INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

- 1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.**
- 2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.**
- 3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.**
- 4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.**
- 5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.**

Xerox University Microfilms

300 North Zeeb Road
Ann Arbor, Michigan 48106

73-26,765

BANTLE, John Albert, 1946-
THE NEUROTROPHIC INFLUENCE ON RNA PRECURSOR
INCORPORATION INTO POLYRIBOSOMES OF
REGENERATING ADULT NEWT FORELIMBS.

The Ohio State University, Ph.D., 1973
Zoology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

THE NEUROTROPHIC INFLUENCE ON RNA PRECURSOR
INCORPORATION INTO POLYRIBOSOMES OF
REGENERATING ADULT NEWT FORELIMBS

DISSERTATION

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

By

John Albert Bantle, A.B., M.S.

* * * * *

The Ohio State University

1973

Reading Committee:

Roy A. Tassava

Thomas J. Byers

Willard C. Myser

Approved By

Advisor
Department of Zoology

ACKNOWLEDGMENTS

Although only one name appears on the title page of a dissertation, it is really a culmination of the efforts of many people. To express thanks to all of the people who in some way contributed to this work would require several pages so I must confine my written appreciation to those who did most to aid my research. I thank Dr. Roy A. Tassava for first posing the problem and for his sound advice throughout the entire study. His encouragement and constructive criticisms aided me greatly. I also wish to extend my gratitude to Dr. Thomas J. Byers and Dr. Willard C. Myser for their advice and critical reading of the manuscript. I must give special thanks to Dr. Byers for the use of his laboratory and equipment during this study.

I also wish to thank Jeanne Manson and Tony Mescher for assisting me with experiments and their very helpful comments. Also, credit must be given to Dwight Hector for his photographic assistance. Most importantly, I must thank my wife, Donetta, who not only typed the rough and final drafts but also tolerated me through this study and encouraged me to finish.

VITA

January 20, 1946.....	Born- Detroit, Michigan
1968.....	A.B., Eastern Michigan University, Ypsilanti, Michigan
1970.....	M.Sc., Eastern Michigan University, Ypsilanti, Michigan
1968-1970.....	Teaching Assistant, Eastern Michigan University, Ypsilanti, Michigan
1970-1973.....	Teaching Associate, The Ohio State University, Columbus, Ohio

PUBLICATIONS

" Effects of Mechanical Vibrations on the Growth and Development of Mouse Embryos." Aerospace Medicine, 42 (10): pp 1087-1091, 1971.

FIELDS OF STUDY

Major Field: Developmental Biology

Studies in Effects of Mechanical Vibrations on Development. Professor Pearl DiMattia

Studies on ribosomal RNA Processing in Sea Urchin Mitochondria. Doctor Lajos Piko

Studies in Amphibian Limb Regeneration. Professor Roy A. Tassava

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	Page ii
VITA.....	iii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
LIST OF SYMBOLS.....	viii
INTRODUCTION.....	1
 Chapter	
I. LITERATURE REVIEW.....	4
II. MATERIALS AND METHODS.....	24
III. RESULTS.....	32
The isolation of undegraded newt polyribosomes	
The influence of denervation on long term (6 h)	
H ³ -Uridine incorporation into newt poly-	
ribosomes	
The influence of denervation on short term (90	
minute) P ³² incorporation into newt poly-	
ribosomes	
The isolation of messenger RNA from newt	
polyribosomes	
The incorporation of amino acid precursors	
into newt polyribosomes of innervated and	
denervated regenerates	
IV. DISCUSSION.....	73
 APPENDIX	
A.....	82
BIBLIOGRAPHY.....	84

LIST OF TABLES

	Page
1. Comparison of results from experiments on 21 day, innervated and denervated newt forelimb regenerates.	79

LIST OF FIGURES

	Page
1. The various stages of newt forelimb regeneration..	5
2. Effects of commercial grade pancreatic ribonuclease and newt ribonuclease on polysome profiles of 14 day old chick embryo leg muscle.....	33
3. Effect of bentonite and diethyl pyrocarbonate on polysome profiles from 14 day chick embryo leg muscle.....	36
4. Labelled amino acid incorporation into undegraded and degraded chick polyribosomes	39
5. Polysome profiles of innervated and denervated 21 day newt forelimb regenerates labelled with H ³ -Uridine for six hours.....	43
6. Polysome profiles of innervated and denervated 28 day newt forelimb regenerates labelled with H ³ -Uridine for six hours.....	45
7. Polysome profiles of innervated and denervated 35 day newt forelimb regenerates labelled with H ³ -Uridine for six hours.....	47
8. Polysome profiles of innervated and denervated 21 day newt forelimb regenerates labelled with P32 for 90 minutes.....	50
9. Polysome profiles of innervated and denervated 28 day newt forelimb regenerates labelled with P32 for 90 minutes.....	52
10. Polysome profiles of innervated and denervated 35 day newt forelimb regenerates labelled with P32 for 90 minutes.....	53
11. Fractionation of polysome gradients in preparation for mRNA analysis of 35 day newt limb regenerates.	56
12. Polysomal RNA profile of 35 day newt forelimb regenerates labelled with P32-NaPO ₄ for 90 minutes.	61

13. P32 labelled material cosedimenting with 21 day forelimb regenerate polysomes.....	Page 61
14. Region 1 polysomal RNA profile of 21 day innervated and denervated newt forelimb regenerates labelled with P32-NaPO ₄ for 90 minutes.....	63
15. Region 2 polysomal RNA profile of 21 day innervated and denervated newt forelimb regenerates labelled with P32-NaPO ₄ for 90 minutes.....	64
16. Polysomal RNA profile of 21 day, innervated and denervated forelimb regenerates labelled with P32-NaPO ₄ for six hours.....	69
17. Polysome profiles of innervated and denervated 21 day newt forelimb regenerates labelled with H ³ -amino acids for one hour.....	71

LIST OF SYMBOLS

CO ₂	Carbon dioxide
DEP.....	Diethyl pyrocarbonate
DNA.....	Deoxyribonucleic acid
EDTA.....	Ethylenediamine tetraacetic acid
ETOH.....	Ethyl alcohol
MSB.....	Muscle standard buffer
RNA.....	Ribonucleic acid
mRNA.....	messenger Ribonucleic acid
rRNA.....	ribosomal Ribonucleic acid
tRNA.....	transfer Ribonucleic acid
RNase.....	Ribonuclease
SDS.....	Sodium dodecyl sulfate
TCA.....	Trichloroacetic acid

INTRODUCTION

In 1823, Todd showed that the amphibian limb failed to regenerate when deprived of an adequate nerve supply. Since that time, the nature of this neural influence on vertebrate limb regeneration has been the subject of many investigations but has not been entirely elucidated. More recently, Singer and Craven (1948) demonstrated that when denervation was performed any time during the first 13 days following amputation, regeneration was precluded and the blastema would regress back to the original amputation site. Thus, any regenerate less than 13 days old was said to be nerve-dependent. However, 13 to 17 day old regenerates varied in their response to denervation. In some cases, regenerates denervated during this period failed to continue regeneration and regressed while others underwent morphogenesis and produced small, well-formed limbs. Regenerating forelimbs of this stage were then said to be undergoing a transition period from nerve-dependence to nerve-independence. After 17 days post-amputation, regenerates were called nerve-independent since subsequent denervation did not prevent morphogenesis of a new limb. Denervation did have an adverse effect on the ultimate size of the completed limb such that the earlier the

denervation, the smaller the final limb formed. Later denervations did not affect the final limb structure although the tissues of the regenerate underwent atrophy.

The ability of the nerve supply to promote regeneration and to maintain the final structure of the regenerate is termed the neurotrophic influence. Attempts have recently been made to characterize the neurotrophic influence biochemically in terms of RNA. protein and DNA synthesis with the hope of explaining the above mentioned observations. This present work is a continuation of this line of research.

More specifically, a workable system was designed by which the neurotrophic influence on RNA synthesis could be analyzed in blastemal tissues of the newt, Notophthalmus (= Triturus) viridescens. Basically, the approach taken was to pulse label polyribosomes and extract them from innervated (control) and denervated forelimb regenerates from various stages of regeneration. Once the polysomes were fractionated on sucrose gradients, they were either analyzed for trichloroacetic acid (TCA) precipitable counts or dissociated and respun on sucrose gradients to determine which RNA species present had incorporated labeled precursors. The major question asked was: Does denervation affect blastemal messenger RNA synthesis? If so, which messenger RNAs are affected? Finally, is ribosomal RNA synthesis also affected by denervation?

Answers to these questions may supply important evidence concerning the mechanisms by which nerves influence the cells of the regenerate.

