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THE EFFECTS OF HYPERTONIC NaCl SOLUTIONS ON
REGENERATION IN LEECHES

A Thesis
Presented to
The Graduate Division
Drake University

In Partial Fulfillment
of the Requirements for the Degree
Master of Arts in Biology

by
Norman B. Erickson
August 1968

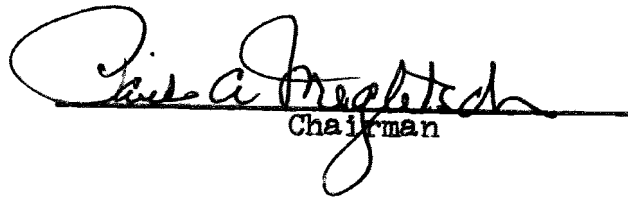
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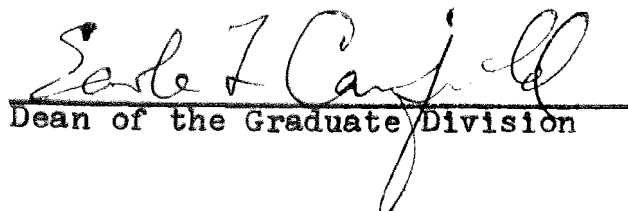
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CHAPTER I

THE PROBLEM

Adult leeches, according to Arthur E. Needham, are completely unable to regenerate, and even wound-healing is incomplete.¹ However, some students of regeneration are of the opinion that animals, such as the leech, that normally do not regenerate might be stimulated to regenerate if the proper chemical or physical stimuli were provided.

I. THE PROBLEM

In the early 1940's, S. Meryl Rose successfully used a hypertonic NaCl solution to stimulate the regeneration of limbs from the amputation stump wounds of adult frogs. Adult frogs normally do not regenerate.² It was the purpose of this study to determine whether or not bisected leeches of the genus Placobdella could be stimulated to regenerate by treating the stump wounds with hypertonic NaCl solutions.

The loss of a limb or major organ in man and other animals that do not regenerate is a permanent loss. An understanding of the healing and regeneration mechanisms of

¹Arthur E. Needham, Regeneration and Wound Healing (London: Methuen and Co., L.T.D., 1952), p. 115.

²S. Meryl Rose, "Methods of Initiating Limb Regeneration in Adult Anura," Northampton, Mass., Smith Collection: Journal of Experimental Zoology, XCV (2), (1944), 149-170.

the tissues of simpler organisms is of vital importance in the medical consideration of wound healing. Do the techniques used to stimulate regeneration in one species that normally does not regenerate have the same effect on another species? Comparative data from such research might provide information about the universality of wound healing and regeneration processes; and could contribute indirectly to insight into the nature of the process of regeneration as well as support or disprove existing theories of regeneration.

II. REVIEW OF THE LITERATURE

As early as the year 1768 biologists such as Lazzaro Spallanzani wondered why certain animals (the frog, e.g.) lost the ability to regenerate body parts as they reached maturity, while others, sometimes closely related (the salamander, e.g.) retained their regenerative powers through life.¹ The question is still unanswered today. What happens to the internal organization of the tissues as they lose their power to regenerate? In such organisms "is the adult organism a fixed structure, incapable of change, or does it still show some of the labile organization so char-

¹Marcus Singer, "The Regeneration of Body Parts," Scientific American, CIC (October, 1958), 80.

acteristic of the egg?"¹

The inability of an organism to regenerate new body parts, such as organ systems and limbs, does not necessarily mean that it is a "fixed structure, incapable of change."² Some ability to replace cells is characteristic of most animals. For example, the hematopoietic tissues of man replace worn out red blood cells at the rate of 8×10^7 cells per second.³ An adult leech will heal its wounds to some degree but is incapable of regenerating new organized parts. It is this limitation, in leeches and in man, that permits them to be considered to have fixed structures, incapable of change.⁴

Recent research indicates that adult organisms that normally do not regenerate can sometimes be induced to regenerate when exposed to certain chemical stimuli. In a series of experiments, Dr. S. Meryl Rose succeeded in stimulating regeneration of frog limbs by repeatedly immersing the exposed limb stumps in a hypertonic NaCl solution.

