Singer (1965) proposed that limb tissues can produce the neurotrophic factor but that under normal conditions the nerve contribution predominates. Supposedly, once innervated, the non-nerve tissues of the limb lose their capacity to produce a sufficient amount of the factor.

The effects of denervation on macromolecular synthesis and enzyme activities have been examined by a number of researchers. DNA, RNA, and protein synthesis all decrease within a few days following the denervation of nerve-independent newt blastemas (Dresden, 1969). By 20 hours post-denervation, the rate of protein synthesis declines to a level 50 to 60% that of control innervated blastemas (Lebowitz and Singer, 1970). Singer and Caston (1972) noted the same pattern for RNA and DNA synthesis following denervation. DNA synthesis is depressed by denervation to a greater extent in the mesoderm of the blastema wound epidermis (Geraudie and Singer, 1978). This type biochemical comparison of wound epidermis and mesodermal core is an important but largely overlooked area in regeneration research.

Morzlock and Stocum (1972) found a 40 to 50% decrease in ribosomal and transfer RNA synthesis 24 hours post-denervation at both nerve-dependent and nerve-independent stages. Manson and Tassava (unpublished) observed a
reduction of $^3$H-uridine incorporation into rRNA 3 days and 7 days following denervation of day 17, nerve-independent newt blastemas. Bantle and Tassava (1974) reported similar results in a study of mRNA associated with free polyribosomes. Denervation reduced the incorporation of RNA precursors into both rRNA and mRNA associated with polyribosomes. Denervation also reduced the incorporation of labeled amino acids into nascent peptides.

Bast, Singer, and Ilan (1979) carried out a more extensive study similar to that of Bantle and Tassava. They found a 34% reduction in the ribosomal content of blastemas denervated for 48 hours relative to contralateral innervated limbs. The ratio of monosomes to polysomes, the polysome profile from sucrose gradients, and the average molecular weight of proteins synthesized were not significantly altered by denervation. This 34% reduction of ribosomes is paralleled by a 40% decrease in amino acid incorporation.

This study by Bast, Singer, and Ilan does not indicate whether the decrease in ribosomal content is a control point in the reduced level of protein synthesis or merely a by-product of some other process such as a reduction in transfer of mRNA. The study does not address the possibility of sub-populations of blastema cells. Is the reduction in rRNA a consequence of some cells dropping to very low levels of rRNA while others maintain relatively normal
levels? This could be the case if the neurotrophic effect is an all or none phenomenon for a given cell as suggested by Tassava and McCullough (1978). The absence of a major shift in the average molecular weight of proteins synthesized does not say anything about alterations of specific proteins. Does denervation alter the proteins produced by stump/blastema cells?

If *Ambystoma* limbs are denervated and amputated on the same day, the level of RNA synthesis, as determined by autoradiography, is the same as in innervated limbs for the first 6 days following amputation (Kelly and Tassava, 1973). This finding indicates that lack of innervation does not affect the quantitative level of RNA synthesis during the first phase of regeneration. Tassava, Bennett, and Zitnik (1974) performed a similar study to examine DNA synthesis and found only slightly lower levels of labeling in denervated limb stumps compared to innervated limb stumps during the first 7 days following amputation.

The observations that RNA and DNA synthesis increased in amputated denervated axolotl limbs but that mitotic indices remained close to zero led directly to the idea of dedifferentiating cells blocking in the $G_2$ phase of the cell cycle (Tassava and Mescher, 1975). Similar results were obtained for DNA synthesis and mitosis in amputated denervated newt limbs (Mescher and Tassava, 1975). Furthermore, microspectrophotometry provided evidence that the
chromosomal complement of denervated limbs went from a 2N to a 4N amount of DNA (Mescher and Tassava, 1975).

Manson (1974) found no difference in the incorporation of $^3$H-leucine of innervated and denervated newt regenerates up to 10 days post-amputation. After day 10, the level of incorporation in denervated limb stumps decreased to the low level of unamputated limbs, while the incorporation in the innervated limb stumps continued to increase. The activity of aspartate carbamyl transferase (ACTase), thymidine kinase and uridine kinase in innervated and denervated limbs 2 to 26 days following amputation was also measured (Manson, Tassava, and Nishikawara, 1976). ACTase is an enzyme in the de novo pathway of DNA synthesis. Thymidine and uridine kinase are in the salvage pathway of DNA synthesis. The pattern for ACTase activity in denervated limbs was unaltered relative to innervated limbs, but the level of activity was decreased 25 to 50%. Likewise, thymidine and uridine kinase of denervated limbs displayed patterns similar to, but 25 to 50% lower than those of innervated limbs. Manson's work indicated that the enzymes examined were affected by denervation but that they probably were not control points for the neurotrophic effect. Levels of DNA polymerase activity of blastemas cultured in vitro also appear to be unaltered by denervation (Dresden, 1973).
Mailman and Dresden (1979) concluded that denervation within 24 hours of amputation does not directly affect collagenase activity at post-amputation intervals up to 42 days, but that denervation does decrease the rate of collagen synthesis to a greater extent than the rate of non-collagen protein synthesis.

Kelly and Singer (1981) noted a 40% denervation induced drop in specific activity of the ATP pool. They attributed this drop to a nerve-related effect on membrane permeability.

Dearlove and Stocum (1974) performed SDS polyacrylamide gel electrophoresis on innervated and denervated newt blastemas at various stages of regeneration and found a wide array of banding differences; however, Dearlove and Stocum used tube gels. Garling and Tassava (1977) noted that complex banding patterns on tube gels are very difficult to compare either visually or by spectrophotometric scans. Because of this difficulty, most or all of the differences that Dearlove and Stocum reported are probably artifactual.

Singer (1978) used $^{35}$S-methionine labeling and two-dimensional electrophoresis to compare the proteins of 48-hour denervated and innervated blastemas. He found no qualitative differences between the autoradiographs. Autoradiographic spots from denervated blastemas were
generally, but not always, fainter than those of innervated blastemas.

Attempts have been made to isolate and identify the neurotrophic factor, but these attempts have been largely unsuccessful. In the early to mid-fifties, investigators speculated that neurotransmitters might have something to do with the neurotrophic effect. Evidence to date has run directly counter to this notion. Infusing sympatin, acetylcholine, and other neurotransmitters does not produce regeneration in a denervated limb (Taban, 1955). Botulinum toxin, the strongest known cholinergic blocking agent, does not stop regeneration when injected intraperitoneally (Drachman, 1971). Singer, Davis, and Scheuing (1960) infused a variety of neuropharmacological drugs into regenerating limbs (mimics, protectors, and blockers of acetylcholine). The results were inconclusive since doses sufficient to obtain a response caused substantial tissue damage. These studies provide evidence that the neurotrophic effect is not directly associated with neurotransmitters.

Deck and Futch (1969) and Burnett, Kary, and Lagorio (1971) claimed to stimulate the formation of small blastemas when they infused denervated limb stumps with blastema extracts. Their work is open to criticism, though, because re-innervation may have occurred during the course of the experiment.
Marcus Singer has made more progress toward isolating the neurotrophic factor than any other researcher. Yet even his results are rudimentary and difficult to repeat. Progress has been hindered by the lack of an adequate bioassay for the neurotrophic factor. Infusing a homogenate or column fraction into a newt limb for several hours is difficult. To infuse for a sufficient number of days to obtain a definite regenerative response is not possible at this time. Singer measures the stimulation of DNA or protein synthesis instead of blastema formation or outgrowth. This is a feasible test, but whether a quantitative increase in DNA or protein synthesis represents the first stage of a true regenerative response is questionable.

By infusing a crude nerve homogenate into mound stage blastemas 48 hours post-denervation, Lebowitz and Singer (1970) obtained a partial recovery of protein synthesis to 75% of the control innervated level. Singer, Maier, and McNutt (1976) found basic brain protein more effective than acidic protein in restoring protein synthesis. The effect was destroyed by heat or trypsin digestion, indicating that the factor may be a protein. Synaptosomal preparations from frog brain were also effective in stimulating recovery of protein synthesis. Thus, whatever it is that Singer and co-workers are isolating is apparently not species specific. DNA synthesis in early regenerates that have been denervated for 48 hours is normally 40% that of innervated
regenerates. Infusing newt brain extracts into denervated blastemas doubles the rate of DNA synthesis relative to uninfused denervated blastemas (Jabaily and Singer, 1977).