LITERATURE REVIEW

Limb regeneration can be divided into regressive and progressive phases (Thornton, 1968). After the trauma of amputation, the regressive phase of regeneration begins and this phase includes: wound healing, the demolition of damaged cells plus cellular debris, and dedifferentiation of stump tissue cells. The progressive phase of regeneration involves the aggregation and proliferation of these dedifferentiated cells, ie. blastema formation and growth, followed by morphogenesis and differentiation of lost limb parts (Thornton, 1968). These stages are not completely sequential and the various developmental processes occurring during limb regeneration overlap considerably (Fig. 1).

The trauma of amputation is important in initiating the entire process of regeneration. Needham (1941) postulated that a "wound release factor" is produced by the limb tissues injured by amputation. This factor initiates tissue dedifferentiation which ultimately supplies mesenchymatous cells for blastemal formation. Results of experiments with the general tissue poison beryllium nitrate, which inhibits regeneration up to three hours post-amputation, have been interpreted as evidence in support

Figure 1. The various stages of newt forelimb regeneration. Data collected from Thornton (1968), Singer (1952) and Powell, (1969). The time intervals have been adjusted to correlate with regeneration at 26°C. Circled numbers indicate days on which experiments were performed in the present study.

of the existence of a wound factor (Thornton, 1949, 1950, 1951). Apparently, beryllium nitrate only destroys injured stump tissues and not normal limb tissue, thus destroying the factor and preventing dedifferentiation from commencing (Thornton, 1949, 1950, 1951).

Between 0-6 hours post-amputation, the amputation wound is healed over by limb skin epidermal cells which migrate distally and medially to form a covering over the wound several cell layers thick. That this "wound epidermis" is important to blastema formation and growth can be demonstrated by repeated removal of the epidermis which completely inhibits regeneration (Thornton, 1957). Treatment of the wound epidermis by ultraviolet irradiation also results in an inhibition or delay of the regenerative process (Thornton, 1968). There is evidence that only "limb" epidermis will support regeneration. Head skin inhibits limb regeneration when it is transplanted in lieu of normal limb skin in Ambystoma talpoideum larvae (Thornton, 1962). The transplanted head skin never thickens into a typical wound epidermis and blastema formation does not occur although epidermal cells migrate normally over the wound surface.

Concomitantly with wound healing, there occurs a 2-3 day period of tissue destruction called the demolition phase. Only the most distal ends of the stump tissues are involved in this degenerative process. Phagocytes

invade the area between stump tissues and the wound dermis. Phagocytic cells remove cellular detritus produced by the degeneration of injured skeletal, muscle and connective tissues (Weiss and Rosenbaum, 1967). Sarcolysis is prevalent as distal muscle tissue loses its striations and fragments of sarcoplasm plus nuclei litter the space between the amputation surface and wound epidermis (Thornton, 1938b).

The environment present during the demolition phase is characteristic of a degenerating system. Circulation is interrupted, hypoxia ensues, and lactic acid levels build up following the anaerobic breakdown of muscle glycogen thus lowering the pH (Schmidt, 1960). In the acid environment of the amputated limb stump, there is four times as much proteolytic enzyme activity as in normal limb tissue (Bromley and Orechowitch, 1934). Johnson and Singer (1964) histochemically demonstrated the presence of lactate dehydrogenase in amputated limb stumps which is also indicative of an acid environment. The dedifferentiation stage of the regressive phase of regeneration begins by the end of the third day with the liberation of mononucleated cells from cartilage, muscle and connective tissue (Thornton, 1938a). These dedifferentiated cells do not die, but are viable and have been shown to synthesize deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins (Hay and Fischman, 1961; Bodemer and

Everett, 1959; Anton, 1965; Kelly and Tassava, 1973). These mononucleated cells lose their specialized characteristics and take on a mesenchymatous appearance. Gradually, as these dedifferentiated cells accumulate, they contribute to the formation of a regeneration bud or blastema and therefore are referred to as blastema cells. Through several series of mitotic divisions, the number of blastema cells increases resulting in the continued growth of the regenerate. Most of the cell proliferation which occurs takes place between days five and 19 of regeneration although mitosis continues at a slower rate even in the later stages of regeneration (Figure 1). Morphogenesis is then the predominant developmental event.

Numerous studies support the view that local stump tissues give rise to the blastema. The earliest evidence was provided by Butler (1935) who transplanted unirradiated limbs of Ambystoma larvae to X-irradiated hosts. Following amputation, these limbs regenerated, but no regeneration was observed from irradiated limbs transplanted to normal hosts. If blastemal cells are not of local origin, then irradiation of limb tissue prior to amputation would not preclude regeneration since unirradiated body cells could move in and support regeneration. However, this does not seem to be the case. Further evidence for the local origin of blastemal cells was provided when short midsegments of limb tissue were irradiated. Amputations through the

irradiated tissue failed to evoke a regeneration response, but amputations just proximal or distal to the irradiated segment resulted in regeneration (Butler and O'Brien, 1942). Also consistent with the theory of local blastemal cell origin is the observation of Chalkley (1954, 1959) that a large number of mitoses can be correlated with specific stump tissues throughout the early stages of regeneration. Chalkley (1954) calculated the contribution of each different stump tissue and concluded that the vast majority of regeneration cells originated from local stump tissue.

The question of whether metaplasia occurs during the course of regeneration has yet to be fully answered. Conflicting evidence has been presented by many authors. Removal of the humerus prior to amputation does not stop the formation of the humerus in the regenerate (Bischler, 1926). This would suggest that at least limited metaplasia does take place. Rose and Rose (1965) labeled adult newt epidermal cells with H^3 - thymidine and followed the labeled cells through five cell generations after amputation. Autoradiographs indicated the presence of labeled muscle and cartilage cells in the differentiated regenerate. Unfortunately, this evidence is not conclusive because the possibility of label transfer was not eliminated. Steen (1968) implanted H^3 - thymidine and triploid labeled cells from skeletal tissue into diploid limb stumps of axolotls (Ambystoma mexicanum). By following the doubly

labeled cells through regeneration, it was demonstrated convincingly that "complete" metaplasia does not occur. Skeletal tissue cells could form connective tissue, cartilage and bone, but not muscle or epidermis. In conclusion, local differentiated stump tissues seem to give rise to dedifferentiated blastemal cells which remain covertly differentiated. After proliferation, they give rise to differentiated tissues of the same general type ("limited metaplasia"). Therefore one could explain Bischler's above mentioned observation by theorizing that although the humerus is removed, other "connective tissue type" cells present in the limb give rise to the new humerus in the regenerate.

The progressive phase of limb regeneration begins between the fourth and eighth days post-amputation, even though the process of dedifferentiation is still taking place. It is significant that the mesenchymatous cells that accumulate bring about the cessation of stump tissue regression (Butler and Puckett, 1940; Schotte, Butler and Hood, 1941). This important tissue interaction leads to the first stage of the progressive phase which commences with the aggregation of blastemal cells beneath a thickened wound epidermis.

The relationship between the wound epidermis and the underlying regeneration blastema is most profound. The experiments involving ultraviolet irradiation and epidermal

cap removal of Thornton (1958, 1968) prompted investigations into the relationship between the epidermal cap and the underlying mesoderm (blastemal cells). Thornton and Sprague (unpublished) removed whole skin or epidermis from axolotl limbs, treated them with Actinomycin D, and replaced the epidermis over untreated mesodermal tissues. These limbs either failed to regenerate when amputated or regenerated imperfectly. It was concluded that some type of DNA-dependent RNA synthesis was needed for the skin to influence regeneration.

Hay and Fischman (1961) showed by autoradiography that DNA synthesis begins in the limb stump as early as four days after amputation which indicates that cell proliferation is an early event in limb replacement. However, the peak of mitotic activity occurs when the blastema is 17 days old (Singer and Craven, 1948). A high level of mitotic activity continues through the cone stage until the regenerate becomes paddle shaped (palette; days 19-20) and then subsides during subsequent morphogenesis and differentiation. The pattern of cell division is not uniform throughout the regenerate. Mitotic activity begins in the tissues of the limb stump during early regeneration phases and then proceeds distally so that by the paddle stage, the mitotic index is highest in the distal half of the regenerate (Chalkley, 1954). This data led Chalkley (1959) to postulate the presence of an apical growth cone

and that cell division is under regional controlling influences.

Blastemal morphogenesis begins around day 19 of regeneration as the cone flattens distally. The resulting paddle-shaped regenerate still contains mitotically active cells, but as regeneration continues cell differentiation replaces proliferation as the predominant developmental process. By day 21, the first digits begin to appear and the regenerate continues to increase in size until by day 28, the limb is somewhat larger and possesses three digits. Usually, the fourth and final digit is formed by day 35 when the regenerate is approximately one half the size of the original limb. From this point on, differentiation is almost complete except for the ossification of the skeletal elements and the laying down of more muscle tissue. Perhaps the slowest tissue to form is bone and even 35 day regenerates show little, if any, ossification.