The stumps were bathed in half saturated NaCl solutions for one and one half minutes four times during the first twenty-four hours, and twice each day thereafter for eight days or until they were killed and fixed. Other

¹Lester G. Barth, Embryology (New York: Holt, Rinehart, and Winston, Inc., Revised Edition, 1953), p. 327.

²Ibid.

³Ibid.

⁴Ibid.

frogs, used as control, did not receive salt baths.

The limb stumps of the experimentals formed a blastema beneath the wound epithelium which then differentiated and regenerated into a new limb.¹ The salt treatments prevented the dermal layer from sealing off the wound area, and may have been instrumental in inducing regeneration.²

Following the hypothesis "that sealed wounds do not regenerate,"³ Rose conducted other experiments designed to prevent normal healing and to encourage regeneration in frogs. The two experimental techniques used consisted of cutting the skin back and preventing it from covering the end of the stump, and feeding the frogs a vitamin deficient diet. Both groups of frogs regenerated new limbs, but the best results were obtained by using the salt treatments.⁴

The fact that the dermal layer inhibited regeneration of the frog limb after amputation is best illustrated by an experiment that involved the amputation of a salamander

¹S. Meryl Rose, "The Effect of NaCl in Stimulating Regeneration of Limbs of Frogs," Journal of Morphology, LXXVII (2), (September, 1945), 119.

²Barth, op. cit., p. 339.

³S. Meryl Rose, "Methods of Initiating Limb Regeneration in Adult Anura," Northhampton, Mass., Smith Collection: Journal of Experimental Zoology, XCV (2), (1944), 149-170.

⁴Ibid., p. 156.

limb. Normally the salamander would regenerate a new limb, but when the skin was drawn across the stump wound no regeneration took place.¹ Perhaps some kind of chemical factor is released by the epidermal tissues that either inhibits the formation of the dermal layer, stimulates regeneration, or a combination of both. It is also possible that the dermal layer presents a physical barrier to a chemical stimulus from the wound epithelium to the internal tissues of the wound stump.²

Another factor in the inability of adult frog limbs to regenerate appears to be related to the effects of nerves and their role in stimulating regeneration. In the frog, if additional nerves from the hind limb are grafted to the front limb stump, the stump regenerates a new limb. If the nerves of a salamander are cut above the limb stump, no regeneration occurs although a stump with a normal nerve supply does regenerate. It is now believed that nerves promote a certain amount of tissue dedifferentiation, providing cells for blastema formation,³ and so promotes regeneration.

It is clear that frogs do not completely lose their

¹Barth, loc. cit.

²S. Meryl Rose, "The Effect of NaCl In Stimulating Regeneration of Limbs of Frogs," Journal of Morphology, LXXVII (2), (September, 1945), 126-127.

³Barth, op. cit., p. 379.

ability to regenerate. Instead it appears that conditions brought about by healing block or inhibit the proliferation and growth of the blastema cells that make regeneration possible. If this block can be removed or overcome by chemical treatments, regeneration can be induced in some forms that normally do not regenerate.¹

Adult leeches do not regenerate, but have some capacity for wound healing. Perhaps such a capacity for healing inhibits regeneration in leeches. A literature search failed to reveal any experiments using leeches that tested the hypothesis that wound healing prevents regeneration, and that techniques used for inhibiting healing can stimulate regeneration. Therefore, should the experiments successfully stimulate leeches to regenerate, the experimental data would indicate the presence of a regeneration mechanism in the tissues of vertebrate and invertebrate organisms that possibly is inhibited by conditions resulting from normal healing; and that by treating the stump wound tissues with chemicals that prevent normal healing, the tissues can be stimulated to regenerate. The failure of the experiments to stimulate regeneration in leeches would indicate that perhaps more than one factor influences the regenerative processes in leeches. Data from such experimentation will pro-

¹Ibid., p. 340.

vide students of regeneration with information needed to determine the basic nature of the processes involved in regeneration.