In vitro culturing of blastemas has also been utilized in the study of the neurotrophic effect. Globus and Liversage (1975) reported some growth and differentiation of explanted, innervated tail regenerates of *Ambystoma*. Liversage and Globus (1977) obtained similar results when they cultured innervated *Ambystoma* forelimbs. Dorsal root ganglia stimulated an almost three-fold increase in the mitotic index of cultured newt blastemas relative to blastemas cultured without dorsal root ganglia (Globus and Vethamany-Globus, 1977). The effect took place across low-porosity filters and displayed a proximo-distal gradient. This indicated that a molecule was responsible for the increase in mitotic activity and not cell contact. Adding insulin to the preceding culture system greatly increased the incorporation of both $^3$H-thymidine and $^{14}$C-labeled amino acid incorporation (Vethamany-Globus, Globus, and Tomlinson, 1978; Globus, 1978), but insulin stimulates DNA and protein synthesis in many culture systems. Choo, Logan, and Rathbone (1978) have been able to maintain in vitro levels of protein synthesis by adding adult newt and chicken embryo brain extracts to culture blastemas, but only for intervals of up to 20 hours. To date, all of the in vitro studies have been flawed by short culture times.
After three days in culture, blastemas deteriorate. It may well be that most cellular activities of the blastemas begin to decline from the moment that they are explanted.

Nerve extracts possess other in vivo and in vitro qualities which may or may not be related to the neurotrophic effect of regeneration. Lentz (1971) noted that extracts of newt sensory ganglia, spinal cord, liver, and nerve all increased the cholinesterase activity of cultured newt muscle. Oh and Markelonis (1978) isolated a protein subfraction from chicken sciatic nerve that was capable of maintaining acetylcholinesterase levels of cultured aneural embryonic or denervated adult chicken muscle. They later purified the protein and named it sciatin. Sciatin is essential for myogenesis and maintenance of muscle in vitro (Oh and Markelonis (1980). These studies and many others on axonal transport hint at a complex array of neurotrophic effects. Teasing out those which relate to regeneration from those that do not will no doubt be a very challenging problem.

The Role of the Wound Epidermis in Regeneration

The function of the wound epidermis is apparently distinct from that of injury. The cells of an amputated limb lacking a functional wound epidermis do not proliferate and do not form a blastema (Mescher, 1976; Loyd, 1978) even though dedifferentiation, replication of DNA, and initial
mitosis do occur as mentioned in the preceding section. Repeated removal of the wound epidermis at daily intervals or treatment of the skin with actinomycin D blocks regeneration (Thornton, 1957). Likewise, irradiating the wound epidermis daily with ultraviolet light prevents the formation of a blastema (Thornton, 1958). Immediate insertion of an amputated newt limb into the coelomic cavity blocks wound epidermis formation and the proliferation of blastema cells; but if insertion is delayed for one or more days, a wound epidermis forms and the inserted limb regenerates (Goss, 1956a and 1956b).

Displacement of the wound epidermis leads to a displaced blastema (Thornton, 1960). This observation led to the idea that the wound epidermis acts by attracting the blastema cells (Thornton, 1960); however, there is an alternative to Thornton's view. If the wound epidermis acts by keeping the cells in the cell cycle as suggested by Tassava and Mescher (1975), then a blastema would naturally form adjacent to the wound epidermis. This would be due simply to local proliferation (Mescher, 1976) rather than to migration (Thornton, 1968). In incomplete skin flap limbs, continued mitotic activity is restricted to the area immediately adjacent to the wound epidermis (Mescher, 1976; Tassava and Garling, 1979).

Several specific functions have been assigned to the wound epidermis: histolysis, debris removal during the
healing phase, and macromolecular secretion. Whether any of these functions directly relate to the more recently suggested proliferative role of the wound epidermis remains to be demonstrated. Taban (1955) discovered that wound epidermis was capable of dissolving blood clots. The blood clot that results from amputation does disappear within a few days after amputation. Acid phosphatase levels are elevated in the wound epidermis during mesodermal cell dedifferentiation (Weiss and Rosenbaum, 1967). Since acid phosphatase is associated with lysosomes, the wound epidermis may be active in histolysis.

Singer and Salpeter (1961) documented the removal of cellular debris through the wound epidermis. They also noted cisternal endoplasmic reticulum in the wound epidermal cells. Cisternal endoplasmic reticulum is often associated with cellular secretion of protein. Chapron (1974) found that $^3$H-fucose accumulated first in the wound epidermis during blastema formation and then moved into the blastema. Chapron suggested that the wound epidermis may act by synthesizing a glycoprotein necessary for regeneration.

Guy and Schmidt (submitted), using SDS polyacrylamide gel electrophoresis, found one band present in epidermis that was absent in mesodermal tissue; but they noted no banding differences between normal epidermis and wound epidermis.
Innervation of the wound epidermis is not essential in order for it to function in regeneration (Sidman and Singer, 1960; Singer and Inoue, 1964). The function of the wound epidermis is clearly required for regeneration, but beyond the evidence that it is involved with cellular proliferation, little is known about how the wound epidermis exerts its influence.

The Cell Cycle and Regeneration

An attempt has been made recently to integrate many of the observations relating to injury, the wound epidermis, and nerves into a unifying hypothesis. Tassava and Mescher (1975) proposed a cell-cycle hypothesis which ascribes the following functions to the three factors: 1) Injury stimulates cells that are in a $G_0$ resting state to dedifferentiate and replicate their DNA. 2) Nerves permit the cells to move from $G_2$ through mitosis. 3) The wound epidermis acts by keeping the cells in the cell cycle for subsequent divisions.

Maden (1978, 1979) recently challenged the $G_2$ block view by presenting mitotic index and microspectrophotometric data that favor a $G_1$ block; however, his method of denervating limbs is open to criticism. Instead of severing the nerves, which is the usual practice, he crushed them. It is generally acknowledged by neurobiologists and neurochemists that crushing nerves is not sufficient to
completely stop axoplasmic flow (Karlstrom and Dahlstrom, 1973).
II. RATIONALE AND AIMS OF THE PRESENT STUDY

Limb regeneration in salamanders is a complex and highly ordered developmental process. Injury, wound epidermis, and nerves are currently three of the most important factors influencing regeneration. Each of these three factors has been studied extensively. Yet in molecular terms, their mechanisms of action remain largely undefined.

Presumably, regeneration is ultimately controlled by the turning on and off of various sets of genes. Consequently, the rationale of this study is based on the assumption that regeneration can be understood in molecular terms.

The general aim of this study is to move in the direction of identifying molecular aspects of regeneration. For reasons that will be elaborated later, polyacrylamide gel electrophoresis and $^{35}$S-methionine gel fluorography were chosen as the initial approach to achieving the general aim.

Thus far, only two papers have been published which attempt to deal with the possibility of specific alterations of electrophoretic protein patterns during regeneration. Dearlove and Stocum (1974) compared the proteins of
innervated and denervated newt blastemas in electrophoretic tube gels. Donaldson, Mason, and Jennings (1974) employed tube gel electrophoresis to compare the protein patterns of regenerating newt limbs, regenerating tails, and healing back wounds. Both studies listed wide-ranging differences in banding patterns. Guy and Schmidt (submitted) compared banding patterns of unamputated limb, wound epidermis, and mesodermal core at various stages of regeneration using gradient slab electrophoresis. Guy and Schmidt noted fewer differences than Dearlove and Stocum or Donaldson et al. None of these studies examined protein synthesis.

The present study was oriented toward first testing the reproducibility of the earlier studies and then second, extending those observations through the use of fluorography to examine protein synthesis. In addition, significant variations from previous experimental designs were included. Slab gels were used to overcome the problem of comparing complex banding patterns between tube gels.

The experiments were designed to answer the following specific questions. All of the questions, with the exception of the third, were addressed in terms of both proteins present at a given time (stainable bands) and proteins being synthesized at a given time (fluorographic bands).

1) Do the protein bands of normal limb tissue differ from those of regenerating limb tissue?
2) Does denervation alter the normal protein banding pattern of regenerating limbs?

3) Does denervation alter the stainable protein banding pattern of unamputated limbs?

4) Is the unique role of the wound epidermis reflected in protein banding patterns that are different from those of mesodermal core?

5) Do the answers to the preceding four questions conflict with or complement the existing evidence in support of the cell cycle hypothesis?

Answers to these questions will, hopefully, further our understanding of the molecular basis of regeneration.
III. METHODS AND MATERIALS

Adult newts, *Notophthalmus viridescens*, were collected from southern Ohio. L-§S-methionine, 1070 Ci/mmol, was obtained from Amersham.

All newts were maintained in aerated tap water in an incubator at 25°C. They were exposed to continuous light and not fed during the experiments. A fungicidal solution of acriflavin and methylene blue was added to the water on occasion for a period of two or three days.

Forelimb amputations were performed through the middle of the radius-ulna and protruding bones were immediately trimmed flush with the amputation surface. Left forelimbs were denervated by severing the third, fourth, and fifth brachial nerves in the shoulder region with sharp watchmaker's forceps. Right forelimbs served as controls. Prior to denervation, the newts were anesthetized in a 1.5 g/l solution of ethyl m-aminobenzoate methanesulfonate (MS 222).

Wound epidermis was separated from the mesodermal blastema core using sharp watchmaker's forceps.

In labeling studies, four hours before tissue collection, each newt was injected intraperitoneally with 20 uCi
of $^{35}$S-methionine in 0.1 ml distilled water. Singer and Caston (1972) have found that incorporation of intraperitoneally injected $^{35}$S-methionine in newts is linear from 1 to 4 hours after injection. In sufficiently advanced regenerates, only blastemal tissue was collected. For earlier stages of regeneration and for denervated limbs, an equivalent amount of distal stump tissue was collected.