Histological sections (personal observations) reveal that cartilage differentiation begins just after day 21. By day 25, some muscle fibers are seen growing in from stump tissue while others arise independently in the regenerate as a result of myoblast fusion (Hay, 1966). The muscle proteins actomyosin and myosin can be detected immunochemically as early as day 21 (Laufer, 1959). This indicates that mesenchymatous blastema cells appear to undertake cell specific protein synthesis several days

before histological differentiation occurs. As muscle tissues form, nerves present in the regenerate establish motor end-plate connections (Lentz, 1969).

When a limb is amputated, nerves as well as blood vessels are severed. Regenerating nerve fibers begin to sprout from the cut ends of the large nerve trunks as early as two days post-amputation (Singer, 1952; Thornton, 1968). New blood vessels grow in from severed ends shortly after the demolition phase and the early regenerate becomes well vascularized. Thus, even early regenerates are well endowed with nerves and blood vessels.

As previously stated in the introduction, denervation of the newt forelimb between the day of amputation and day 13 of regeneration results in a complete cessation of development. Denervation between days 13 and 17 has a variable effect in that some blastemata continue to grow and undergo morphogenesis while others regress. After day 17, denervation does not affect the morphogenesis of the blastema, but the completed regenerate undergoes atrophy (Singer, 1952; Powell, 1969). In the newt, denervation is typically performed by transecting the third, fourth and fifth brachial nerve trunks which contain both sensory and motor fibers.

It makes no difference as to the type of nervous tissue which innervates the blastema. Karmen and Singer (1959) were able to obtain limb regeneration after

implanting isolated, brachial sensory ganglia into denervated newt regenerates. This data also indicates that a central nervous connection is not needed for regeneration. Thornton (1956) implanted spinal cord segments together with a forelimb onto the dorsal tail-fins of Ambystoma larvae. After spinal nerve fibers innervated the deplanted limb, subsequent amputation caused a regeneration response. Even hindbrain segments could elicit a response and these experiments indicated that the effect of nerves on regeneration is not specific with regard to the type of nervous tissue.

Although the nerve effect is not qualitative in nature, it does appear to be quantitative. Singer (1946a, b; 1947a, b) severed various combinations of the three brachial nerve trunks which supply each forelimb and was able to show that the newt forelimb could regenerate with only about one-third of its total nerve supply left intact. This was an average of 9.6 fibers for each $100\mu^2$ of amputation surface and represented a "threshold quantity" since fiber numbers below this concentration were not sufficient for regeneration. Later, Peadon and Singer (1965) discovered that larval stages of the newt have the ability to regenerate with fewer nerve fibers than the adult. It was concluded that the neural threshold varies throughout the life cycle of the urodele amphibians.

Singer (1951) suggested that non-regenerating animals,

such as frogs, fail to replace lost limbs because the number of nerves present at the amputation surface is below the "threshold" value. In proof of this view, Singer (1950, 1954) deviated intact sciatic nerves into forelimbs of young adult frogs thus adding to the normal supply of fibers provided by the third, fourth and fifth brachial nerves. Subsequent amputations resulted in regeneration responses from super-innervated limbs but not from contralateral control limbs. Nerve fiber counts indicated that only in the super-innervated limb was the nerve fiber "threshold" reached. The addition of extra nervous tissue in limbs to obtain regeneration is even possible in the taxonomically distinct opossum although only new-born animals have been used in these experiments (Mizell, 1968; Mizell and Isaacs, 1970).

Interestingly, the south African clawed frog, Xenopus laevis, regenerates a long spike-like protuberance in spite of the fact that the number of nerve fibers present at the amputation surface falls significantly below the neural threshold. Rzehak and Singer (1966) investigated this case and found the cross sectional area of the nerve fibers to be much greater in Xenopus than in all other species examined. This finding led them to modify Singer's original theory and propound a modified theory which takes into account the quantity (cross sectional area) of nervous tissue present at the amputation site rather than just the

number of nerve fibers. Egar and Singer (1971) used the electron microscope to detect small nerve fibers in the adult newt regenerate not previously seen with the light microscope (Singer, 1952). It was determined that there are actually 20 fibers per $100\mu^2$ amputation surface instead of 9.6 fibers per $100\mu^2$. However, the amount of neuroplasm present at the amputation surface increased only by 3% because the fibers detected by the electron microscope were small and unmyelinated.

Typically, the nature of the neurotrophic action on amphibian limb regeneration has been investigated by depriving the limb of its nerve supply (Singer, 1952). Changes detected in the denervated regenerates are then compared to controls and in this manner the influence of the nerve supply on regeneration can be inferred. Replacement therapy studies and reinnervation experiments have also yielded valuable data concerning the neurotrophic effect.

Regenerates of adult newts kept at $25 \pm 0.2^\circ\text{C}$ typically end nerve-dependence on day 12, enter a transition period on day 13 and become nerve-independent by day 17 of regeneration (Singer and Craven, 1948). Sperry (1971), however, determined that regenerates of young (12 cm) axolotl larvae (21.5°C) were nerve-dependent on day seven post-amputation and became nerve-independent by day 12. It is most important to realize that during any point of

the nerve transition period, some regenerates are nerve-independent while others are nerve-dependent and for any one regenerate the actual transition period may be much shorter than several days.

When the 10 to 12 day nerve-dependent blastema of the adult newt is denervated, tissue edema ceases by one day post-denervation and the regenerate becomes characterized by an opaque and wrinkled appearance. By the second day following denervation, a cessation of growth can be noted macroscopically and shortly thereafter the epidermis covering the blastema becomes thin. Regression of the blastema commences by 3-4 days post-denervation with some atrophy also seen later in the stump tissues (Bryant, Fyfe and Singer, 1971).

Bryant et al, (1971) determined from ultrastructural studies that the blastema loses up to 50% of the typical dedifferentiated blastema cells four days following denervation. Phagocytic cells, which comprise 6-8% of the blastema, remain unchanged in number in spite of continued nerve deprivation while pale and vacuolar cells increase in number. These pale and vacuolar cells may be formed from the autophagocytosis of common blastema cells (mesenchymatous cells) (Bryant et al., 1971). Other changes following denervation include the decrease in the number of cell layers in the overlying epidermis, the slow formation of a basement membrane and an increase in the amount of

connective tissue matrix (Bryant et al., 1971).

Popeila (1972) noticed that when denervation occurs on the day of amputation in Ambystoma larvae, the formation of a blastema is precluded. Instead, numerous collagen containing cells appear while stump tissues regress. This regression is probably due to autophagocytosis and the removal of cells by macrophages. Experiments such as these indicate that nerves are not only needed to initiate regeneration but also to maintain the blastemal cell population once it is formed.

Mitotic activity of the blastemal cell is also under neurotrophic control. Newt forelimbs denervated on days 13, 15 or 17 respond by undergoing a burst of mitotic activity shortly after neural deprivation (Singer and Craven, 1948). Following this temporary burst of cell division, there occurs a sharp decline in the mitotic index to very low levels. Both the burst of mitotic activity and its ultimate decline are seen only in denervated limb regenerates and not in controls.

Although cell numbers and mitotic index decline in denervated limbs, the ultrastructure of the typical undifferentiated blastema cell is apparently unchanged. The typical blastema cell found in 10 to 17 day regenerates of clumped chromatin in the nucleus, and possesses abundant mitochondria. A characteristic feature of this cell type

is an extensive network of rough endoplasmic reticulum with numerous laminated cisternae (Salpeter and Singer, 1960; Norman and Schmidt, 1967). Denervation of both the adult newt forelimb (Bryant et al., 1971) and larval Ambystoma forelimb (Popeila, 1972) have no apparent effect on the ultrastructure of the common blastema cell. Whether or not denervation causes changes in the type of biochemical activities of these cells has yet to be ascertained.

Denervation of the newt forelimb regenerate profoundly influences the amount of biosynthesis of many important macromolecules. Dresden (1969) denervated nerve-independent, paddle stage regenerates and sampled for total DNA, RNA and protein synthesis at various intervals following nerve deprivation. He determined that RNA synthesis begins to decline sharply seven hours post-denervation and levels off by 40 hours to 50% of control levels. Subsequently, DNA and protein synthesis begin to fall off at 38 hours and DNA synthesis does not stabilize until 78 hours post-denervation. Stabilized synthesis of DNA in the denervated limb is only 30% of control values while protein synthesis is 80%.