CHAPTER II

MATERIALS, METHODS, HISTOLOGICAL TECHNIQUES

Although the experiments in this thesis resemble those performed by S. Meryl Rose, the materials and methods used in this research differ slightly as they had to be adapted for use on an aquatic invertebrate animal.

I. MATERIALS

The organism used in this thesis project was the turtle leech Placobdella parasitica, a freshwater leech commonly found on snapping turtles. They varied in length from $\frac{1}{4}$ to $1\frac{1}{2}$ inches when contracted, and were obtained from a biological supply firm.

Other materials used were stainless steel razor blades, a wooden block, NaCl tablets--physiological grade, and distilled water. The razor blades and wooden block were used to bisect the leeches, and the NaCl tablets and distilled water were used to prepare the 10%, 15%, and 20% hypertonic saline solutions used throughout the experiments.

Fresh water taken from an artesian well was used to maintain the leeches during the experiments. The water had not been treated with any chemicals of any kind, and was classified as hard water containing dissolved carbonates of calcium and magnesium.

II. METHODS

The organism Placobdella parasitica was maintained in a large clay crock filled with pure fresh well water. The leeches were not fed during this time, nor were any aquatic plants kept with them. Each day the dying and dead individuals were removed and maintained separately in another container. The water was changed frequently to keep contamination at a minimum. During the experiments, the bisected leeches were kept in clean glass jars filled with fresh well water. The water in each jar was changed during each salt treatment, and once a week after the salt treatments were terminated.

Razor blades were used to bisect the leeches. After the leeches had been removed from the water, they were allowed to creep over the surface of a cutting block. When fully extended, they were cut in half by drawing two sharp stainless steel razor blades together and towards each other in a scissor-like motion, or by pressing one blade downward on the leech and drawing the blade across it as though cutting a piece of meat with a sharp knife.

The leeches were given salt treatments by pouring the salt solution into a glass jar containing the bisected leeches, and allowing the solution to remain in contact with them for one minute. After this interval, the salt solution was removed by pouring the leeches and solution into a

strainer. The leeches and jar were rinsed with fresh well-water, and the leeches were returned to fresh water, where they remained until the next treatment or until the experiment was terminated six weeks later.

The techniques used during the second series of experiments were the same except that the leeches were exposed to the salt solutions for thirty seconds. Leeches that did not survive the salt treatments during the first few days after bisection were fixed in Zenker's fluid after they failed to respond to stimuli and were considered to be dead. The other survivors were killed and fixed when the salt treatments ended.

III. HISTOLOGICAL TECHNIQUES¹

The techniques used in the preparation of the bisected leeches for histological analysis were taken from The Manual of Histologic and Special Staining Technics, Armed Forces Institute of Pathology, Washington, D. C., 1957 Edition. All discussion involving technical information pertaining to the methods used in the preparation of the leech tissue for histological studies appear in this paper

¹The author wishes to acknowledge the assistance given to him by the professional staff of the pathology labs at the Swedish American Hospital, Rockford, Illinois; and to give special thanks to Dr. Paul A. Van Pernis, M. D. and Mr. Robert Martinelli, Technician, for their assistance in the preparation and interpretation of the slides used in thesis research project.

as it appears in the manual unless specified otherwise.

Preparation of Tissues -- Fixation, Dehydration, Embedding

The bisected leeches were killed and fixed by placing them in Zenker's fluid. Although not recommended, the fixed leeches were stored in Zenker's solution until they were processed for embedding in paraffin. Tissues are usually kept in Zenker's solution for eight to twelve hours to avoid overfixing; fortunately the quality of the slides proved to be excellent. The Zenker's solution used to fix the leech tissues was prepared in the following way:

Zenker's Fluid

Distilled water.....	1000 cc.
Mercuric chloride.....	50 gm.
Potassium dichromate.....	25 gm.
Sodium sulfate.....	10 gm.

