EFFECTS OF DEIONIZED WATER ON LIMB REGENERATION OF THE RED-SPOTTED NEWT
(Notophthalmus viridescens)

by

KATHRYN BUSS

SENIOR HONORS THESIS

Submitted in Partial Fulfillment of Requirements for the College Scholars Program
North Central College

May 24, 1995

Approved:

First Reader - Thesis Supervisor

Approved:

Second Reader - CSP Faculty Member

I certify that a complete copy of this thesis has been deposited with me for the college records.

College Librarian

(Credit for the Honors Thesis shall not be granted by the Registrar until the Registrar’s Office has received a copy of this form with all three signatures.)
INTRODUCTION

Limb regeneration in vertebrates, the process of growing new pectoral and pelvic appendages to replace damaged or missing ones, is an ability unique to salamanders (Hay, 1966). This unique ability has been thoroughly examined for decades in an attempt to understand both the components involved and also the mechanisms through which these individual elements interact to accurately reconstruct limbs. By emphasizing one aspect of the process over another, the anomaly of limb regeneration can be approached from several different directions. One should first focus on the actual sequence of events that compose the regenerative process and the time required for each event. Other important considerations include the tissues involved in each of these stages and the ways in which they are interacting. In numerous studies emphasis has been on the role of nerves (Thornton, 1938; Singer, 1942a, b, 1943); however, research has also suggested that electrical currents may play a key role in the regenerative process (Becker, 1961; Borgens et al., 1977, 1979).

Several studies (Hay, 1966; Mescher & Tassava, 1975; Browder et al., 1991; Schmidt, 1966) established the sequence of events in and the time frame of limb regeneration. According to Schmidt (1966), limb replacement can be divided into four phases. The first phase takes place during the first 24 hours after a limb is wounded. Repair is initiated by adjacent epidermis spreading to seal the open wound (Browder et al., 1991). These epidermal cells then undergo a series of rapid divisions "...to form a thickened wound epithelium which lacks both the prominent basement membrane and the fibrous dermal layer of normal adult newt skin..." (Mescher & Tassava, 1975). This thick covering
over the wound site is known as the epidermal cap and is necessary for continuing the process of regeneration.

The second phase of limb regeneration occurs between 24 hours and 15 days (Schmidt, 1966). All of the cells in the wounded area begin to lose their specialized characteristics as degradative enzymes break them down into undifferentiated mesenchyme (Bryant et al., 1987). This process is called dedifferentiation. By approximately the sixth day these mesenchymal cells have formed a hillock known as the regeneration blastema (Mescher & Tassava, 1975).

During the third stage, from days fifteen to twenty five, the blastema undergoes a change in its appearance and size. Rapid mitotic divisions increase the number of cells in the developing blastema and therefore cause it to lengthen. The blastema eventually assumes a characteristic shape which defines this as the conical stage (Schmidt, 1966). It is from this aggregate of cells that the future limb will develop.

Stage four occurs between days 27 and 70 (Schmidt, 1966). During this time period, known as the paddle stage, the blastema flattens and undergoes tissue differentiation (Hay, 1966). "The blastema proliferates rapidly and generates the new limb structures..." (Browder et al., 1991). This process of differentiation must replace cartilage, bone, muscles, blood, and nerves, all at the proper time and location.

Since the regeneration blastema is such a key structure in the regenerative process, several studies have been conducted to determine the source of these blastema cells. It is agreed that the wounded limb cells themselves dedifferentiate to form the blastema (Hay, 1966; Goss, 1969; Thornton, 1938). However, further studies by Bryant and others (1987)
have demonstrated that dermis cells local to the area of wounding form up to forty-three percent of the blastema. Schwann cells and connective tissue cells contribute to the blastema also (Butler, 1933; Thornton, 1938). Satisfactory explanations are still being sought for this phenomenon.

If the cells which compose the regeneration blastema are altered to a mesenchymal form, how does the blastema determine which are the proper limb segments to regenerate? Many models have been proposed over the years to explain the regeneration of only the missing limb structures, but the most widely accepted explanation is the polar coordinate model. According to this model, cells contain information about their position within the limb with respect to a proximal-distal axis and the limb's circumference (Bryant et al., 1987). When a limb is wounded, cells with dissimilar positional values become juxtaposed. The process by which these cells from different locations create cells which will possess these "missing values" is called intercalation (Browder et al., 1991). Although this model can be used to explain the regeneration of the correct limb segments when amputation occurs at various levels, it is now thought that positional information is carried by cell surface proteins (Gilbert, 1994). This model, called the differential adhesion hypothesis, proposes that cells possess different cell adhesion molecules (CAMs) on their surfaces. These CAMs interact with the extracellular matrix to localize cells of the same tissue type and to separate different tissues.