Liebowitz and Singer (1970) reported that in 10-13 day newt forelimb blastemata, denervation results in a rather transient increase in the amount of protein synthesis approximately 5 hours following severance of the

nerve supply. Later, Singer and Caston (1972) detected an increase in RNA synthesis four hours post-denervation and an increase in DNA production three hours later. After this initial increase in RNA, protein and DNA synthesis, all values declined to levels much lower than controls. Singer and Caston (1972) suggested that following denervation, neurotrophic substances are quickly released from the severed nerve trunks and this initiates the burst of RNA synthesis seen shortly thereafter. The RNA synthesized causes an increase in protein translation some of which is involved in DNA replication. As DNA synthesis increases, cell proliferation ensues and the mitotic index rises (Singer and Craven, 1948). As the severed nerve trunks degenerate, the supply of trophic substances becomes exhausted, and synthesis of macromolecules and cell proliferation decline accordingly (Singer and Caston, 1972).

Kelly (1971) correlated the initiation of regeneration in amputated, larval Ambystoma limbs with increases in RNA synthesis and mitotic activity. Denervation on the day of amputation inhibited RNA synthesis only slightly while cell proliferation and blastema formation were completely inhibited. It appears that the trophic action of the nerves on the cells of the regenerate is one of controlling the biosynthesis of important macromolecules responsible for the aggregation of blastemal cells and their

subsequent proliferation. Finally, the maintenance of the structures formed during regeneration also seems to be under neurotrophic control (Thornton, 1970).

These data indicate that the nerve supply may act directly on blastemal cells by repressing and activating selected genes. Although good evidence for this possibility is lacking in the field of limb regeneration, important related facts have accrued during the study of cross reinnervation effects on muscle tissue (See Guth, 1968 for review). Samaha et al, (1970), using cats, surgically shifted the nerve supply of a fast muscle to a slow muscle. The nerve supply of the slow muscle was then implanted in the fast muscle. Subsequently, the fast muscle assumed the physical and biochemical characteristics of the slow muscle while the slow muscle took on characteristics of the fast muscle. Self-reinnervated controls did not alter their typical features. The most interesting result was that in cross-reinnervated muscles, a change occurred in the proteins found closely associated to the muscle protein myosin. For example, in slow muscle innervated by a fast muscle nerve supply, the characteristic "closely associated proteins" for slow muscle disappeared while the "closely associated proteins" indicative of fast muscle were detected by disc gel electrophoresis. Later, Guth et al. (1971) showed that this cross reinnervation of muscle served to initiate

the synthesis of these different proteins. Subsequent denervation of both fast and slow muscles does not result in a cessation of the synthesis of these closely associated proteins. In this particular case, the nerve supply clearly influences the gene activity in the tissue it innervates.

The neurotrophic activity seen in newt forelimb regenerates seems to be mediated chemically although its exact nature has eluded investigators for years. Taban (1955) injected acetylcholine and other neurodynamic substances into denervated limbs but did not obtain a regeneration response. However, Singer (1960) perfused newt stumps with substances which blocked the acetylcholine mechanism and achieved blockage or delay of regeneration, but it is possible that these substances were toxic to the blastemal cells. Burnett et al. (1971) perfused frog limbs and denervated newt limbs with extracts of newt blastemata and observed some growth while Deck and Futch (1969) found that the ribonucleoprotein fraction of blastemal extracts permitted limited blastemal cell aggregation and growth. Lebowitz and Singer (1970) demonstrated that heat sensitive nerve extracts could maintain protein synthesis in denervated mound stage blastemata for several hours.

In summary, it may be concluded that the entire process of regeneration is at least partially under neurotrophic control. This control seems to be mediated mainly by chemical means and may act via the genes of the

blastemal cells and overlying epidermis.

Although past experiments have shown that RNA synthesis appears to be inhibited by denervation, there have been no attempts to study mRNA synthesis in this system. If mRNA synthesis is directly affected by denervation, it would lend support to the theory that the neurotrophic influence is one of genetic control. It would also provide information as to the extent the regeneration process is dependent on nerves.

METHODS AND MATERIALS

Newts (Notopthalmus viridescens) were collected from the Shawnee State Forest near Portsmouth, Ohio and stored at 4°C until use. Two days prior to amputation, the newts were divided into groups of 15 per plastic container and allowed to acclimate to incubator conditions of 26± 1°C in aerated tap water with aqua aid added to inhibit fungal growth. Due to the extremely large number of animals used in the course of this study, the animals were not fed. However, regeneration occurred normally nonetheless (Tassava, 1969).

After the acclimation period, newts were anesthetized in MS-222 (ethyl m-amino benzoate methanesulfonate, Eastman) and both forelimbs of each newt were amputated just proximal to the elbow. The protruding bone was then pared back to the amputation surface. The newts were allowed to regenerate to the appropriate stage of regeneration. Newts which regenerated poorly were eliminated from the experiment. When infection was apparent, a solution containing penicillin G (20 I.U./cc aerated H₂O) and streptomycin sulfate (20 µg/cc aerated H₂O) was temporarily added to the water.

The three stages of regeneration chosen for experimentation

were the late paddle-early digit stage (day 21), the 2-3 digit stage (day 28) and the 3-4 digit stage (day 35) (Figure 1). Only later nerve-independent stages were chosen because a large quantity of regenerating limb tissue was needed to detect radioisotope label incorporation. Later in the study, it was determined that mRNA synthesis in the late paddle-early digit stage (day 21) was very sensitive to denervation so that most of the subsequent experimentation was performed on this stage.

Two days prior to each of the regeneration stages the right forelimbs of the experimental animals were denervated by transecting the third, fourth and fifth brachial nerves at the shoulder region. The two day interval between denervation and polysome extraction was chosen because total RNA synthesis in paddle stage regenerates has been shown to decrease maximally two days after denervation (Dresden, 1969). The success of the denervation operation was routinely tested by removing the left (control) and right (denervated) regenerates and allowing regeneration to take place again. In all cases, the left limbs regenerated while the right limbs did not, thus indicating that denervation was complete. Sham-operated controls were not used because experiments by Singer and Caston (1972) showed that incorporation of labelled precursors into DNA, RNA and protein did not differ significantly between unoperated limbs and those that were sham-operated.

On the appropriate day of regeneration (two days post-denervation), newts were intraperitoneally injected with either (1) H^3 -Uridine (New England Nuclear- 24 Ci/m mole) for six hours, (2) P^{32} - $NaPO_4$ for 90 minutes or (3) an H^3 -amino acid mixture (New England Nuclear-15 Ci/m mole) for 90 minutes. The six hour incorporation period resulted in H^3 -Uridine labelling of all species of RNA; ribosomal RNA, transfer RNA, heterogeneous nuclear RNA and messenger RNA (Morzlock and Stocum, 1971). The 90 minute incorporation period resulted in P^{32} - $NaPO_4$ labelling of heterogeneous nuclear RNA and messenger RNA only (Heywood and Nwagwu, 1969). P^{32} was used in this case in order to obtain detectable activity in the heterogeneous nuclear and messenger RNA species. Messenger RNA labelled with H^3 -Uridine was not radioactive enough in this system for consistent detection in all regions of the polysome gradient. The H^3 - amino acids were used as a precursor for nascent peptides on polyribosomes and served as an indicator of the level of protein synthesis.

Following the incorporation period, left (control) and right (denervated) regenerates were collected and pooled separately. The regenerates (each group was processed separately) were immediately immersed in ice-cold muscle standard buffer (MSB- 0.25M KCl, 0.01 M $MgCl_2$, 0.01 M tris, pH 7.4). MSB was employed because the high molarity of the KCl prevented the co-precipitation of

the larger-sized polysomes (Heywood et al., 1967). All subsequent operations were performed at 0°-4°C to prevent any nucleases from degrading the polysomes.

The collected tissue was greatly homogenized with not more than five strokes of a loose-fitting Dounce style homogenizer to minimize the mechanical breakage of polysomes (Heywood et al., 1967). Because of the potential problem of releasing contaminating, labeled, co-sedimenting RNA from nuclei and of releasing nucleases from lysosomes, the use of detergents was avoided (Venkatesan and Steele, 1972; Olsnes, 1970; Heywood et al., 1967). Consequently, the polysome extraction technique used liberated free, nonmembrane bound polysomes only.

Included in the homogenization buffer were the ribonuclease inhibitors bentonite (Jacoli, 1968) and diethyl pyrocarbonate (DEP-Venkatesan and Steele, 1972:). Bentonite (Fisher) was first purified by three separate 10K x g centrifugations in distilled water followed by overnight stirring in the presence of 0.1 M EDTA. The bentonite was then washed several times to remove all traces of EDTA and finally heat dried. The bentonite was powdered and 0.1 mg was added for each milliliter of homogenization buffer. Diethyl pyrocarbonate (Eastman) was added to the homogenization buffer immediately prior to use at a concentration of 0.02%. This concentration was selected because at higher concentrations DEP is degraded to CO₂

and leads to a lowering of pH in low molarity tris buffers (Vankatesan and Steele, 1972). However, 0.02% DEP did not cause the pH to be lowered in this system.