Tissues were homogenized on ice in 1 ml of a 10 mM TrisHCl, 1 mM EDTA buffer at pH 8.0. The homogenate was divided into two 0.5 ml aliquots which were quick-frozen in a dry ice-isopropyl alcohol bath. Following lyophilization, samples were stored in a freezer at -20°C.

Protein was quantitated by following the method of Esen (1978) with minor modifications. First, 200 ul of sample buffer (see Table 1) was added to each vial of lyophilized protein and placed in an 80°C water bath for 2 min. Samples of 5 ul were spotted onto 0.16-mm-thick No. 1 Whatman chromatography paper supported by a peg-style polypropylene test tube rack. Bovine serum albumin was used for the standard in concentrations that ranged from 0 to 50 ug per 5 ul spot in 5 ug increments.

After 5 min of drying, the paper was stained for 15 min in a solution of 65% distilled water, 25% isopropyl alcohol, and 10% acetic acid (v,v,v) with 0.1% (w/v) of Coomassie Brilliant Blue R-250. The paper was destained
for 5 min in several changes of distilled water and left to dry on the test tube rack for several hours.

The stained spots were cut out and placed in scintillation vials with 3 ml of 1% (w/v) SDS. The dye was eluted overnight and the following day was measured on a Gilford 240 spectrophotometer at 600 nm.

SDS polyacrylamide slab gels were made according to a formulation taken from the Cold Spring Harbor Laboratory Methods Manual. This was based, in turn, on Laemmli (1970) and Anderson, Baum, and Gesteland (1973). Table 2 lists the specifics of the gel and running buffer composition.

The gels were 10% acrylamide and the buffer system was discontinuous. The slab gel was 3 mm thick with a 3 cm stacking gel and a 12 cm running gel. Protein sample volumes of 20 to 100 ul containing 50 to 60 ug of protein were layered on the gels. Gels were run at approximately 1.2 watts for the stacking gel and 10 watts for the running gel. Molecular weight standards (BioRad) were run concurrently and included myosin (200,000 daltons), B-galactosidase (116,500 daltons), phosphorylase b (94,000 daltons), albumin (68,000 daltons), and ovalbumin (43,000 daltons). After fixing, gels were stained with Coomassie Brilliant Blue R-250. Table 3 lists the fixing, staining, and destaining solutions. Gels were photographed before drying.

Fluorography was carried out according to Bonner and Laskey (1974). The gel was immersed in 20 times its volume
of Me$_2$SO for 1 hour with a change to fresh Me$_2$SO after the first 30 min. The gel was then placed in 4 volumes of 2,5-diphenyloxazole (PPO) in Me$_2$SO (22.2% w/v) for three hours. Prior to drying, gels were soaked for 1 hour in two changes of a 1% glycerol, 10% acetic acid solution (v,v) to help reduce cracking of the gel. X-ray film (Kodak X-Omat R film) was photosensitized according to Laskey and Mills (1975). The film was kept in a low-temperature freezer (-80°C) for the 10- to 14-day exposure period.

Paraffin histology was performed on the wound epidermis and mesodermal core in one of the experiments. The tissue was fixed in Carnoy's (75% EtOH, 25% acetic acid (v,v) and dehydrated in serial EtOH solutions. Toluene was used as the exchange reagent between alcohol dehydration and paraffin infiltration. The tissues were sectioned at 10 um and stained with hematoxylin and eosin.

Five experiments were carried out. In most of the experiments, tissue of 10 limb stumps or blastemas was pooled, homogenized, and divided into two equal aliquots.

In the first experiment, newts were denervated on the day following amputation and distal limb stumps and/or blastemas collected on days 9 and 18 post-amputation and at the subsequent regeneration stages of mound, cone, palette, 3-digit, and 4-digit. These stages are easily identifiable and commonly used in regeneration work. The cone stage represents the transition period from a nerve-dependent to
a nerve-independent regenerate (Singer and Craven, 1948).

A second experiment was run along similar lines to the first. Days 9 and 18 were duplicated and day 5 was added. Three-day post-amputation, innervated limbs were also examined.

In the third experiment, the regenerates were denervated three days prior to tissue collection. Days 9 and 18 and the 4-digit stage were sampled. In all these experiments, left limbs were denervated and right limbs used as normal regenerates.

Taken together, these first three experiments supplied the data for answering the first two questions of the Rationale and Aims: 1) Do the protein bands of normal limb tissue differ from those of regenerating limb tissue? 2) Does denervation alter the normal protein banding pattern of regenerating limbs?

In the fourth experiment, the left limb was denervated and tissue from the middle of the radius-ulna limb region on the denervated left and the innervated right was collected on days 9, 18, and 27 post-denervation. This experiment was designed to detect any alteration of stainable bands by denervation without amputation.

In the fifth experiment, the wound epidermis of mound stage blastemas was separated from the underlying mesodermal core. Proteins of wound epidermis, mesodermal core, and whole blastema and stump immediately proximal to the
blastema, along with unamputated limb tissue, were then electrophoresed. Histology was performed on both wound epidermis and mesodermal core in order to check for the degree of separation between the two tissues. The intent of this experiment was to determine if the role of wound epidermis in regeneration is reflected in a banding pattern different from that of mesodermal core.

The sixth experiment was a comparison of the banding patterns of normal limb tissue, peripheral nerve, brain, kidney, and liver. This was done in order to obtain some idea of how many detectable limb bands were common to all tissue types.
TABLE 1

Sample Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithiothreitol</td>
<td>0.77 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>1.0 M TrisHCl pH 6.8</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>0.2% BPB in EtOH</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>Water to make</td>
<td>50.0 ml</td>
</tr>
</tbody>
</table>
### TABLE 2

Running Gel, Stacking Gel, Running Buffer

#### Running Gel--10% Acrylamide:

<table>
<thead>
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<th>Component</th>
<th>Volume</th>
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<tr>
<td>30% Acrylamide</td>
<td>30.0 ml</td>
</tr>
<tr>
<td>1% Bisacrylamide</td>
<td>11.7 ml</td>
</tr>
<tr>
<td>1.5 M TrisHCl pH 8.8</td>
<td>22.5 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.45 ml</td>
</tr>
<tr>
<td>Water</td>
<td>24.0 ml</td>
</tr>
<tr>
<td>10% AMPS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
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</tr>
</tbody>
</table>

#### Stacking Gel:

<table>
<thead>
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<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>3.34 ml</td>
</tr>
<tr>
<td>1% Bisacrylamide</td>
<td>2.6 ml</td>
</tr>
<tr>
<td>1.0 M TrisHCl pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Water</td>
<td>11.36 ml</td>
</tr>
<tr>
<td>10% AMPS</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
</tr>
</tbody>
</table>

#### Running Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>144.0 g</td>
</tr>
<tr>
<td>Trizma base</td>
<td>30.0 g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>25.0 ml</td>
</tr>
<tr>
<td>Water to make</td>
<td>5.0 l</td>
</tr>
<tr>
<td>TABLE 3</td>
<td></td>
</tr>
</tbody>
</table>

Fixing Solution, Staining Solution, Destaining Solution

**Fixing Solution:**
- Acetic acid 10.0%
- Methanol 50.0%

**Staining Solution:**
- Acetic acid 10.0%
- Methanol 50.0%
- Coomassie Brilliant Blue 0.2%

**Destaining Solution:**
- Acetic acid 7.0%
- Methanol 10.0%
IV. RESULTS

The first three experiments did not demonstrate any qualitative differences in either stainable bands or fluorographic bands between innervated amputated and denervated amputated limbs. All of the post-amputation and post-denervation times displayed the same banding pattern.

A set of stainable banding differences was consistently present between amputated stumps/blastemas and unamputated limbs regardless of whether or not the amputated limb stump was denervated or innervated. One band (band e) was present in unamputated limbs but absent (or greatly reduced) in amputated limb stumps/blastemas (46,000 daltons). Four bands (bands a, b, c, and d) were present in amputated limb stumps/blastemas but absent (or greatly reduced) in unamputated limbs (92,000, 62,000, 60,000, and 48,000 daltons).* One of these four bands (92,000 daltons) was faint and often did not show up clearly on photographs, but it was always present in the amputated limb stumps/blastemas. Stump tissue immediately adjacent to blastemas

*On a given gel lane, a band might be greatly reduced rather than totally absent relative to another lane. For ease of reference, the terms present or absent are used with the understanding that absent may imply greatly reduced in a given instance.
also displayed the five banding differences. The five differences were present as early as 3 days post-amputation and persisted through the 3-digit and 4-digit stages of regeneration (Figures 1, 3, 5, 7, 9, and 11). A total of 24 stainable bands was present in unamputated limbs and 27 bands in amputated limbs.

Figure 3 shows a stained gel in which the banding is slightly sharper than in the other gels. This gel is representative of how the lanes are arranged in other gels (Figures 1, 5, 7, 9, and 11) and illustrates the five banding changes found in all lanes that contain amputated limb tissue. Lanes 2 and 12 contain the protein molecular weight markers. Although many bands are present in these two lanes, the five most prominent bands are assumed to be the five standard proteins.