The next line of inquiry focused on a component which would consistently be involved with regeneration. Previously, nerves were considered to be unimportant in the regeneration process (Barfuth, 1901; Uranovsky, 1936). However, this hypothesis was
rejected when it was found that throughout most of regeneration, "...the nerve is present in the form of regenerating twigs and trunks among the wound and regenerate tissues" (Thornton, 1938). It has since been ascertained that nerves are a critical component of the regenerating limb (Samarajew, 1939a, b). Studies by Singer (1946) showed that there is a set number of nerve fibers required to continue regeneration, and that this number was relative to the size of the limb. If half or more of the amputation surface is innervated, full regeneration will occur. No regeneration will occur if less than one-third of the amputation surface is innervated.

Researchers then sought to learn which particular nerves were most important. Although the initial studies indicated the importance of sympathetic nerves (Walter, 1919; Schotte & Butler, 1941; Weiss, 1925), further research demonstrated that both sensory and sympathetic nerves are necessary for accurate regeneration, even though sensory nerves alone can support regeneration in a less accurate fashion (Singer, 1942a, b, 1943). The next logical inquiry involves the mechanisms by which these nerves induce and sustain limb regeneration. Without the presence of nerves within roughly the first two weeks, a regenerate will not form. Furthermore, a regenerating limb will deteriorate if nerves are removed after the limb had already begun to emerge. Studies by Weiss (1925) and Singer and Egloff (1949) demonstrate the relationship between the presence of nerves and both the size of the regenerate and the efficiency of the process. Singer and Cravin (1948) further show a drop in mitosis when nerves are removed from regenerating limbs. The mechanism involved here is still under investigation, although several researchers are of the opinion that a chemical emanation from the nerves is implicated (Schotte and Butler, 1941; Singer, 1943;
Further studies about the nerves’ functions in regeneration demonstrated the association of nerves with a bioelectrical field, thus implying that electrical currents might be involved in the regeneration process (Becker, 1961). This research established a relationship between these currents and growth and tissue repair. Therefore, these electrical impulses may be an early mechanism by which data can be distributed within the regenerate by means of nervous tissue. Since Becker discovered these electrical currents in regenerating limbs, subsequent studies have sought to better define the role of electrical impulses in limb regeneration. Development of an ultrasensitive vibrating probe used in studies by Borgens et al. (1977) allowed for the observation that "... large steady currents leave the end of the stump for a week or two after amputation". This study further demonstrated that "... these regeneration currents are sodium dependent and nerve independent" (Borgens et al., 1977). Furthermore, this research showed that a current with a density of 10-100 Å/cm emerges from a newt’s forelimb stump for approximately a week after wounding (Borgens et al., 1977). A comparable level of electrical charge can be applied to frogs to cause them to regenerate (Borgens et al., 1979). With skin batteries as strong as these, the currents produced are capable of "... traversing some part of the stump to move and localize certain developmentally critical macromolecules electrophoretically" (Borgens et al., 1977).

The next step in the process was to identify the source of these currents. Many studies concluded that the electrical currents produced in regenerating limbs were due to influx of sodium ions (Borgens et al., 1977, 1979). "Most of the current pumped by the skin into the stump undoubtedly consists of sodium ions, whereas the current leaving the cut
end of the stump is driven by ... this sodium pump" (Borgens et al., 1977). These electrical charges can be reduced by either reducing the availability of environmental sodium or by using amiloride to block sodium channels (Borgens et al., 1977, 1979). Environmental sodium is therefore considered to be one of the most critical and influential components in the process of limb regeneration.

My study further explores the effects of environmental sodium on limb regeneration of the red-spotted newt, Notophthalmus viridescens. My hypothesis is that if environmental sodium ions are a critical factor in limb regeneration of the red-spotted newt, then newts maintained in deionized water will either show no regeneration or a slower rate of regeneration than the newts maintained in ionized water.

METHODS AND MATERIALS

I tested the water used for this project for both the presence of sodium and also relative ion concentrations. A flame test of the water produced a characteristic yellow color if sodium was present (Wagner, 1987). I determined the relative ion concentrations using a protocol by Angelici (1969) and a Markson (model 1096) Digital Conductivity Meter.

I anesthetized 50 red-spotted newts (N. viridescens) using a 0.2% solution of MS222, and amputated their anterior right and posterior left limbs. Twenty-five randomly chosen newts were placed into individual 4.5" diameter culture dishes filled halfway with deionized water (experimental group). The remainder were placed in similar dishes filled halfway with spring water from the college pond (control group). (Individuals in this group were switched to aged tap water at the end of week three, due to illness.) I maintained both groups on a 14 light: 10 dark cycle, changing their water daily and feeding them blackworms three times.
weekly. At set intervals over a seven week period, I randomly selected individuals from each group, photographed them, and then removed either their front or hind limb blastemas. These samples were then placed in Gilson’s fixative overnight and then rinsed with 70% ethanol.