Polysome gradients were prepared by centrifuging the homogenates at $10K \times g$, 10 minutes, $0^{\circ}C$ using a SS-34 rotor in a Sorvall RC-2B centrifuge. The pellets were discarded and 0.1 mg bentonite was added for each milliliter of post-mitochondrial supernatant. The supernatants were separately layered on top of linear 15-40% sucrose gradients in MSB with 0.02% DEP added immediately prior to gradient formation. Following centrifugation at 25,000 rpm for two hours at $0^{\circ}C$, using a Spinco SW 25.1 rotor in a Beckman L2-65B preparative ultracentrifuge, the gradients were fractionated using Isco equipment while the optical density was recorded at 254 nm. Depending on the experiment being performed, gradient fractions were either precipitated with trichloroacetic acid (TCA) and analyzed for radioactivity or the polysomes were dissociated with a sodium dodecyl sulfate (SDS)-EDTA buffer and respun on another set of sucrose gradients. The second set of gradients separated out the various species of RNA contained in the polysome fractions and these were also subjected to TCA precipitation and analysis for radioactivity.

If the polysome fractions were being analyzed for TCA precipitable counts, then 1.2 ml fractions were

collected from each gradient and made 5% with respect to cold TCA. The resultant precipitates were caught on 24mm, 0.45 μ pore size nitrocellulose filters (Millipore) and the filters were washed with five ml of cold TCA followed by cold 95% ethanol. The filters were air dried and placed in scintillation vials with 10 ml of toluene based scintillant (4 grams Omnifluor; New England Nuclear; 1 liter toluene). The vials were counted in a Packard Tri-Carb liquid scintillation spectrometer and background counts were subtracted from the total, the degree of quench being consistent for all vials prepared in this manner. If the isolated polysomes were being analyzed for labelled mRNA, then two gradients were run for control regenerates and two for denervated regenerates. Each gradient was divided into a "heavy" and "light" polysome region (Figure 11) and these two regions were pooled from two gradients. The combined heavy and light polysome regions from control and denervated regenerates were caught on 0.45 μ Millipore filters (previously washed in SDS, rinsed in H₂O and finally washed in MSB). The filters were rapidly washed with 0.01 M MgCl₂, 0.01 M tris-HCl (pH 7.4) and then placed in small plastic Petri dishes containing 0.8 ml of buffer (0.5% SDS, 0.005 M EDTA, 0.02 M sodium acetate and 0.04 M tris-HCl, pH 7.8) for one-half hour. The released RNA in the buffer was layered on a 17 ml, 10-30% sucrose gradient in the SDS-EDTA buffer and spun

using an SW-27 rotor (5/8" x 4" buckets) in a Beckman L2-65B preparative ultracentrifuge at 27,000 rpm for 16 hours at 20°C. The gradients were fractionated using Isco equipment and the optical density read at 254 nm. The 0.4 ml fractions collected were processed for TCA precipitable counts as previously described.

Early in the study it was determined that the newt system contained relatively low levels of polysomes and that ribonuclease was present in the preparations. Consequently, it was necessary to test the effects of newt ribonuclease on polyribosomes and to prove that the ribonuclease inhibitors bentonite and DEP were effective in totally inhibiting newt ribonucleases. The chick embryo leg muscle system was chosen as a test system because it is characterized by high levels of non-membrane bound polysomes and no free ribonucleases (Heywood et al., 1967; Heywood and Rich, 1968; Heywood and Nwagwu, 1968; Heywood et al., 1968; Heywood, 1969; Heywood and Nwagwu, 1969).

In all test cases, chick muscle polysomes were prepared in the following manner. Fourteen day old chick embryo leg muscle was intramuscularly injected with isotopes. After the incorporation period, 0.8-1.0 gram of muscle tissue was dissected free from skin and bone under cold MSB. Following homogenization with a Dounce homogenizer, the homogenate was usually divided into three

aliquots. One aliquot served as a control, the second was combined with newt homogenate and the third was treated with commercial grade bovine pancreatic ribonuclease (Nutritional Biochemicals). The homogenates were then processed for polysome fractionation as previously described.

RESULTS

1. The isolation of undegraded newt polyribosomes

A major goal of this study was to extract undegraded polyribosomes from newt forelimb regenerates and to separate P^{32} labeled messenger RNA from these polysomes. The greatest disadvantage of this system proved to be the small size of the regenerate which necessitated using large numbers of late stage regenerates. Although the gentle homogenization technique used in this study minimized leakage of ribonuclease from lysosomes and rapidly labeled RNA from nuclei, it was also inefficient in breaking the blastemal cells resulting in lower yields. Consequently, it was necessary to use chick embryo leg muscle polysomes (Heywood et al., 1967; Heywood and Nwagwu, 1969) in an indirect test to determine whether ribonuclease from newt preparations was completely inactivated during polysome isolation.

Figure 2 shows the effects of ribonuclease on polysomes extracted from 14 day old chick embryo leg muscle. Graph A represents an undegraded pattern of chick muscle polyribosomes from tissue which contained little, if any, ribonuclease activity (Heywood et al., 1967). The polysome part of the profile contained distinct peaks well into the middle of the profile and the optical density was generally

Figure 2. Effects of commercial grade pancreatic ribonuclease and newt ribonuclease on polysome profiles of 14 day old chick embryo leg muscle. One gram of unlabeled, 14 day chick embryo leg muscle was homogenized in 1.5 ml of MSB using 5 strokes of a Dounce style homogenizer (loose-fitting). Twenty three day (post-amputation) newt forelimb regenerates (0.25 g) were homogenized similarly in 0.4 ml of MSB. No ribonuclease inhibitors were used in this experiment. Following centrifugation at 10K x g, 10 minutes, 0°C, both supernatants were divided into aliquots, variously combined and layered on sucrose gradients as described in Methods and Materials. A: Control pattern of 14 day chick embryo polysomes using 0.5 ml of 10K x g chick supernatant combined with 0.2 ml of MSB. B: Pattern of 14 day chick embryo polysomes using 0.5 ml of 10K x g chick supernatant with 0.2 ml of MSB containing 50 μ g bovine pancreatic ribonuclease (commercial). C: Pattern of 14 day chick embryo polysomes using 0.5 ml of 10K x g chick supernatant with 0.2 ml of newt 10K x g supernatant. D: Pattern of 23 day newt forelimb regenerates using 0.2 ml of newt 10K x g supernatant with 0.5 ml of MSB.

high throughout the scan. Although the 75S monosome peak extended off the graph, the dimer shoulder immediately to the left was relatively small (dimer to monomer -0.33). This pattern is indicative of an undegraded profile (Vankatesan and Steele, 1972). Graph B shows the effect of commercial grade bovine pancreatic ribonuclease on the polysome profile of chick embryo leg muscle. The optical density in the polysome part of the scan was greatly lowered over that seen in Graph A and the monosome peak was greatly increased. This was probably due to the added ribonuclease cleaving messenger RNA strands holding individual ribosomes together. If all the interconnecting mRNA was cleaved, then only liberated ribosomes remained, as indicated by the high 75S monosome peak. Graph C indicates that newt preparations contained a quantity of ribonuclease. The dimer peak was higher with respect to the monosome peak (dimer to monomer ratio -0.47) while the heavier polysome regions appear to be depressed. Although there were distinct peaks in the lighter portion of the gradient, these peaks were somewhat higher than those seen in Graph A. They were probably due to the partial cleaving of heavier polysomes by ribonuclease resulting in fragments which then sedimented with the smaller polysomes. Graph D is the polysome profile from 23 day newt forelimb regenerates. Due to the scarcity of material much less homogenate could be layered on the gradient as compared to the chick material in

Graphs A, B and C. The small amount of UV absorbing material made it impossible to determine whether polysomes were present in the gradient and, if so, whether they were intact or degraded by ribonucleases.

It was important then to find an acceptable method of determining whether ribonuclease activity was completely inhibited during the isolation procedures for the newt polyribosomes. Thus, if newt polysome preparations were added to H^3 -Leucine labelled chick polysomes in the presence of bentonite and DEP, the effectiveness of ribonuclease inhibitors could be tested. The O.D. and counts per minute profiles of the newt plus chick polysome preparation should show no difference from the chick only profiles if all ribonuclease activity was inhibited. However, it was necessary to first prove that the ribonuclease inhibitors used did not adversely affect the resultant polysome profile.