Add 5 cc. of glacial acetic acid to 95 cc. of Zenker's fluid before use.

After fixation, the tissues of the bisected leeches were washed in running water for 24 hours before dehydration, clearing, and embedding. Leech tissues were processed for embedding in paraffin using the routine fixation and dehydration procedure used at the Swedish American Hospital pathology lab, Rockford, Illinois, on an Autotechnicon Machine. The machine was programmed for the following schedule:

<u>Step</u>	<u>Solution</u>	<u>Timing</u>
1.	10% formaldehyde in 70% alcohol (90 ml R-OH, 10 ml formaldehyde).....	3 - hrs.
2.	95% ethyl alcohol.....	3 - 1 hr. changes
3.	acetone.....	2 - 1 hr. changes
4.	xylene.....	3 - 1 hr. changes
5.	melted Paraplast.....	1 hr.
6.	embed in paraplast (paraffin)	

Before embedding in paraplast, each bisected half was divided into two sections by a cut along the vertical longitudinal axis, and then each division was placed into the mold with the cut surface facing downward.

Sagittal sections were cut at a thickness of six microns, placed on a glass slide, baked in an oven for ten minutes at a temperature of approximately 150 degree Centigrade, and cooled to room temperature. At this point the sections were ready for staining.

Preparations for Staining¹

Solutions.

Weigert's Iron Hematoxylin

Solution A

Hematoxylin.....	1.0 gm.
Absolute alcohol.....	100.0 cc.

¹Armed Forces Institute of Pathology, Manual of Histologic and Special Staining Technics (Washington, D. C.: 1957 Edition), p. 66.

Weigert's Iron Hematoxylin

Solution B

29% ferric chloride.....	4.0 cc.
Distilled water.....	95.0 cc.
Hydrochloric acid, concentrated.....	1.0 cc.

Working Solution

Equal parts of Solution A and Solution B.

van Gieson's Solution

Acid fuchsin, 1% aqueous solution....	2.5 cc.
Picric Acid, saturated aqueous solution.....	97.5 cc.

Lugol's Solution (Weigert's Modification)

Potassium iodide.....	2 gm.
Iodine Crystals.....	1 gm.
Distilled water.....	100 cc.

5% Sodium Thiosulfate Solution

Sodium thiosulfate.....	5 gm.
Distilled water.....	100 cc.

Staining Procedure¹

1. Xylene..... 2 - 2 minute baths
2. Absolute alcohol..... 2 - 2 minute baths
3. 95% alcohol..... 6 dips
4. 50% alcohol..... 6 dips
5. Rinse in distilled water
6. Lugol's solution..... 5 minutes
7. Wash in water
8. 5% Sodium thiosulfate solution .. 1 - 3 minutes
9. Wash in running water
10. Stain in Weigert's hemotoxylin solution .. 10 min.
11. Wash in distilled water
12. Counterstain in van Gieson's solution 2 min.
13. Absolute Alcohol..... 2 - changes
14. Xylene..... 2 - changes

¹Ibid.

15. Mount in Adams Histoclad, a synthetic mounting medium.

When using the van Gierson's Stain for Collagen Fibers Technique, collagen fibers should appear red, muscle and cornified epithelium tissue yellow, and the cell nuclei should be blue to black.¹

¹Ibid.

CHAPTER III

THE EXPERIMENTS

A series of four experiments, patterned after those used by S. Meryl Rose to stimulate regeneration in adult frogs, was used to determine the effects of hypertonic NaCl solutions on leech regeneration. In each of the four experiments, each bisected leech was exposed to a 10%, 15%, or 20% hypertonic NaCl solution for sixty seconds. The number of and time interval between each salt solution treatment varied with each experiment. The series of experiments made an attempt to find the best possible combination of variables (the number of treatments, frequency of treatments, a 5%, 10% or 15% hypertonic NaCl solution e.g.) that possibly could inhibit healing and stimulate regeneration. These four experiments in the following discussion are referred to as Series I.