Lanes 1, 3 to 6, and 11 contain unamputated limb tissue. Lanes 7 to 10 contain amputated limb tissue. The four bands present in amputated limbs, but not in unamputated limbs, are present in lanes 7 to 10, but are absent in lanes 1, 3 to 6, and 11. These four bands are lettered a, b, c, and d. Conversely, the one band, lettered e, that is present in unamputated limbs, but absent in amputated limbs, is present in lanes 1, 3 to 6, and 11, but absent in lanes 7 to 10.

Lanes 7 and 8 contain limb tissue collected 9 days post-amputation. The limb tissue of lane 7 was denervated
1 day post-amputation. The limb tissue in lane 8 was the contralateral innervated limb that was amputated and collected on the same days as the limb tissue in lane 7. Lanes 9 and 10 are arranged similarly to lanes 7 and 8, but were collected 18 days post-amputation. The limb tissue in lane 9 was denervated 1 day post-amputation; and lane 10 contains tissue from the contralateral innervated limb.

The most important aspect of lanes 7 to 10 is the absence of any banding differences between denervated amputated limbs and innervated amputated limbs.

The gels in Figures 1, 5, 7, 9, and 11 have the same basic layout as the gel in Figure 3. Standard protein markers are usually in the far left lane and in the last or next to last lane on the far-right lane of the gel. Lanes with unamputated limb tissue are on the left side of the gel, and amputated limb tissues are to the right of the lanes with unamputated limb tissue. Lanes with amputated limb tissue are arranged in pairs. Each pair represents a post-amputation time or post-amputation stage. The left lane of each pair contains the denervated limb tissue, and the right lane of each pair contains the innervated limb tissue.

Numbering of lanes and lettering of bands for fluorographs is the same as for the corresponding gel. Also, the important observations and comparisons to be made on
Figures 1, 5, 7, 9, and 11 are the same as those made on Figure 3.

Fluorography did not indicate any consistent qualitative differences in synthesis between innervated amputated and denervated amputated limb stumps/blastemas (Figures 2, 4, 6, 8, 10, and 12). Overall levels of synthesis in limb stumps 18 days post-amputation that had been denervated on the day following amputation seemed surprisingly high relative to same-day innervated limbs (Figure 4, lane 9). General levels of labeling dropped off for denervated limbs at later post-amputation dates compared to innervated amputated limbs (Figures 8, 10, and 12).

Denervating the limb without amputating produced no stainable banding differences up to 27 days post-denervation (Figure 13). In Figure 13, standard protein markers are in lane 1, and unamputated limb tissue is in lane 2. Lanes 3 to 10 also contain unamputated limb tissue, but the limb tissues in lanes 3, 5, 7, and 9 were denervated 9, 18, 27, and 9 days respectively prior to collection. Unamputated denervated limbs were paired on the gel with contralateral unamputated innervated limbs--lane 3 with 4, 5 with 6, 7 with 8, and 9 with 10. The important comparisons to be made on this gel are between these pairs. It was, therefore, not possible to detect nerve proteins by subtraction (i.e. denervation). However, when peripheral nerve alone was examined, band differences were detectable
compared with limb tissues (Figure 18).

Only one stainable banding difference (Figure 14, band f, 57,000 daltons) was found between wound epidermis and mesodermal core—a band present in wound epidermis (lane 4) but absent in mesodermal core (lane 5). This same band was present in whole skin (lane 7) but not in unamputated limb mesoderm (lane 8). Hence, it is assumed at this point that the band is due to a specialized epidermal protein and not to a regeneration specific protein of the wound epidermis. Likewise, there were no differences between wound epidermis (lane 4) and mesodermal core (lane 5) on the fluorograph except for the previously mentioned band f (Figure 15). This one band consistently showed up on fluorographs of unamputated limbs (Figures 2, 4, 6, 8, 10, 12, and 15), whole limb skin (Figure 15, lane 7), blastemas (lane 3), and wound epidermis (lane 4), but not on fluorographs of unamputated limb mesoderm (lane 8). On the stained gel and on the fluorograph, wound epidermis and mesodermal core both displayed the five banding changes found in amputated limb tissue (Figures 14 and 15, lanes 4 and 5).

Histological examination of the isolated wound epidermis and mesodermal core indicated an almost completely clean separation of the two tissues (Figures 16 and 17). No epidermal cells adhered to the mesodermal core and less than 5% of the cells in the separated wound epidermis were
mesodermal. Thus, the similarities in the banding patterns noted above are real and are not due to cross-contamination of cells.

Appreciable stainable banding differences were present between unamputated limb tissues (Figure 18, lane 2) and other tissues--peripheral nerve (lane 3), brain (lane 4), kidney (lane 5), and liver (lane 6). In the fluorograph, aside from the two bands in unamputated limbs that are usually detectable, only one other tissue, liver (Figure 19, lane 6), labeled sufficiently to produce any bands.

The results of stainable banding differences are summarized on Table 4.
### TABLE 4

Summary of Results—Stainable Bands

<table>
<thead>
<tr>
<th></th>
<th>Bands subtracted</th>
<th>Bands added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>relative to</td>
<td>relative to</td>
</tr>
<tr>
<td></td>
<td>unamputated</td>
<td>unamputated</td>
</tr>
<tr>
<td></td>
<td>innervated limbs</td>
<td>innervated limbs</td>
</tr>
</tbody>
</table>

#### Amputated, innervated—
Days 3, 5, 9, 18 post-amputation, and mound, cone, palette, 3-digit, and full regenerate stages

|                         | 1     | 4     |

#### Amputated, denervated 1-day post-amputation—
Days 5, 9, 18 post-amputation, and mound, cone, palette, 3-digit, and full regenerate stages

|                         | 1     | 4     |

#### Amputated, denervated 3 days prior to collection—
Days 9 and 18 post-amputation and full regenerate stage

|                         | 1     | 4     |

#### Unamputated, denervated—
Days 9, 18, 27 post-denervation

|                         | 0     | 0     |

#### Amputated, innervated—

- mound stage
  - whole blastema
    - immediately proximal to blastema
  - wound epidermis
  - mesodermal core
  - stump tissue

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>4</th>
</tr>
</thead>
</table>
V. DISCUSSION

Proteins are the molecules which most directly influence the structure and function of cells and, therefore, are of great developmental significance. During regeneration of a salamander limb, there are seen such developmental phenomena as wound healing, dedifferentiation, cell proliferation, morphogenesis, and redifferentiation (Hay, 1966; Thornton, 1968). Tissue-tissue interactions can also be seen in that the wound epidermis (Mescher, 1976) and adequate nerve fiber numbers (Singer, 1946, 1952) are needed for the process of regeneration to occur. A knowledge of protein changes during normal regeneration and effects of experimental manipulation on these proteins will lead to a greater understanding of the entire process. Results from the present molecular approach will complement those from the early histological studies and more recent cellular studies on regeneration.

The results of the present study are a beginning in this direction. Several important aspects of the regeneration process were successfully examined with regard to the presence or absence of specific proteins. These fall into three categories: 1) proteins of regenerating limbs
relative to unamputated limbs, 2) the effect(s) of denervation on limb stump/blastaema proteins and on unamputated limbs relative to innervated limb stump/blastaema proteins, and 3) proteins in the wound epidermis relative to the underlying blastemal mesoderm.

One-dimensional SDS gel electrophoresis and fluorography were chosen as the methods for studying the proteins. This choice was a reasonable one given that no specific proteins important to regeneration have yet been identified and characterized. The present study was, therefore, aimed at the proteins that were extractable from whole stump/blastaema tissue using an SDS sample buffer. Most of the membrane proteins probably were solubilized by the SDS treatment employed. However, the extraction of membrane proteins by SDS is influenced by a number of variables (Maddy, 1976; Helenius and Simons, 1975). Hence, some of the proteins may have been extracted only partially or not at all.

The presence or absence of proteins was examined by staining and the synthesis of proteins by fluorography using $^{35}$S-methionine. None of the previous studies involving electrophoresis of proteins during limb regeneration (Dearlove and Stocum, 1974; Donaldson et al., 1974; Guy and Schmidt, submitted) dealt with protein synthesis.

None of the protein isolation, electrophoretic, or fluorographic techniques had been previously employed in
our laboratory. Although slab gel electrophoresis and fluorography are widely used techniques, it took considerable effort to develop a reliable procedure. The small size of the tissue sample also presented a challenge. About 60 newt blastemas were required to make 0.1 g of wet tissue. Refinements of the methods made it possible ultimately to obtain several gel runs from 10 pooled distal stump tips or limb blastemas. Each gel, therefore, represents the average of several pooled blastemas or limb stumps.