Various ribonuclease inhibitors were tried to prevent degradation of the newt polysomes. Rat liver ribonuclease inhibitor (Triplett et al., 1970), macaloid, bentonite and diethyl pyrocarbonate were used with success, but the latter two inhibitors proved most effective when used in combination with each other. Figure 3 shows that bentonite and diethyl pyrocarbonate did not affect the polysome profile of the chick embryo leg muscle. Graph A is the control profile using no ribonuclease inhibitors and represents an undegraded polysome profile. Graph B represents the

Figure 3. Effect of bentonite and diethyl pyrocarbonate on polysome profiles from 14 day chick embryo leg muscle. Three equal aliquots of unlabeled, 14 day chick embryo leg muscle were homogenized and processed for polysomes as described in Methods and Materials. A: Control pattern without ribonuclease inhibitors. B: Pattern with 0.1 mg bentonite added to each milliliter of homogenization buffer. C: Pattern with 0.02% diethyl pyrocarbonate added to the homogenization buffer and the sucrose gradient.

pattern obtained from chick muscle homogenized in the presence of bentonite. Following centrifugation, bentonite was again introduced into the 10K x g supernatant to inhibit polysomal breakdown. As the supernatant was layered on the sucrose gradient, the bentonite slowly sedimented through the gradient but this did not change the resultant profile in any way as this graph is identical to Graph A. Graph C is the pattern of chick muscle polysomes obtained using 0.02% diethyl pyrocarbonate. Although this level of DEP was very low and not entirely effective in inhibiting ribonuclease alone, it could be added to the gradient whereas bentonite would not stay in suspension due to centrifugation.

Venkatesan and Steele (1972) pointed out the dangers of using DEP to inhibit ribonuclease in polysome preparations. DEP rapidly breaks down in aqueous solutions to CO₂ and ethanol. The CO₂ in solution lowers the pH and disrupts both polysomes and monosomes. Only higher concentrations of tris prevent the lowering of pH, but if the tris concentration is too high, polysomes aggregate together and coprecipitate. The 0.01 M concentration of tris used in this study was high enough to keep the pH constant despite degradation of the 0.02% DEP, but not so high that polysome aggregation occurred.

Figure 4 shows the optical density profile of H³-Leucine labelled polysomes obtained from chick leg muscle (Graph A) and chick leg muscle plus 23 day newt forelimb

Figure 4. Labeled amino acid incorporation into undegraded and degraded chick polyribosomes. ■■■■ optical density (254 nm), ●●●● net counts per minute, H^3 -Leucine. Two 14 day chick embryos were intramuscularly injected with H^3 -Leucine. After 5 minutes of incorporation, the leg muscle was dissected free of skin and bone and homogenized in 0.5 ml of MSB in the presence of 0.1 mg/ml bentonite and 0.02% DEP. The homogenate was centrifuged at 10K x g, 10 minutes, 0°C. Twenty three day (post-amputation) newt forelimb regenerates were processed similarly, but without ribonuclease inhibitors. The 2-10K x g supernatants were variously combined and layered on sucrose gradients as described in Methods and Materials. A: Control pattern of labeled, 14 day chick embryo polysomes using 0.5 ml of 10K x g chick supernatant with 0.2 ml of MSB. B: Pattern of labeled 14 day chick embryo polysomes using 0.5 ml of 10K x g chick supernatant with 0.2 ml of newt 10K x g supernatant. C: Pattern of labeled 14 day chick embryo polysomes using 0.5 ml of 10K x g chick supernatant with 0.2 ml MSB containing 50 ug bovine pancreatic ribonuclease.

regenerates (Graph B) which were isolated in the presence of the RNase inhibitors bentonite and DEP. Graph C shows the effect of commercial grade ribonuclease on chick leg muscle polysomes in the absence of the inhibitors. Graphs A (control) and B (chick plus newt polysomes preparations) showed virtually no differences throughout the entire scans thus indicating that ribonuclease activity was nonexistent. Graph C exhibits a greatly heightened monosome peak (75S) compared to Graphs A and B while the polysome portion of the gradient is greatly depressed.

The H^3 -Leucine label was used to further test the effectiveness of the two inhibitors in inactivating ribonuclease. Proteins are made on polysomes and not on individual ribosomes (Rich et al., 1963). Thereafter, if labelled amino acid precursors are administered for a short five minute labelling period, the bulk of the TCA precipitable counts representing nascent peptides should be seen in the polysome regions of the gradient and not in the monosome region (Heywood et al., 1967). A labelling period longer than five minutes would allow some ribosomes to complete protein synthesis on the messenger RNA and become free ribosomes thus possibly carrying some of the labelled peptide chains into the monosome region of the gradient. Degradation of polysomes by ribonuclease would also cause monosomes containing labelled peptides to be produced.

Graphs A and B of Figure 4 are equivalent in terms of

the counts per minute profiles. The monosome peaks (75S) of both showed very little incorporated radioactivity while polysome regions of both gradients incorporated far higher quantities of H^3 -Leucine. These patterns were consistent with those obtained by Heywood et al. (1967). Graph C is the pattern which results when bovine ribonuclease was added to the 10K x g chick leg muscle supernatant. In Graph C, there were few counts seen in the polysome region while many counts (153 counts per minute) were located in the 75S monosome peak. This data indicates that ribonuclease completely cleaved the mRNA holding the ribosomes together thus liberating single ribosomes which sedimented with their labelled nascent chains in the monosome peak. Graph B of Figure 4 then provided important evidence that newt ribonuclease activity could be controlled by a combination of gentle homogenization, pH, cold temperatures and the inhibitors bentonite and DEP. Since ribonuclease was controlled and the danger of mechanical breakage of polysomes minimized by the gentle homogenization technique used, it was plausible that the polysome profiles of newt forelimb regenerates were undegraded.

2. The influence of denervation on long term (6 hr) H^3 -Uridine incorporation into newt polyribosomes

The long-term labelling experiments were performed to determine the extent to which denervation affects precursor incorporation into monosomal and polysomal RNA of 21, 28, and 35 day regenerates. Denervations were per-

formed on days 19, 26, and 33 respectively so that the denervation time was two days in each case. The six hour incorporation period has been shown to label messenger, ribosomal and transfer RNA in newt forelimb regenerates (Morzlock and Stocum, 1971). Since rRNA is the predominant RNA species present, the six hour labelling data mainly represents radioactivity in rRNA. Consequently, the long labelling period data indicated the effect of denervation on ribosomal RNA incorporation assuming that a sufficient number of labelled monosomes were incorporated into polysomes during the six hour time period. The total optical densities in the polysome profiles derived from innervated and denervated regenerates were equalized and the net counts per minute were adjusted accordingly for comparative purposes.

H^3 -Uridine incorporation into polysomes of denervated 21 day regenerates was greatly decreased when compared to contralateral controls (Figure 5). The monosome peak (75S) of the denervated pattern showed a 49% decrease in the amount of radioactivity when compared to the monosome peak of the control (Table 1). The polysome part of the profile extending from the left side of the 75S monosome peak through the denser regions of the gradient showed a 55% decrease in incorporation by polysomes of denervated regenerates compared to controls (Figure 5). This 55% decrease in isotope incorporation into polysomes of

Figure 5. Polysome profiles of innervated and denervated 21 day newt forelimb regenerates labeled with H^3 -Uridine for 6 hours. — optical density (254 nm), - - - net counts/minute control (left) limbs, • • • net counts/minute denervated (right) limbs. Right forelimbs of 155 newts were denervated 19 days post-amputation while left limbs served as contralateral controls. On day 21, each newt was intraperitoneally injected with 35 μ Ci of H^3 -Uridine for 6 hours. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The optical density of the polysome profile from denervated regenerates was adjusted to match that of the control profile and the net counts/minute were correspondingly adjusted.

denervated 21 day regenerates was about the same for all regions of the polysome profile which indicated that denervation adversely affected ribosomal RNA incorporation into all sizes of polyribosomes equally.

This data suggest that if fewer labelled monosomes were in the denervated profile compared to the controls, then these labelled monosomes had less of a chance to take part in polysome formation since there was a proportionately greater number of unlabelled monosomes competing for a binding site on the messenger RNA. In both innervated and denervated profiles, the monosome peaks (optical density) were very large suggesting that there were ample monosomes available for protein synthesis and that this was not a limiting factor in polysome formation.