A second series of experiments similar in design and technique to the first were performed. These experiments differed from the first series only in that the bisected leeches were exposed to the salt solutions for thirty seconds. In the following discussion these experiments are referred to as Series II.

I. SERIES I

For each of the four experiments, fifteen leeches were divided equally into three experimental groups and designated as groups 1, 2, and 3. Then the leeches from each group were bisected and treated with one of the hypertonic salt solutions. The group number of the experimental organism, then, indicates whether a 10%, 15% or a 20% NaCl solution is being used in an attempt to induce regeneration in leeches.

Because experiments I - IV were performed simultaneously, five leeches were used as a control group for the entire first series of experiments. The controls, referred to as group 4 throughout the following discussions, were bisected and placed in a jar containing fresh well water that was used to maintain the leeches during the experiments. They remained in the fresh water until the experiments of that series were terminated. At this time they were killed, fixed, and made into slides for histological analysis.

To provide an adequate basis of comparison between the experimentals and controls, a large healthy leech was kept in well water during the experimental period, and was then bisected after it had been killed and fixed. This control was used as a standard to determine the extent of healing that had occurred on the experimentals after they had

been bisected.

Experimental Procedure - Experimentals

Using the salt treatment techniques described previously, the first series of experiments exposed the bisected leeches to the salt solutions for sixty seconds per treatment.

Experiment I. The bisected leeches were given salt treatments every six hours for the first twenty-four hours, and then every twelve hours for the next six days. Leeches from group 1 were treated with a 10% salt solution, group 2 with a 15% salt solution, and group 3 with a 20% NaCl solution.

Experiment II. Salt treatments were given every six hours for the first twenty-four hours, and then the leeches were allowed to live in fresh well-water until the experiment was terminated. Leeches from group 1 were treated with the 10% NaCl solution, group 2 with the 15% salt solution, and group 3 with a 20% NaCl solution.

Experiment III. Salt treatments were administered every six hours for the first twenty-four hours, and then once every twenty-four hours for the next six days. Again group 1 was treated with a 10% NaCl solution, group 2 with a 15% salt solution, and group 3 with a 20% NaCl solution.

Experiment IV. The bisected leeches were given salt treatments once every twenty-four hours for seven days. Group 1 was treated with a 10% NaCl solution, group 2 with a 15% NaCl solution, and group 3 with a 20% NaCl solution.

Termination of Experiments

The controls and the leeches that survived the first series of salt treatments were maintained undisturbed in fresh well water for two months. They were observed during this period for evidence of regeneration. Then the control and experimental organisms were killed, fixed, and made into slides for histological study.

II. SERIES II

The second series of experiments used the same experimental procedures on the experimental and control organisms. Series II differs from Series I only in that the bisected leeches were exposed to the NaCl solutions for thirty seconds, and that the experimental and control groups each contained eight leeches.

The survivors and controls from the second series of experiments were killed and fixed at the end of the seven day experimental period. Unlike the first series of experiments, leeches that died during the salt treatments but were still intact were also fixed and made into slides for tissue studies.

CHAPTER IV

DATA AND INTERPRETATION OF DATA

I. DATA

Visual Observations - Series I and II

Immediately after bisection, each leech contracted violently and drew its body up into a ball. At the same time it sealed off the stump wound by drawing the brown pigmented dermal layer of tissue together over the exposed surface from the dorsal, lateral, and ventral surfaces. When disturbed, the leeches would extend their bodies, thereby drawing the protective outer layer of tissues off of the stump wound and exposing the viscera and open stump wound. The wounds appeared speckled except for the major viscera which appeared as dark rings. There was no visible layer of tissue covering the stump wound at this time.