A total of 26 gels were run in order to produce the data for this study. This included, in many instances, duplicate gels to check for reproducibility of banding patterns. One hundred separate tissue samples were collected. This study greatly exceeded previous electrophoretic studies in the scope and thoroughness of the experimental design. A second denervation time, 1 day post-amputation, was added to the Dearlove and Stocum design (1974). The experiment using denervation without amputation had not been done prior to this study. Also, the examination of non-limb newt tissues is unique to this study. With the exception of the very preliminary results of Singer (1978), protein synthesis during regeneration had not been investigated by gel autoradiography or fluorography.
Normal Regeneration

A total of 27 bands were obtained from amputated limbs and 24 bands from unamputated limbs. In regenerates, one band present in unamputated limbs disappeared (band e) and four bands not present in unamputated limbs appeared (bands a, b, c, and d) (Figures 1, 3, 5, 7, 9, and 11). Hence, five differences appeared in stainable and fluorographic bands during normal regeneration relative to unamputated control limbs. These differences were present at 3 days post-amputation (Figure 1) and were maintained through 10 weeks post-amputation (Figure 11). This is an important finding because it indicates that regeneration initiates major qualitative changes in the proteins synthesized by the limb and that these changes are quite stable during the full course of regeneration. This is all the more striking since many proteins in cells are not present in sufficient quantities to be detected by one-dimensional gel electrophoresis. Amputation is sufficient to greatly stimulate protein synthesis in the relatively quiescent limb cells.

The molecular weights of the five banding changes related to amputation were compared to the molecular weights of common structural proteins. Several of the common structural proteins have molecular weights either well above or below those of the most significant bands (a to e)
resolved in this study—myosin—200,000, tubulin—120,000, and histones—11,000 to 21,000 (Lehninger, 1975) as compared to 92,000, 62,000, 60,000, 48,000, and 46,000 for bands a to e. Rabbit G-actin, though, has a molecular weight of 46,000 and this corresponds exactly to band e. Band e is the band present in unamputated limbs and absent in amputated limbs. This would imply that band e could be actin; however, band e is still absent in palette and 3-digit stage regenerates (Figure 9, lanes 6 to 9) and in full regenerates (Figure 11, lanes 5 to 8). Actin should be present again by the late stages of regeneration at which time muscle redifferentiates; and, therefore, band e is probably not actin.

Stump tissue immediately proximal to the blastema had an essentially identical banding pattern relative to that of blastema (Figures 14 and 15, lanes 3 and 6). It is well known that this area of the limb stump includes proliferating mesenchymal cells (Chalkley, 1954).

Several bands from unamputated limb tissue were usually present on fluorographs (Figures 2, 4, 6, 8, 10, and 12). Only one band from unamputated limb tissues was present on the fluorograph in Figure 15. This one band (Figure 14, band f) is associated with epidermis (lane 4) but not mesoderm (lanes 5 and 8). The fluorographic bands of regenerating limbs generally matched the intensity of the stainable bands (Figures 1 to 12, 14, and 15). This
indicates that cellular proliferation entails a generalized increase in protein synthesis. The intensity of protein synthesis appeared to peak about day 18 post-amputation and to gradually decline thereafter (Figures 2, 4, 8, 10, and 12). It should be noted that although most bands are found in both amputated and unamputated limbs, they are synthesized to a significant extent only in amputated limbs (Figures 2, 4, 6, 8, 10, 12, and 15).

These stable, highly reproducible banding changes of regenerating limbs are in marked contrast to those found by Dearlove and Stocum (1974). They obtained 24 bands from unamputated limbs. By 8 days following amputation, 8 of the 24 bands present in unamputated limbs had disappeared and no new ones had appeared. Eight days post-amputation in the Dearlove and Stocum study corresponds to the 5-day group in their data tables. This is because the day or stage of each of their groups refers to the day of denervation rather than the day of collection. Tissues from denervated and innervated limbs were always collected 3 days following denervation. By day 13 post-amputation, Dearlove and Stocum reported the appearance of 5 regeneration unique bands and the reappearance of 1 of the normal unamputated limb bands absent at day 8 post-amputation. Likewise, Donaldson et al (1974) found considerable shifting of bands at different times during limb regeneration relative to unamputated limb tissue. Their gel photographs
show considerable blurring; and, therefore, their graphic presentation of bands may not be merited.

Normally, a detailed comparison of the results from both of the studies just cited would be in order; however, my experience strongly indicates that most of the banding differences found by Dearlove and Stocum and also by Donaldson, Mason, and Jennings are artifactual. Both of the above studies employed tube gels. I found that the banding patterns on 5 to 10 cm tube gels are simply too complex and variable from one tube to the next for accurate comparisons to be made either by sight or by spectrophotometric scans. Many banding pattern differences from one point in regeneration to the next relative to unamputated limbs (Garling and Tassava, 1977) disappeared when slab gels were employed.

Guy and Schmidt (submitted) compared protein banding patterns of unamputated limb tissue, whole skin, epidermis, muscle, regenerates of various stages, mesodermal core, and wound epidermis. They used gradient SDS polyacrylamide slab gel electrophoresis. Using this technique, they obtained about 60 bands, approximately twice as many as obtained by Dearlove and Stocum (1974), Donaldson et al. (1974), or by this researcher. In contrast to the two previous studies, Guy and Schmidt found no bands unique to regeneration. Likewise, in contrast to the present study, Guy and Schmidt found no bands unique to the effect of
amputation with or without regeneration.

Although Guy and Schmidt did use slab gels, it appears that their study suffers from the same basic defect as that in the two previous studies. Most of the comparisons are made between slab gels rather than within given slab gels. The photographs indicate marked variation in the length of gel runs. The increased number of bands would make comparisons between gels even more difficult than in the two previous studies.

Most of the results of the Guy and Schmidt study conflict with the present results. Even the molecular ranges of banding differences are largely noncongruent. All of the banding changes noted by Guy and Schmidt are in the range of 84,000 to 100,000 daltons and greater. The five banding differences noted in this study were 46,000, 48,000, 60,000, 62,000, and 92,000 daltons.

Aside from using a gradient gel and making comparisons between gels, two other sources of possible variation are apparent. First of all, in the Guy and Schmidt study, the time from harvesting the regenerates to adding the denaturing sample buffer was at least an hour and quite possibly longer. This included an incubation time of 30 min at 22-24°C. It is not possible to tell from the protocol whether adequate controls for possible protein degradation were included. Secondly, the SDS was not added to the sample until after homogenization and centrifugation. Thus a
number of membrane proteins may have escaped being extracted in detectable amounts.

The present study and the Guy and Schmidt study do agree in that both noted a band present in whole skin or wound epidermis that is absent in mesodermal tissue.

As previously stated, the five banding differences found on slab gels were constant for all post-amputation stages examined. One might speculate that the five banding differences reflect basic differences between rapidly proliferating and non-proliferating cells; however, the effect of denervation which will be discussed next does not support this hypothesis.

**Denervation**

The denervation times were selected for the following reasons. Dearlove and Stocum (1974) denervated 3 days prior to collecting for all of the post-amputation times that they observed. This same denervation time was used in the present study to test the reproducibility of Dearlove and Stocum's results. In these experiments, limbs were allowed to regenerate to various stages before denervation. The other denervation time, 1-day post-amputation, provided the opportunity to examine long-term denervation effects and to insure that residual neurotrophic factor in the cut nerve was not influencing the results. It is important to note that denervation 1-day post-amputation results in no
histologically visible regeneration (Mescher and Tassava, 1975). Significantly, denervation at either time did not qualitatively alter the banding pattern on either the stained gels or on the fluorographs, but denervation did decrease the overall intensity of fluorographic bands (Figures 1 to 12). Amputated, denervated stumps/blastemas displayed the same five banding differences found in amputated, innervated stumps/blastemas. However, denervating the limb without amputating did not induce the five changes (Figure 13). Taken together, these two experiments indicate that denervation is neither sufficient nor necessary to produce the five banding differences detected by this system. The same five banding differences occurred and remained for the latest post-amputation time sampled, 10 weeks post-amputation (Figure 11).

If the neurotrophic factor is a protein specific to or abundant in nerves (Singer, 1978), one might expect a denervated, unamputated limb to display the loss of one or more bands compared to innervated, unamputated limbs. The fact that this loss of bands was not seen (Figure 13) may merely indicate that the neurotrophic factor is present in very small quantities. Of course, all speculation at this point about the neurotrophic factor must include the possibility that it is a complex of factors.

It would appear that a significant amount of cellular proliferation is not required in order for the banding
changes to occur since the mitotic index in amputated, denervated limbs remains near zero (Tassava and Mescher, 1975; Mescher and Tassava, 1975). The cell cycle hypothesis emphasizes the independence of dedifferentiation from innervation (Mescher, 1976; Loyd, 1978; Tassava and Mescher, 1975). Since injury alone is sufficient to promote dedifferentiation, any banding differences due to injury should manifest independently of innervation. The results are consistent with this prediction. The following conclusions can be made from the analysis of banding patterns of innervated and denervated amputated limbs. Amputation initiates the synthesis of almost all of the protein bands of unamputated limbs and the synthesis of 4 new protein bands not found in unamputated limbs. The synthesis of these 4 new protein bands is not dependent on innervation and, hence, is independent of cellular proliferation and blastema formation. One band normally present in unamputated limbs is lost or greatly reduced following amputation.