Subsequently, I prepared these samples for microscopic observation. I dehydrated the samples in a graded series of ethanol solutions (70%, 85%, 95%, 100%) and then cleared the tissues using a series of solutions of absolute ethanol / Hemo De (2:1, 1:1, 1:2, 100% Hemo De). I then embedded the samples in paraffin using standard techniques (Brauer, 1955). These blocked samples were cut into sections 10 μm thick using a rotary microtome. I affixed these sections to glass slides using Haupt’s adhesive and 0.5% formalin. I allowed the slides to dry on a warming tray and then deparaffinized them with toluene. Next I rehydrated them using a series of decreasing concentrations of ethanol (100%, 95%, 70%, 50%, 30%, 15%), ending with water. I then stained the slides using Mallory’s triple stain, affixed the coverslips to them using Permount, and viewed them using a light microscope.

RESULTS

The water tests yielded the expected results (Table 1). A yellow flame was produced by the tap water and spring water, and not by the deionized water. This indicated that sodium was present in the first two types of water and not in the third. The conductivity tests yielded a values of $7.02 \times 10^{-6} \Omega$ for the deionized water, $2.77 \times 10^{-4} \Omega$ for the tap water, and $1.02 \times 10^{-3} \Omega$ for the spring water. These values indicate that there was a higher concentration of ions in the spring and tap water than there was in the deionized water (Angelici, 1969).
The regenerating limbs were examined at both the gross anatomical and the microscopic levels. I found that both groups of animals followed the basic sequence of regenerative events described above. Differences in the rate of regeneration were seen between the two groups, especially in the later stages.

At the end of week one, I saw no gross anatomical differences between the regenerates of the newts maintained in deionized water and those maintained in spring water. Samples from both groups (Figures 1 and 2) showed the typical blastema expected at this stage. In both cases, the wound has been completely sealed by the epidermis, and the slightly rounded tips of the regenerates indicate the presence of the epithelial cap.

The samples from weeks two through four displayed no obvious gross anatomical differences. Samples from this time period exhibited both the expected lengthening of the regenerating limb and also the formation of a conical shape typical of this stage.

I saw distinct differences between the samples from week 5.5. The experimental group showed a greatly lengthened regenerate; however, it showed no formation of digits (Figure 3). In comparison, the control group exhibited both the lengthened appendage and also the beginning of digit formation (Figure 4).

The regenerates from the control group at week seven displayed further development and greater differentiation of the digits (Figure 5). Unfortunately, no comparative samples were available from the experimental group at this stage, due to the deaths of the animals.

My microscopic examination of samples from the experimental group from week one revealed typical blastema formation (Figure 6). This sample had a thick outer layer of cells known as the epidermal cap, and lacked a basement membrane. The other tissues in the
regenerate had not yet begun to dedifferentiate. All of these characteristics were also seen in the week one sample from the control group (Figure 7).

Tissue dedifferentiation was clearly present in the week two samples from the experimental group (Figure 8). The tissue which appears red in this figure was muscle losing its differentiation and becoming more mesenchymal-like. (Mesenchyme is the portion of the mesoderm that produces connective tissue, blood vessels, and the lymphatic system.) Another example of dedifferentiation was found in the week two samples from the control group (Figure 9). This micrograph represents a stage similar to that observed in figure 8, with mesenchyme being centrally located (blue tissue) in figure 9. Again, little difference was seen between these samples.

Samples from the experimental group from week four displayed the beginning of tissue differentiation (Figure 10). The concentrated blue areas in this figure represent places where bones are developing in the limb regenerate. Bone formation was also seen in the week four samples from the control group (Figure 11). Although this is a cross section, the two dark blue areas of concentrated bone are still clearly visible.

Significant microscopic differences between the two groups were observed in the week 5.5 samples. The samples from the experimental group at that time demonstrated further development of the bone organizational centers (Figure 12). When this figure is compared to a control sample from the same week (Figure 13), the differences are obvious. The control sample was clearly at a more advanced stage.

**DISCUSSION**

Both groups of newts followed the sequence of regenerative events previously
described (Schmidt, 1966). At the end of the first week both groups showed typical blastema formation (Figures 1 and 2). I observed tissue dedifferentiation at the end of week two (Figures 8 and 9). By week four, the regenerates showed the beginnings of tissue differentiation in the form of bone organizational centers (Figures 10 and 11). At weeks 5.5 and seven, individuals from the control group had begun digit formation as predicted by the proposed timetable of regenerative events (Schmidt, 1966).