Within two days to a week, the viscera of the controls were barely visible through a translucent layer of tissue that appeared to cover the stump wound. All of the experimentals had the same general appearance, although to different degrees. At the same time many of the experimentals developed some of the conditions described in the frogs used for the experiments conducted by S. Meryl Rose. Some of the leeches became rigid, many lost normal pigmentation, and the stump wound area of most became swollen and

distended. Many appeared to shed their outer skin before and during the experiments.

Externally, there was no evidence of a blastema or regeneration of any kind other than the formation of the translucent layer of tissue over the stump wound.

Data - Histological Analysis

Data obtained from histological examination of the stump wound region of the controls and experimental organisms is limited to a qualitative nature because of a limited number of slides that contained good sagittal cuts through the tissues of the stump wounds. Enough good slides were made to enable a comparative study of the degree of healing between the bisected leeches of Series I and II and the controls. It was also possible to examine the slides to determine whether or not regeneration had occurred.

The histological data will be limited to a description of a typical control from Series I, a typical experimental organism from Series I, and a typical experimental organism from Series II.

Using slides containing good sagittal sections of controls and experimental organisms from both series or experiments, the histological examination of the stump wound tissues did not reveal any difference in tissue types found in the scar tissues. The only differences found were in the degree of healing between the controls and experimentals of

Series I and the experimentals of Series II.

The scar tissues of the control and experimental organisms did not have a consistent geometric shape as some bulged outward from the stump wound while others appeared concaved. However, they were all formed primarily from connective and epithelial tissues.

The thickness of a mass of scar tissue over the stump was never uniform, but usually varied between 20 and 100 per cent of the normal thickness of adjacent normal connective tissues. Scattered throughout the connective tissue covering the stump wound were tiny glands that stain yellow because of their epithelial origin. The same glands are seen in normal adjacent connective tissues.

The outer epidermal covering of the stump wound is one cell in thickness, and usually continuous with adjacent epithelial tissues in 90 per cent of leeches examined. In the other 10 per cent of the leeches examined, the epidermis was continuous with the normal adjacent epidermal tissue but in places there appeared clear areas and gaps caused by poor cutting and artifacts resulting from fixation and staining techniques.

The stump wounds of the organisms examined from the second series of experiments show evidence of healing, but none of the stump wounds examined were completely sealed by a layer of connective and epithelial tissues. Connective

tissue is more in evidence over the stump wounds with only scattered pieces of epithelial tissue over the stump wound surface.

Although the slides made were not designed to determine the origin of the epithelial-epidermal tissue that forms over the stump wound, a slide made of leeches taken from group 2 of experiment 3, Series II, clearly shows the gut endodermis everting outward and over the connective tissues that are already migrating over the exposed surface.

The next two pages contain a summary of the mortality rates suffered by the controls and experimental organisms during the salt treatments (the first seven days of the experiments). The following information should prove helpful in evaluating the data.

Because the leeches were purchased from a biological supply firm, it is not known what possible variables (bad water, disease, chemicals in their native water supply, e.g.) they might already have been exposed to prior to their use in the experiments discussed in this thesis.

The leeches used in Series I., experiments II., III., and IV., had been without food for one month. Experiment I. was conducted using leeches that had not been fed for three months.

The shipment of leeches used for Series II. suffered a 10-15 per cent mortality rate before the experiments

TABLE I

A DAILY ACCOUNT OF THE TOTAL PER CENT OF LEECH HALVES THAT DIED DURING THE SALT SOLUTION TREATMENTS; THE EXPOSURE TIME USED WAS ONE MINUTE PER TREATMENT