Dearlove and Stocum (1974) reported a multitude of differences in stainable bands between innervated and denervated stumps/blastemas during various stages of regeneration. However, for the same reasons just cited above in the section on normal regeneration, their differences were probably artifactual.

The absence of detectable qualitative differences between innervated-amputated and denervated-amputated limb
stumps/blastemas by either staining or fluorography was unexpected. These results tend to support Singer's (1978) view that the neurotrophic effect is quantitative rather than qualitative. Singer has argued that the innervated limb responds to amputation by increasing the synthesis of pre-amputation proteins as opposed to synthesizing new proteins unique to regenerating limbs. However, qualitative neurotrophic effects could be below current levels of detection.

Previous studies would strongly suggest that the effects of neurotrophic factor are qualitative in nature. The existence of the nerve-dependent period of regeneration (Singer and Craven, 1948) points toward a qualitative effect. If the nerve merely exerts a quantitative effect, then one might expect a smaller regenerate with denervation at early stages of regeneration, but not the regression which is actually observed (Singer and Craven, 1948; Schotte and Butler, 1941, 1944). Likewise, Tassava and McCullough (1978) found no significant difference in the total cell cycle time in limbs denervated just prior to the nerve-dependent/independent transition period although the mitotic index of the denervated limbs dropped off rapidly during the period examined. It would seem that a purely quantitative neurotrophic effect would result in an increased cell cycle time rather than the apparent all-or-none cycling. Given the present restrictions of
methodology in limb regeneration work and the identification of specific neurotrophic factors by other researchers such as sciatin by Oh and Markelonis (1980), it appears premature to argue that the neurotrophic effect of regeneration is purely quantitative in nature.

The five injury related differences may represent necessary preparation for cellular proliferation. Denervation may block one or more events that normally follow the injury-triggered changes. Injury stimulates both the five banding differences and generalized synthetic activity. The generalized synthetic activity appears to fall off after about day 18 post-amputation in the denervated limbs (Figures 4, 6, 8, 10, and 12). The synthetic activity is always greater in innervated than denervated limbs but is considerable in both through day 18 post-amputation relative to unamputated limbs (Figures 2, 4, and 6). Hence, amputation alone stimulates the synthesis of proteins present in four bands that are normally absent in unamputated limbs (or present in only trace amounts) along with the synthesis of all but one of the bands normally present in unamputated limbs. Only two or three of the bands in unamputated limbs are normally detectable on fluorographs. The one band in unamputated limbs that is most frequently detected by fluorography is probably keratin since this band is present in skin (Figure 14, lane 7) but not mesoderm (lane 8).
One might speculate that injury induces the cells to prepare for a proliferative signal from the nerves, whether or not nerves are present. This preparation includes enhanced levels of protein synthesis. The enhanced level of synthesis apparently cannot be maintained past day 18 post-amputation without nerves; however, the qualitative changes in banding patterns are very stable. Qualitative stainable banding differences are still present in denervated limbs 10 weeks post-amputation (Figure 11). That these stable differences may be directly related to preparation for proliferation is supported by the work of Tassava and Loyd (1977). They observed that reinjured skin graft limbs amputated 5 weeks prior to reinjury regenerated markedly faster than amputated, non-grafted limbs.

It may be that denervation acts by adversely affecting the permeability or receptivity of the cell membrane to substances that are always present in the interstitial space. The study by Kelly and Singer (1981) on phosphate uptake by innervated and denervated limbs is in accord with this possibility, but provides no evidence that the membrane changes are either critical or primary to the neurotrophic effect.

Singer has definitely made greater strides toward delineating the nerve factor of regeneration than anyone else. Yet the specific role of nerves is still unknown (Lebowitz and Singer, 1970; Singer et al, 1976; Jabaily and
Singer, 1977). Singer (1978) implies, but does not completely confirm, that a protein(s) is responsible for the nerve effect. The work of Tassava (Tassava and Mescher, 1975; Mescher and Tassava, 1975) has further illuminated how the nerve factor may act relative to the cell cycle, but its exact nature and mode of action is still a mystery.

It should be re-emphasized that no qualitative changes in protein synthesis could be seen on the fluorographs after denervation. However, it is possible that nerves have a qualitative effect on the proteins synthesized, but that the effect is not detectable with the methodology used. Differences might show up on two-dimensional gels. Singer (1978) has claimed that there are no qualitative denervation related differences on two-dimensional gel autoradiographs; but he examined only one stage, early bud blastema, and used a short, 48-hour denervation period.

Although a number of biosynthetic rates drop off quickly after denervation (Dresden, 1969; Lebowitz and Singer, 1970; Singer and Caston, 1972; Morzlock and Stocum, 1972), Tassava and McCullough (1978) found that the mitotic index in nerve-dependent larval axolotl (Ambystoma mexicanum) limbs does not start to drop off significantly until 4 days post-denervation. Similarly, Bryant, Fyfe, and Singer (1971), using electron microscopy, did not detect significant alterations in the blastema until 3 to 4 days post-denervation. It is an open question, then, as to
whether or not the 48-hour denervation time employed by Singer is adequate to detect a qualitative neurotrophic effect. Would a regenerate, denervated during a period of high proliferative activity, show protein differences on two-dimensional gels 6 days following the denervation?

It may also be that the asynchronous nature of the blastema cell population masks differences. The S phase of newt blastema cells accounts for about 80% of the total cell cycle time (Grillo, 1972; Laux, 1980). Hence, approximately 80% of the cells in an innervated blastema are S phase cells. If the neurotrophic effect is related to a specific phase of the cell cycle, for example G2, protein differences in that phase could easily go undetected by either one or two-dimensional gels. Ideally, one would like to compare the proteins of G2 blocked cells in denervated limb stumps with those of G2 cells in innervated limbs, a comparison not technically possible at this time.

A study by Al-Bader, Orengo, and Rao (1978) provides an example of G2 dependent protein differences. HeLa cells can be irreversibly arrested in the G2 phase with a brief exposure to a nitrosourea compound. Two-dimensional gels indicate that these arrested cells as well as S phase cells lack at least 9 proteins that are present in G2-synchronized cells. Arrested cells fused with synchronized G2 cells are able to divide in synchrony with the G2 cells in about 50% of the cases, but mitosis of the fused cells
is delayed. The delay is dose dependent; that is, the ratio of arrested nuclei to synchronized $G_2$ nuclei determines the degree of delay. Similar studies with regenerating limbs will not be possible until synchronizing techniques are developed.

One of the aims of this study was to attempt to delineate a banding difference or set of differences which correlated with innervated, amputated limbs versus denervated, amputated limbs. Such a difference could be used as a starting point for the development of an effective bioassay for the nerve factor. If innervation and denervation correlated with the presence or absence of a specific band(s), then conceivably micro-injection into the blastema of a homogenate containing the presumptive neurotrophic factor might produce the normal innervated banding pattern. While a banding pattern is not the equivalent of regeneration, it would be a step up from using less specific stimulation of DNA and protein synthesis (Lebowitz and Singer, 1970; Singer et al, 1976; Jabaily and Singer, 1977) as a bioassay. Unfortunately, no denervation related differences were detected.

The nerve problem may well be very difficult to unravel. Sixty mound stage blastemas have a wet weight of only 0.1 g and limb nerve tissue is even more difficult to accumulate in workable quantities than blastemas. Certainly micro-biochemical methods will have to be employed.
The great advances that have been made in isolating the neurotrophic factor of myogenesis indicate that although neurotrophic problems are difficult, they are not insolvable. Oh and Markelonis (1980) have purified a protein called sciatin from adult chicken sciatic nerves that is essential for myogenesis and muscle maintenance in vitro. This protein has a molecular weight of 86,400 ±600 as determined by conventional equilibrium sedimentation and contains 11% sugar by weight (Markelonis, Kemerer, and Oh, 1980). They have demonstrated through antiserum immunoprecipitation that sciatin is a normal component of chicken embryo extract (Oh and Markelonis, 1980). Popiela and Ellis (1981) partially purified a similar protein from adult chicken ischiatic-peroneal nerve of 80,000 daltons (by electrophoresis) that promoted in vitro muscle cell proliferation and differentiation.

It is interesting that the 92,000 dalton injury related band present in amputated limbs exactly matches a band present in newt peripheral nerve (Figure 18, lane 3). Perhaps this is a neurotrophic protein supplied to muscle and/or other tissue that is necessary both for maintenance and proliferation of limb tissue. It could be that the protein is relatively stable, is taken up avidly by injured tissue, but for some reason must be supplied continuously up to the nerve-independent stage in order for regeneration to occur. If this is the case, it would be important to
denervate the limb several days prior to amputation and determine if the 92,000 dalton band is present in the injured tissue. Absence of the band would indicate that perhaps injury normally stimulates an increased uptake of that protein by the limb cells from the peripheral nerves rather than its synthesis within the limb cells. However, this band is present in two fluorographs (Figures 2 and 4) of limbs that were denervated 1 day following amputation, but not labeled and collected until 5 to 18 days later. This suggests that either the same protein is synthesized by denervated amputated limb tissue or that denervated amputated limbs synthesize a different protein of approximately the same molecular weight.