The polysome profiles of 28 day (Figure 6) innervated and denervated (denervation on day 26) regenerates demonstrated about the same pattern as seen in the 21 day profile (Figure 5). The 75S monosome peak of the denervated regenerates was depressed by 68% compared to the control which was an even greater difference than that detected for innervated and denervated 21 day regenerates. The polysome part of the 28 day profile was depressed by only 43%. Most of the difference was seen in the lighter regions of the polysome part of the profile (60% depression) while there was very little difference seen in the denser regions of the

Figure 6. Polysome profiles of innervated and denervated 28 day newt forelimb regenerates labeled with H^3 -Uridine for 6 hours. — optical density (254 nm), - - - net counts/minute control (left) limbs, ••••• net counts/minute denervated (right) limbs. Right forelimbs of 160 newts were denervated 26 days post-amputation while left limbs served as contralateral controls. On day 28, each newt was intraperitoneally injected with 35 μ Ci of H^3 -Uridine for 6 hours. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The optical density of the polysome profile from denervated regenerates was adjusted to match that of the control profile and the net counts/minute were correspondingly adjusted.

profile (26% depression). Depression of precursor incorporation was again noted in all regions of the denervated pattern with respect to the control. However, this depression was not as uniform as seen in the 21 day experiment (Figure 5). In Figure 6, the light polysomes (Region 1, fractions 10-19) were depressed uniformly by about 60% in each fraction, but the heavy polysomes (Region 2, fractions 1-9) showed much less percent depression as the polysomes became increasingly larger (toward the left of the gradient).

The least differences seen between denervated and control polysome patterns were those obtained from 35 day innervated and denervated regenerates (Figure 7). The 75S monosome peak of the denervated profile (denervation on day 33) was only depressed by 26% compared to the control. A slightly greater difference was observed in the polysome regions of the gradient as there was a 28% decrease in the amount of label incorporation in the denervated profile as compared to the control. More interestingly, the counts in the light region of the denervated profile (Region 1, fraction 10-18) were only depressed by 22% compared to the control while the counts in the denser region (Region 2, fractions 1-9) were decreased by 33%. The percent depression seen in 35 day denervated regenerates was not uniform throughout the profile as there was very little decrease in incorporation seen in polysomes located in the middle of the gradient (Figure 7). The very heavy and very light

Figure 7. Polysome profiles of innervated and denervated 35 day newt forelimb regenerates labeled with H^3 -Uridine for 6 hours. — optical density (254 nm), — — — net counts/minute control (left) limbs, • • • • net counts/minute denervated (right) limbs. Right forelimbs of 115 newts were denervated 33 days post-amputation while left limbs served as contralateral controls. On day 35, each newt was intraperitoneally injected with 30 μ Ci of H^3 -Uridine for 6 hours. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The optical density of the polysome profile from denervated regenerates was adjusted to match that of the control profile and the net counts/minute were correspondingly adjusted.

polysomes of denervated regenerates showed the greatest amount of depression compared to controls.

The results of the six hour, H^3 -Uridine incorporation experiments were made more difficult to interpret because of the basis of comparison used. Since the O.D. profiles of each experiment had been equalized to serve as a basis of comparison, it was not possible to use the O.D. profiles as a means of determining whether denervation decreased the number of polysomes present in newt regenerates (although in these particular experiments the O.D. was too low to be indicative of polysome number and type). Also, the equalization of innervated and denervated profiles did not allow the comparison of O.D. and net counts per minute profiles between 21, 28, and 35 day experiments since there was no common basis for this type of comparison. Accordingly, all other experiments performed in this study were standardized on the basis of 15 mg of protein present in the 10K x g supernatant using the basic technique of Lowry et al. (1951). This permitted the interpretation of optical density data from polysomes extracted from innervated and denervated regenerates (if the O.D. was high enough) of a single experiment as well as TCA precipitable counts. The results of experiments performed on different stages of regeneration could also be compared on this basis.

3. The influence of denervation on short-term (90 minute) P^{32} incorporation into newt polyribosomes

A series of experiments using 21, 28 and 35 day

regenerates were performed employing P^{32} for 90 minutes as an RNA precursor. This short labelling period allowed the incorporation of the isotope into only heterogeneous nuclear RNA and messenger RNA types. Late in the study, it was discovered that some soluble RNA was also labelled but this was only a small fraction of the total amount of isotope incorporated. Essentially, messenger was the only type of RNA that was labelled to a great extent and the radioactivity in polysome profiles of short term labelled regenerates primarily represented messenger RNA incorporation into polysomes.

A two day denervation period greatly decreased the amount of P^{32} incorporation into polyribosomes of denervated 21 day regenerates when compared to contralateral controls (Figure 8). The entire part of the denervated profile was depressed by 46% compared to the control (Table 1). However, in this experiment, the lighter weight polysomes (Region 1, fractions 10-18) showed a greater difference between denervated and innervated (63%) than the heavier weight polysomes (29%) in the denser regions of the profile (Region 2, fractions 1-9). The percent depression of incorporation into polysomes of denervated 21 day regenerates was highest near the 75S monosome peak (81%) but steadily decreased at a constant rate as the polysomes became larger so that by fraction 3 (Figure 8) the difference between innervated and denervated profiles was only 10%. This

Figure 8. Polysome profiles of innervated and denervated 21 day newt forelimb regenerates labeled with P^{32} for 90 minutes. — optical density (254 nm), — — — counts/minute control (left) limbs, • • • • counts/minute denervated (right) limbs. Insert in upper left hand corner shows an expanded scale of the net counts/minute for fractions 2 through 15. Right forelimbs of 100 newts were denervated 19 days post-amputation while left limbs served as controls. On day 21 each newt was intraperitoneally injected with 100 μ Ci of P^{32} - $NaPO_4$ for 90 minutes. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The Lowry test for protein was performed on the 10K x g supernatant of both innervated and denervated samples. The optical density and net counts/minute were then adjusted to a standard of 15 mg of 10K x g supernatant protein for comparison.

indicated that although all parts of the 21 day denervated polysome profile were depressed relative to controls, the reduction was not a uniform one as was the case in the 21 day long term experiment (Figure 5).

The polysome profiles of 28 day innervated and denervated regenerates (Figure 9) showed much less incorporation of P^{32} into polysomes (90 minute label) than in the 21 day experiment (Figure 8). However, the amount of depression seen in the polysome part of the 28 day denervated profile compared to the control was greater (56%) than that seen in the short term 21 day experiment (46%-Table 1). In the 28 day experiment, heavy polysomes (Region 2, fractions 1-9) showed a 66% depression in the denervated pattern and this difference was about the same for each fraction of Region 2. On the other hand, the light polysomes (Region 1, fractions 10-18) demonstrated a 46% reduction in the denervated pattern which was not uniform throughout. The greatest amount of depression was seen in fractions 14-18 of Region 1 (60%) while only about 28% depression was noted in fractions 10-14 of Region 1.

Both the innervated and denervated polysome profiles of 35 day regenerates demonstrated much more incorporation of isotope after 90 minutes of labelling (Figure 10) than either the 21 (Figure 8) or 28 day (Figure 9) short term labelling experiments. The denervated 35 day polysome profile was only depressed by 24% compared to the control

Figure 9. Polysome profiles of innervated and denervated 28 day newt forelimb regenerates labeled with P^{32} for 90 minutes. — optical density (254 nm); - - - counts/minute control (left) limbs, • • • counts/minute denervated (right) limbs. Insert in upper left hand corner shows an expanded scale of the net counts/minute for fractions 2 through 15. Right forelimbs of 100 newts were denervated 26 days post-amputation while left limbs served as controls. On day 28 each newt was intraperitoneally injected with 100 μ Ci of P^{32} - $NaPO_4$ for 90 minutes. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The Lowry test for protein was performed on the 10K x g supernatant of both innervated and denervated samples. The optical density and net counts/minute were then adjusted to a standard of 15 mg of 10K x g supernatant protein for comparison.

dense **FRACTION NUMBER** **light**
 Figure 10. Polysome profiles of innervated and denervated 35 day newt forelimb regenerates labeled with P^{32} for 90 minutes—optical density (254 nm), — counts/minute control (left) limbs, • • • counts/minute denervated (right) limbs. Insert in upper left hand corner shows an expanded scale of the net counts/minute for fractions 2 through 15. Right forelimbs of 50 newts were denervated 33 days post-amputation while left limbs served as controls. On day 35 each newt was intraperitoneally injected with 65 μ Ci of P^{32} - $NaPO_4$ for 90 minutes. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The Lowry test for protein was performed on the 10K x g supernatant of both innervated and denervated samples. The optical density and net counts/minute were then adjusted to a standard of 15 mg of 10K x g supernatant protein for comparison.

(Figure 10) and both heavy (Region 2, fractions 1-9) and both light (Region 1, fractions 10-18) polysomes were equally depressed. Differences in isotope incorporation between particular denervated and innervated fractions were somewhat variable throughout the profile and no consistent pattern was seen.

In all three short term labelling experiments, both innervated and denervated optical density profiles matched within 10% at any one point after standardization to 15 mg of protein. For this reason and because machine profile drawing errors of 5-10% were common, only one optical density profile was drawn on the graphs representing both innervated and denervated patterns. Differences in optical density profiles greater than 10% were considered to be significant in all experiments performed in this study.