Experiment	Group #	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<u>Exp. I. NaCl</u> given every 6 hrs. for first 24, every 12 next 6 days	1	0%	0%	M	M	M	^{*80} 50%	100%
	2	0%	0%	0%	M	50%	60%	100%
	3	0%	0%	0%	M	50%	100%	100%
	4	0%	0%	0%	0%	0%	0%	0%
<u>Exp. II. NaCl</u> Sol. every 6 hrs. for first 24 hrs. then fresh H ₂ O 6 days	1	0%	0%	0%	0%	0%	0%	0%
	2	0%	0%	0%	0%	0%	M	25%
	3	0%	0%	0%	0%	M	M	28%
	4	0%	0%	0%	0%	0%	0%	0%
<u>Exp. III. NaCl</u> every 6 hrs.-1st 24 hrs.-1 every 24 hrs. for six days	1	0%	0%	M	M	⁵⁰ 75%	100%	100%
	2	0%	0%	M	M	90%	100%	100%
	3	0%	0%	M	M	90%	100%	100%
	4	0%	0%	0%	0%	0%	0%	0%
<u>Exp. IV. NaCl</u> every 24 hrs. for 7 days	1	0%	0%	0%	0%	M	M	60%
	2	0%	0%	M	M	100%	100%	100%
	3	0%	not tested - no data					
	4	0%	0%	0%	0%	0%	0%	0%

Note:

M - moribund, dying. The organism was considered to be absolutely dead when it failed to respond to external stimuli over a period of two days.

*80

50% - 80 to 50% mortality rate

TABLE II

A DAILY ACCOUNT OF THE TOTAL PER CENT OF LEECH HALVES THAT
DIED DURING THE SALT SOLUTION TREATMENTS: THE EXPOSURE
TIME USED WAS 30 SECONDS PER TREATMENT

Experiment	Group #	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
<u>Exp. I. NaCl</u> every 6 hrs.- 1st 24 hrs., every 12 hrs. next 6 days	1	0%	M	M	10%	45%	60%	60%
	2	0%	M	20%	^{*20} 50%	50%	terminated	
	3	0%	M	60%	terminated-killed-fixed			
	4	0%	M	M	40%	⁴⁰ 60%	terminated	
<u>Exp. II. NaCl</u> every 6 hrs. first 24 hrs. fresh H ₂ O next 7 days	1	0%	M	⁵⁰ 100%	70%	-	-	-
	2	0%	M	⁵⁰ 100%	80%	-	-	-
	3	0%	M	⁵⁰ 100%	100%	-	-	-
	4	0%	M	M	40%	⁴⁰ 60%	terminated	
<u>Exp. III. NaCl</u> 6 hrs. for 24 hrs.-once every 24 hrs. for six days	1	0%	0%	M	30%	30%	35%	term.
	2	0%	0%	M	10%	⁵⁰ 80%	terminated	
	3	0%	0%	M	M	⁵⁰ 80%	terminated	
	4	0%	M	M	40%	⁴⁰ 60%	terminated	
<u>Exp. IV. NaCl</u> 1-time every 24 hrs. for 7 days	1	0%	0%	30%	³⁰ 50%	50%	50%	50%
	2	0%	M	M	10%	10%	M	20%
	3	0%	0%	M	M	M	M	10%
	4	0%	M	M	40%	⁴⁰ 60%	terminated	

Note:

M - moribund, dying. The organism was considered to be absolutely dead when it failed to respond to external stimuli over a period of two days.

^{*20}
50% - 20 to 50% mortality rate

began; the cause of death was unknown. The experiments were started as soon as the leeches appeared healthy, and the sick and dying were discovered and removed.

Many of the leeches used as experimentals and controls in Series II. were extremely small -- less than 0.25"

II. INTERPRETATION OF DATA

There was no evidence of regeneration in any of the data obtained either through visual observation during the experimental period or through histological analysis. There was no evidence of blastema formation, dedifferentiation, or differentiation of tissues into new structures.

Comparative studies of the stump wound tissues of the controls and experimentals of Series I. and II. indicate that throughout the experimental period the salt treatments did not stop or change normal healing patterns to any great extent, and that if given enough time the leech will completely seal off the stump wound with scar tissue made of connective and epithelial tissues. One slide prepared from a leech from the second series of experiments gave support to the statement that leeches heal or seal off wounds by the eversion of the gut endodermis over the exposed stump wound area.