Searching for denervation induced changes in synthesis or activity of specific enzymes is a possible approach to identifying the neurotrophic effect. The enzymatic approach, though, is difficult because there is no basis for making a reasonable prediction as to which enzymes are the critical ones.

Manson et al (1976) looked for denervation related, critical enzymes. Their results are basically compatible with the current study. Amputation alone was sufficient to increase the activity of aspartate carbamyl transferase (ACTase), thymidine kinase, and uridine kinase. These enzymes are involved in de novo and salvage synthetic pathways of nucleic acid precursors. The degree of increase
was less in denervated limbs than innervated limbs, but the pattern was the same in both cases. At no time did activity in denervated limbs fall below unamputated limb baselines.

The results of Manson et al. (1976) are consistent with the view expressed above on injury related banding differences and is worth re-emphasizing. That is, whether or not nerves are present, injury stimulates the cells of the limb to prepare for cellular proliferation. This synthetic activity is also stimulated in denervated amputated limbs but is not manifested in cell division and blastema formation.

Likewise, a re-examination of transcriptional events is a possible approach; but again, the basis for selecting a particular aspect of the transcriptional process is lacking. Bantle and Tassava (1974) observed a decrease of precursor incorporation into both messenger and ribosomal RNA when nerve-independent regenerates were denervated. As Bantle and Tassava noted, whether or not this effect was directly or indirectly related to a neurotrophic influence is not known. Dernervation appeared to uniformly depress synthesis of mRNAs of all sizes but as with both present and previous research, subtle but important differences may have gone undetected. It could be that denervation affects a very specific post-transcriptional control point and that this in turn depresses the rate of RNA precursor
incorporation. Given our relatively meager knowledge of what governs the cell cycle in eukaryotes (Hochhauser, Stein, and Stein, 1981), or prokaryotes for that matter, it is obvious that delineating regeneration in biochemical terms is going to be a long-standing problem.

Wound Epidermis

One might expect, based on previous research, that there would be protein differences between the wound epidermis and mesodermal core. Also, aside from having different functions in regeneration, wound epidermis and mesodermal core are entirely different tissue types, ectoderm and mesoderm respectively. However, with the exception of one skin-related protein band (Figure 14, band f) found in normal whole skin (lane 7) and in wound epidermis (lane 4), there were no banding differences between the wound epidermis and mesodermal core (lane 5). Keratin is the most likely candidate for the 57,000 dalton skin-related protein (Fuchs and Green, 1978). This would be consistent with the fact that newt skin epidermis is a proliferating tissue (Hoffman and Dent, 1977). It could be argued then that all the bands visible by staining in mesodermal core and wound epidermis are housekeeping proteins. However, mesodermal core and wound epidermis both displayed the five amputation related banding differences (Figures 14 and 15). Also, there are several significant banding differences between
mesodermal core/wound epidermis and other tissues such as peripheral nerve, brain, kidney, and liver (Figure 18). It would appear that either mesodermal core and wound epidermis have specialized proteins in common, including injury-related proteins, or that the specialized proteins are synthesized in one tissue and transported to the other.

Chapron (1974) observed movement of $^3$H-fucose from the wound epidermis to the mesodermal core. It could be that the wound epidermis produces a glycoprotein required for regeneration, but that the glycoprotein is rapidly transported to the mesodermal core. Conversely, the mesodermal core may rapidly transport proteins to the wound epidermis. Thus, the wound epidermis and mesodermal core would have the same banding pattern. It would be worth while to use the time course of events in the Chapron study and to use labeled fucose in a fluorographic gel experiment to see if the movement of a specific labeled band(s) from wound epidermis to mesodermal core could be detected.

In any case, mesodermal core and wound epidermis, with the exception of one band, apparently end up with equivalent banding profiles. A feasible and important experiment would be to see if the five banding differences can be produced separately in the two tissue types. Will trauma to mesoderm without breaking the skin produce the banding changes? If epidermis is scraped off the dermis without breaking the dermis, will the banding changes occur in the
new epidermis? For that matter, does regenerating lens or liver display the same five changes of amputated limbs? If injured mesoderm displayed the five changes in the absence of a wound epidermis, this would further indicate that the role of the wound epidermis in regeneration is distinct from that of injury (Tassava and Mescher, 1975; Mescher, 1976; Tassava and Garling, 1979).

According to the cell cycle hypothesis, the wound epidermis is responsible for keeping the cells in the cell cycle once they have begun to proliferate (Tassava and Mescher, 1975). This role for the wound epidermis is supported by the studies of Mescher (1976) and Loyd (1978) in which they found that the lack of a functional wound epidermis prevented continued proliferation but not initial cellular dedifferentiation, DNA replication, and mitosis. However, the mechanism by which the wound epidermis acts remains speculative. The mechanism could be a molecular message such as Chapron's work (1974) suggests or something as simple as altering the local electrolyte balance. The present work indicates that if there are differences in the proteins synthesized by wound epidermis and mesodermal core, they are either too subtle to be detected with the methods used or are obscured by transport between the two cell types.
Future Studies

The strength of electrophoretic techniques in limb regeneration lies in the variety of electrophoretic approaches and in the practical applicability of electrophoresis to small amounts of complex tissues. Many electrophoretic techniques, such as micro-electrophoresis, are largely or entirely untapped. Much remains to be done with electrophoresis using different techniques (stains, two-dimensional gels), comparing different species, and adding other experimental designs (internal limb injury with regression, re-injury of skin flap limbs).

Also, improved techniques in other areas of biochemistry may re-open previous avenues of approach, such as the RNA work of Bantle and Tassava (1974). Perhaps major controlling factors other than injury, wound epidermis, and nerves will be discovered in the future.

Injury, nerves, and wound epidermis are all essential for regeneration (Mescher, 1976; Singer, 1952). Tassava and Mescher (1975) proposed a hypothesis which related injury, nerves, and wound epidermis to phases of the cell cycle. In this cell cycle hypothesis, injury stimulates the cells to dedifferentiate and replicate their DNA, nerves permit cells to enter mitosis, and the wound epidermis keeps cells in the cell cycle for additional divisions. The current work strongly supports the idea that dedifferentiation is stimulated by injury and is independent of
innervation. The experimental designs and techniques of the current work did not bring forth any clues as to how nerves and wound epidermis exert their effects. More sophisticated techniques will be required to detect the specific effects of nerves and wound epidermis. The current work has opened the way for a better understanding of injury in regeneration using presently available methods.

What role(s) these five injury-related banding differences do or do not play in regeneration is an open question. Larval limbs that are denervated and amputated or denervated and internally injured (by breaking the radius and ulna) regress to the shoulder region (Thornton, 1954; Thornton and Kramer, 1951). It would be interesting to see if these regressing limbs display the same banding changes as regenerating limbs. Likewise, it would be valuable to know if stripping off limb skin without amputation or if injuring non-limb regions of the body produce the same five banding changes. Would the banding changes occur if the formation of the wound epidermis is blocked?

Summary

In the current study, SDS polyacrylamide gel electrophoresis and $^{35}$S-methionine gel fluorography were used to answer the following questions: 1) Do the protein bands of normal limb tissue differ from those of regenerating limb tissue? 2) Does denervation alter the normal protein
blanding pattern of regenerating limbs? 3) Does denervation alter the stainable protein banding pattern of unamputated limbs? 4) Is the unique role of the wound epidermis reflected in protein banding patterns that are different from those of mesodermal core? 5) Do the answers to the preceding four questions conflict with or complement the existing evidence in support of the cell cycle hypothesis?

The results were clear, highly repeatable, and can be summarized very concisely. 1) With the techniques that were employed, amputation of newt limbs produced five differences in electrophoretic bands obtained from stumps/blastemas relative to normal limb tissue. 2) The qualitative differences in banding patterns were the same for stained bands and fluorographic bands. 3) The differences were stable a) over time (up to 10 weeks post-amputation), b) in innervated, amputated limb stumps/blastemas versus denervated, amputated stumps/blastemas, and c) in wound epidermis versus mesodermal core. 4) Denervation of unamputated limbs did not produce any banding differences.

From these results, it can be concluded that injury is sufficient to either initiate or greatly increase the synthesis of four protein bands that are not normally present (or present only in very small amounts) in uninjured limbs and to cause the disappearance of a protein band normally found in unamputated limbs. These synthetic changes appear to be independent of the neurotrophic effect and, hence, of
cellular proliferation and blastema formation. This study, in conjunction with the existing literature, indicates that injury has a qualitative effect on regeneration whereas no qualitative molecular effect has yet been associated with nerves. The results of the current study are in accord with the injury portion of the cell cycle hypothesis since the injury-related differences are independent of innervation. The remainder of the results neither support nor conflict with the nerve and wound epidermis elements of the cell cycle hypothesis.