It was at later stages that I observed distinct differences between the control and experimental groups. The differences between these two groups were first noted at the end of week four. I first noticed bone organization centers then (Figures 10 and 11). The most obvious difference between the two groups was observed in the week 5.5 samples at both the gross anatomical and the microscopic levels. At this stage digits were visible in the control group and lacking in the experimental group (Figures 4 and 3). At the microscopic level, I saw bone organizational centers in the experimental group (Figure 12), while entire bones had formed in the control group (Figure 13). These results support my hypothesis.

Even though differences were noted between the regenerates of the newts maintained in deionized water and those maintained in spring water, I had expected to see such differences during the entire process. One potential explanation for the lack of difference between the two groups at earlier stages is the fact that it is impossible to remove all of the sodium ions from the newts’ environment. Even though I cleaned the culture dishes daily and refilled them with clean water, there are still other ion sources. The uric acid and skin secretions from the newts, as well as the ions from the blackworms that they ate, are ion sources which can not be completely removed. It is possible that these minor sources of ions
might have created a concentration of sodium large enough to support the production of the sodium-dependent electrical currents across the wounded limb which are thought to stimulate regeneration.

It is also possible, although doubtful, that the presence of other ions in the water of the control animals might have affected the regenerative process. Ionized water contains not only sodium ions but also calcium, magnesium, iron, and other ions in small concentrations. One could hypothesize that it is these other ions, and not sodium, that are inducing regeneration; however, the results of my flame test indicate that sodium is absent in the deionized water and present in the other waters. Furthermore, since the deionized water had a conductivity of $7.02 \times 10^{-6} \Omega^{-1}$, and since ions create conductivity, there were some ions other than sodium in this water. It is likely that these same ions would also be present in the tap and spring waters. However, the only established type of ion which differed between the two groups was sodium. Furthermore, a study by Borgens and others in 1977 demonstrated that the electrical current produced across the regenerate was greatly reduced by either reducing the availability of environmental sodium, or by applying amiloride to the limb immediately after wounding. No other ions except for sodium were implicated in this study.

The results of my study demonstrate that, when compared to newts maintained in ionized water, newts maintained in deionized water show both gross and microscopic differences in their limb regenerates. Since the only type of ion that was proven to be different between these two treatments was sodium, and since a previous study has implicated the sodium ion in regeneration (Borgens et al., 1977), it is likely that environmental sodium ions were the critical factor in limb regeneration. In order to verify this conclusion, further
studies could be conducted in which a sodium channel blocker was applied to the amputation site immediately after surgery. This would prevent all sodium from entering the wounded limb through this surface. If no regeneration occurred, then it could be stated that sodium is the required environmental ion which stimulates limb regeneration in newts.
<table>
<thead>
<tr>
<th>TYPE OF WATER</th>
<th>FLAME TEST</th>
<th>CONDUCTIVITY TEST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized</td>
<td>No flame</td>
<td>$7.02 \times 10^{-6}$ $\Omega$</td>
</tr>
<tr>
<td>Tap</td>
<td>Yellow flame</td>
<td>$2.77 \times 10^{-4}$ $\Omega$</td>
</tr>
<tr>
<td>Spring</td>
<td>Yellow flame</td>
<td>$1.02 \times 10^{-3}$ $\Omega$</td>
</tr>
</tbody>
</table>

Table 1. Results of flame and conductivity tests.
Figure 1. Front limb of an experimental group regenerate at week 1. This is a typical blastema.
Figure 2. Front limb of a control group regenerate at week one. This is a typical blastema.
Figure 3. Front limb of an experimental group regenerate at week 5.5. Note the lack of digits.
Figure 4. Front limb of a control group regenerate at week 5.5. Note the presence of digits.
Figure 5. Hind limb of a control group regenerate at week 7. Note the further differentiation of the digits.
Figure 6. Sagittal section of an experimental group regenerate at week 1. (100X) Note the thick epidermal cap [EC] and the absence [A] of the basement membrane [BM] near the tip (left). This is a typical blastema.
Figure 7. Sagittal section of a control group regenerate at week 1. (100X) Note the epidermal cap [EC] and the absence [A] of the basement membrane [BM] at the tip (left). This is a typical blastema.
Figure 8. Sagittal section of an experimental group regenerate at week 2. (35X) Note the muscle [M] losing its differentiation.
Figure 9. Sagittal section of a control group regenerate at week 2. (35X)
Note the mesenchymal tissue [M] in place of previously differentiated tissue.
Figure 10.  Sagittal section of an experimental group regenerate at week 4. (35X) Note the concentrated blue areas representing bone formation [B].
Figure 11.  Cross section of a control group regenerate at week 4.  (35X)  
Note the two blue areas representing bones forming [B].
Figure 12. Sagittal section of an experimental group regenerate at week 5.5. (35X) Note the blue area which represents a bone forming [B].
Figure 13. Sagittal section of a control group regenerate at week 5.5. (35X) Note the advanced stage of bone development [B].
WORKS CITED