The mortality rates were very high in both series of experiments. The cause is probably the combined effects of

the dehydrating effects of the NaCl solutions and infection and disease. In some instances, such as in Experiment I of Series I., the amount of stored food reserves available in the organism would effect its ability to survive, and therefore effect the mortality rates. Although the mortality rates were high, the survivors would still have shown the effects, if any, of hypertonic NaCl solutions on leech regeneration.

Lastly, it is apparent that the techniques used in this thesis research failed to stimulate regeneration in leeches. But this does not mean that leeches cannot be stimulated to regenerate. The question that should be asked at this point is, "Why did a technique that was used successfully to stimulate regeneration in adult frogs fail to stimulate regeneration in the leech Placobdella parasitica?"

CHAPTER V

DISCUSSION

Many factors should be considered when attempting to account for the failure of the techniques used to stimulate regeneration in leeches. Therefore, reference should be made at this time to a basic hypothesis stated previously -- that rapid, natural healing might inhibit regeneration. If this hypothesis is correct, then the healing that occurred over the stump wounds during and after the salt treatments would offer a reasonable explanation for the failure of the leeches to regenerate.

It is very possible that the failure of the salt solutions to stimulate regeneration is not due to the failure of techniques designed to cause the physiological changes necessary to stop healing, but because the general anatomy and defensive reflexes of the leech prevented the salt solution from making contact with the exposed tissues of the wound. It is very possible that during the salt treatments the stump wound was too well protected by the dermal tissue layer the leech had drawn over the stump, and that this defensive reflex allowed normal healing to progress free from the potential effects of the hypertonic salt solution.

Another possibility that should be considered is that the process of regeneration in organisms that normally do

not regenerate might be dependent on more than one form of a chemical inducer, and perhaps even different chemical stimuli in different organisms in different situations. This theory was illustrated when another research team repeated Rose's experiments and found that when the nerves leading to the stump wound were cut, the frogs failed to regenerate -- even when salt treatments were continued.¹ Perhaps there are chemical mechanisms within the leeches that require a combination of stimuli to stimulate regeneration, and that the salt treatments did not trigger the same response that occurred in the frog.

Another factor that should be taken into consideration is the fact that the leech is an aquatic organism subject to dehydration in the presence of a hypertonic salt solution. An animal that has been bisected is already subjected to enough stress without having to cope with osmotic problems. The author believes that this is one of the major reasons for the high mortality rates experienced in the experimental groups throughout the experiments, and the osmotic problems could have effected the ability of the leech to regenerate as well.

The author feels that the salt treatments failed to stimulate regeneration in leeches because of the healing that had occurred, the failure of the salt treatments to

¹Marcus Singer, "The Regeneration of Body Parts," Scientific American, CIC (October, 1958), 88.

stop the healing due to the layer of tissue the leech had drawn over the wound, and the possibility that the wrong chemical stimuli was used.

I. RECOMMENDATIONS FOR FURTHER STUDY

It is apparent that the techniques used in this research failed to stimulate leeches to regenerate, but the results suggest the possibility that other techniques could be developed and used to induce regeneration in *Placobdella*. Therefore, the following ideas for future research and study are offered for thought and consideration. If healing does inhibit regeneration in leeches, then perhaps a technique could be devised to prevent the leech from drawing outer tissues over the stump wound. One way to accomplish this would be to remove enough of the dermal tissues so that the stump tissues would be exposed directly to the salt solutions. Using this technique, it would be interesting to see if exposing the gut endodermis and other stump tissues directly to the salt solutions will stop the eversion of the gut endodermis out and over the stump wound and stimulate regeneration.

Another new and interesting field of research in regeneration is the field of "wound hormones," or as they are also named "regeneration promoting factors." At the present time, not much is known about wound hormones except

that all are direct or indirect products of injuries that lead to regeneration.¹

Once the regeneration-promoting factors are identified and their general properties established, it would be most interesting to determine their effects on the regeneration of bisected leeches.

¹Arthur E. Needham, Regeneration and Wound Healing (London: Methuen and Co., L.T.D., 1952), p. 115.

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