The results obtained conflict with the three previous electrophoretic studies on limb regeneration in newts (Dearlove and Stocum, 1974; Donaldson et al., 1974; Guy and Schmidt, submitted). The methodology used in the current study greatly reduced the potential for misinterpretation of banding patterns relative to the three previous studies.

The present examination of protein differences is potentially of great importance for the following reasons: The results are consistent with the view that normally inactive genes are expressed during regeneration. The signals turning the genes on or off are likely to be proteins and certainly a high proportion of the final gene products are proteins as well.

At the level of resolution used, amputation itself, rather than innervation or type of regenerate tissue, was the deciding factor in observed protein banding
differences. The relationship of these differences to injury and/or regeneration remains to be seen.
Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of innervated and denervated stumps/blastemas. All denervations were performed 1 day post-amputation. Collection times refer to days post-amputation. (1) Standard protein markers. (2) Unamputated mid-radioulnar limb tissue. (3) Day 3 innervated stump tissue. (4) Day 5 denervated stump tissue. (5) Day 5 innervated stump tissue. (6) Day 9 denervated stump tissue. (7) Day 9 innervated stump/blastema tissue. (8) Day 18 denervated stump tissue. (9) Day 18 innervated stump/blastema tissue. (10) Unamputated mid-radioulnar limb tissue. Letters A to E mark the levels of the five amputation related banding differences.
Figure 1.
Figure 2. Fluorograph of the gel in Figure 1. Numbers and letters correspond to those of Figure 1. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph.
Figure 2.
Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of innervated and denervated stumps/blastemas. All denervations were performed 1 day post-amputation. Collection times refer to days post-amputation. (1) Unamputated mid-radial-ulnar limb tissue. (2) Standard protein markers. (3) Unamputated mid-radial-ulnar limb tissue. (4) Unamputated mid-radial-ulnar limb tissue. (5) Limb tissue proximal to the elbow from day 9 innervated limbs. (6) Limb tissue proximal to the elbow from day 18 innervated limbs. (7) Day 9 denervated stump tissue. (8) Day 9 innervated stump tissue. (9) Day 18 denervated stump tissue. (10) Day 18 innervated stump/blastema tissue. (11) Unamputated mid-radial-ulnar limb tissue. (12) Standard protein markers. Letters A to E mark the levels of the five amputation related banding differences.
Figure 4. Fluorograph of the gel in Figure 3. Numbers and letters correspond to those of Figure 3. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph.
Figure 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of innervated and denervated stumps/blastemas. All denervations were performed 3 days prior to collection. Collection times refer to days post-amputation. (1) Standard protein markers. (2) Unamputated mid-radioulnar limb tissue. (3) Limb tissue proximal to the elbow from day 9 innervated limbs. (4) Limb tissue proximal to the elbow from day 18 innervated limbs. (5) Day 9 denervated limb stump. (6) Day 9 innervated limb stump. (7) Day 18 denervated limb stump. (8) Day 18 innervated limb stump-blastema. (9) Standard protein markers. (10) Unamputated mid-radioulnar limb tissue. Letters A to E mark the levels of the five amputation related banding differences.
Figure 5.
Figure 6. Fluorograph of the gel in Figure 5. Numbers and letters correspond to those of Figure 5. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph.
Figure 7. Sodium dodecyl sulfate-polyacrylamide gel elec-
trophoresis of innervated and denervated stumps-blastemas. All
denervations were performed 1 day post-amputation. Limbs
denervated 1 day post-amputation did not produce any
visible regeneration, but are still identified by the stage of
the innervated contralateral limb; for example, cone
stage denervated stump tissue. (1) Standard protein mark-
ers. (2) Unamputated mid-radioulnar limb tissue. (3) Limb
tissue proximal to the elbow from mound stage innervated
limbs. (4) Limb tissue proximal to the elbow from cone
stage innervated limbs. (5) Mound stage denervated stump
tissue. (6) Mound stage innervated blastema tissue.
(7) Cone stage denervated stump tissue. (8) Cone stage
innervated blastema tissue. (9) Standard protein markers.
(10) Unamputated mid-radioulnar limb tissue. Letters A to
E mark the levels of the five amputation related banding
differences.
Figure 7.
Figure 8. Fluorograph of the gel in Figure 7. Numbers and letters correspond to those of Figure 7. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph.
Figure 8.
Figure 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of innervated and denervated stumps/blastemas. All denervations were performed 1 day post-amputation. Limbs denervated 1 day post-amputation did not produce any visible regeneration, but are still identified by the stage of the innervated contralateral limb; for example, palette stage denervated stump tissue. (1) Standard protein markers. (2) Unamputated mid-radioulnar limb tissue. (3) Unamputated mid-radioulnar limb tissue. (4) Limb tissue proximal to the elbow from palette stage innervated limbs. (5) Limb tissue proximal to the elbow from digit stage innervated limbs. (6) Palette stage denervated stump tissue. (7) Palette stage innervated blastema tissue. (8) 3-digit stage denervated stump tissue. (9) 3-digit stage innervated blastema tissue. (10) Standard protein markers. (11) Unamputated mid-radioulnar limb tissue. (12) Unamputated mid-radioulnar limb tissue. Letters A to E mark the levels of the five amputation related banding differences.
Figure 10. Fluorograph of the gel in Figure 9. Numbers and letters correspond to those of Figure 9. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph. The unamputated mid-radioulnar limb tissue in lanes 2 and 11 incorporated insufficient isotope to form any clear images.
Figure 11. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of innervated and denervated stumps/blastemas. (1) Standard protein markers. (2) Unamputated mid-radioulnar limb tissue. (3) Limb tissue proximal to the elbow from full regenerate stage innervated limbs. (4) Limb tissue proximal to the elbow from full regenerate stage innervated limbs. (5) Full regenerate stage denervated stump tissue. Denervation performed 1 day post-amputation. (6) Full regenerate stage innervated tissue. (7) Full regenerate stage denervated tissue. Denervation performed 3 days prior to collection. (8) Full regenerate stage innervated tissue. (9) Standard protein markers. (10) Unamputated mid-radioulnar limb tissue. Letters A to E mark the levels of the five amputation related banding differences.
Figure 11.
Figure 12. Fluorograph of the gel in Figure 11. Numbers and letters correspond to those in Figure 11. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph. The unamputated mid-radioulnar limb tissue in lane 10 incorporated insufficient isotope to form any clear images.
Figure 12.
Figure 13. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of innervated and denervated unamputated limbs. All times refer to days post-denervation. (1) Standard protein markers. (2) Unamputated mid-radioulnar limb tissue. (3) Day 9 denervated mid-radioulnar limb tissue. (4) Day 9 innervated mid-radioulnar limb tissue. (5) Day 18 denervated mid-radioulnar limb tissue. (6) Day 18 innervated mid-radioulnar limb tissue. (7) Day 27 denervated mid-radioulnar limb tissue. (8) Day 27 innervated mid-radioulnar limb tissue. (9) Day 9 denervated mid-radioulnar limb tissue. (10) Day 9 innervated mid-radioulnar limb tissue.
Figure 14. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole blastema, wound epidermis, mesodermal core, whole skin, and unamputated limb mesoderm. All regenerating tissues were taken from mound stage regenerates. (1) Unamputated mid-radioulnar limb tissue. (2) Standard protein markers. (3) Whole blastema. (4) Wound epidermis. (5) Mesodermal core. (6) Distal stump tissues immediately proximal to the blastema. (7) Whole skin from the mid-humeral region of the limb. (8) Mesoderm tissues from the mid-humeral region of the limb. (9) Standard protein markers. (10) Unamputated labeled mid-radioulnar limb tissue. Letters A to E mark the levels of the five amputation related banding differences. Letter F marks the skin-specific band.
Figure 14.
Figure 15. Fluorograph of the gel in Figure 14. Numbers and letters correspond to those in Figure 14. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph. The mesoderm tissues from the mid-humeral region of the limb in lane 8 incorporated insufficient isotope to form any clear images.
Figure 16. Light micrograph of a representative 10 um section through the mesodermal core of a mound stage blastema. No wound epidermal cell contamination was evident in serial sections. Hematoxylin-eosin stain 150 X.

Figure 17. Light micrograph of a representative 10 um section through the wound epidermis of a mound stage blastema. Very minimal mesodermal cell contamination (arrow) was revealed in serial sections. Hematoxylin-eosin stain 135 X.
Figure 18. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of various newt tissues. (1) Standard protein markers. (2) Unamputated mid-radioulnar limb tissue. (3) Peripheral nerve. (4) Brain. (5) Kidney. (6) Liver. (7) Standard protein markers. Letter A marks the level of the 92,000 dalton band in peripheral nerve that is also present in amputated limb tissue.
Figure 18.
Figure 19. Fluorograph of the gel in Figure 18. Numbers and letters correspond to those in Figure 18. Standard protein marker and unlabeled tissue lanes are not numbered in the fluorograph.
Figure 19.


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