4. The isolation of messenger RNA from newt polyribosomes

The second major goal of this study was to liberate labelled messenger RNA from polyribosomes and separate it from ribosomal and transfer RNA species which make up the rest of the polysomal RNA. The technique employing "Millipore" nitrocellulose filters to catch polyribosomes during the fractionation of polysome gradients and the subsequent dissociation with deproteinization of the polyribosomal complex using an SDS-EDTA buffer was developed by Heywood and Nwagwu (1969). The deproteinized, liberated polysomal RNA is layered on a second sucrose gradient (10-30% sucrose

in SDS-EDTA buffer) and spun to separate the different types of polysomal RNA. The gradient is then fractionated, the optical density recorded at 254 nm, and the fractions analyzed for the presence of TCA precipitable counts indicative of labelled RNA. The major advantage of this technique is that particular regions of the polysome gradient can be specifically analyzed for labelled mRNA of a certain type i.e. the mRNA which codes for the muscle protein myosin (Heywood et al. , 1967; Heywood and Nwagwu, 1969).

Because large amounts of material were needed in order to detect labelled mRNA, 35 day regenerates were used in an initial experiment exploring the feasibility of this technique using newt material (Figure 11). The 10K x g supernatant was layered on four separate polysome gradients which were spun and fractionated into two different regions containing both light (Region 1) and heavy (Region 2) polysomes. The four fractions of Region 1 were pooled as were the Region 2 fractions. The pooled Region 1 and 2 fractions were then filtered, dissociated and respun on second sucrose gradients which were then analyzed for optical density and TCA precipitable counts (Figure 12). The optical density profiles of Regions 1 and 2 indicate the presence of 28 and 18S ribosomal RNA and a small peak of 4-5S RNA which proved that polysomes were definitely present in the gradient and secondly, there were more

Figure 11. Fractionation of polysome gradients in preparation for mRNA analysis of 35 day newt forelimb regenerates. Newts (153) were injected with 40 μ Ci each of P^{32} -NaPO₄ for 90 minutes after 35 days of forelimb regeneration. Following incorporation, both forelimb regenerates of each newt were removed, homogenized and spun at 10K x g for 10 minutes at 0°C. The 10K x g supernatant was divided equally among 4 separate gradients and centrifuged using a SW 27 rotor (1" x 3½" buckets) in a L2-65B ultracentrifuge at 25,000 rpm for 2 hours at 0°C. Each gradient was fractionated into 2 regions. Region 1 included all "light" polysomes from the dimer region to midway in the polysome gradient while Region 2 included all "heavy" polysomes from midway in the polysome gradient to the bottom of the tube. The 4 Region 1 fractions of the 4 gradients were combined together as were all Region 2 fractions. These 2 combined fractions were then processed as described in the legend of Figure 12.

Figure 12. Polysomal RNA profile of 35 day newt forelimb regenerates labelled with P^{32} -NaPO₄ for 90 minutes. optical density (254 nm), net counts/minute. Region 1 (light) and Region 2 (heavy) fractions were collected and combined from 4 polysome gradients as described in Figure 11. Both regions were separately filtered onto 0.45 u pore size Millipore filters at 20°C. An SDS-EDTA buffer (0.8 ml) was used to dissociate the polysomal RNA off the filters and the 2 buffer fractions were layered on 17 ml, 10-30% sucrose gradients in the SDS-EDTA buffer. Following centrifugation at 26,000 rpm for 17 hours at 20°C using a SW 27 rotor (5/8" x 4" buckets), the gradients were fractionated into 0.4 ml fractions were precipitated with cold 5% TCA, filtered onto 0.45 u Millipore filters and washed with cold 95% ethanol. The filters were then dried and counted.

polysomes present in Region 1 than Region 2. The profile of TCA precipitable counts (Figure 12) in Region 1 shows that several peaks of radioactivity were present throughout the scan suggesting that several mRNAs were released from the polysomes in the region. The S values of these peaks ranged from about 10-32S while the S values of the single peak of radioactivity seen in Region 2 was about 36S. These data were consistent with the theory that polysomal size is dependent on the length of the messenger RNA since the ribosomes space themselves out equally along the messenger RNA. Therefore, the larger mRNAs have more ribosomes attached to them than smaller mRNAs and thus sediment at a faster rate (Heywood et al., 1967).

It was noted, however, that a considerable number of counts remained on the "Millipore" filters used in collecting the polysomes prior to dissociation. This may indicate that the dissociation process was incomplete and that some mRNAs were still tightly bound to the filters. Both Region 1 and 2 profiles were characterized by a persistent low background of counts throughout the gradient suggestive of labelled contaminating material which co-sediments with the polysomes in sucrose gradients. This made it necessary to determine the extent to which P^{32} labelled polysome profiles were contaminated by labelled cosedimenting material.

There is evidence that labelled, heterogeneous

nuclear RNA is released from nuclei during homogenization due to mechanical rupturing of the nuclei or the action of detergents on the nuclear membrane (Plagemann, 1969; Baltimore and Huang, 1968; Olsnes, 1970). This labelled material forms non-specific aggregates and cosediments along with polyribosomes thus adding its radioactivity to that present in polysomal RNA. Lee and Brawerman (1971) used 0.01 M EDTA in the homogenization buffer to dissociate rat liver polysomes prior to centrifugation. Therefore, the only material which sedimented into the gradient was the labelled contaminating RNA which was not dissociated. The amount of radioactivity from the EDTA-treated gradient was then subtracted from the polysome gradient which had been layered with an equal amount of undissociated 10K x g supernatant. The difference was considered to be the amount of label incorporated into polyribosomes.

This experiment was repeated using 21 day newt forelimb regenerates labelled with P^{32} - $NaPO_4$ for 90 minutes (Figure 13). Graph A shows incorporation of P^{32} into polysomes with the insert showing an expanded scale of fractions 2 through 15. Graph B shows the amount of isotope incorporated into the contaminating material with the insert depicting an expanded scale of fractions 2 through 15. A comparison of the inserts revealed that the pattern of labelled contamination followed the pattern of polysome

Figure 13. P^{32} labeled material cosedimenting with 21 day forelimb regenerates. — optical density (254nm), --- net counts/minute. Both forelimbs of 80 newts were amputated and 21 days later each newt was intraperitoneally injected with 60μ Ci P^{32} -NaPO₄ for 90 minutes. Following incorporation, the regenerates were collected and homogenized in 2 ml of MSB. The homogenate was centrifuged at $10K \times g$, 10 minutes, 0° C and the resultant supernatant was divided equally in half. One ml of MSB was added to the one half while the other received 1 ml of MSB containing 0.02M EDTA. The two samples were then layered on gradients, spun and processed for TCA precipitable counts as described in Methods and Materials. A: Polysome profile, B: EDTA treated profile showing counts/minute due to labeled cosedimenting material. Inserts show expanded scales of counts/minute from fractions 2 through 15.

labelling and that 82% of the TCA precipitable counts represented messenger RNA while the remainder of the counts were due to contaminating material. In the lighter regions of the polysome profile, the difference between label in polysomes and contaminating material was greater than heavier regions, but the total amount of contaminating material present in the lighter regions was greater than that seen in the heavier regions of the gradient.

Since the 21 day denervated polysome profiles of both short (Figure 5) and long term (Figure 8) labelling experiments showed a large decrease in isotope incorporation compared to controls, it was decided that further experiments should be conducted on this stage of regeneration. Region 1 (light) and Region 2 (heavy) polysomes were collected separately from polysome gradients of 21 day denervated and control regenerates labelled with P^{32} for 90 minutes. The polysomes were caught on "Millipore" filters, dissociated and respun on sucrose gradients (Figures 14 and 15).

The optical density profile seen in Figure 14 illustrates the pattern of polysomal RNA extracted from control (Graph A) and denervated (Graph B) regenerates. Comparison of the two graphs revealed a 13% decrease in the amount of 28S and 18S ribosomal RNA in the denervated profile compared to the control. This suggested that there were 13% fewer polysomes present in Region 1 of the denervated poly-

Figure 14. Region 1 polysomal RNA profile of 21 day innervated and denervated newt forelimb regenerates labeled with P^{32} - $NaPO_4$ for 90 minutes. — optical density (254 nm), --- net counts/minute. Polysomes from innervated and denervated 21 day forelimb regenerates were prepared as described in Figure 8 except that 158 newts were used in this experiment with each newt receiving 58 μ Ci of P^{32} - $NaPO_4$ for 90 minutes. Instead of a TCA precipitation, region 1 (light polysomes) were collected from 2 control (innervated) gradients and pooled as were region 1 fractions from 2 denervated gradients. These fractions were then processed for polysomal RNA as described in Methods and Materials. A: innervated profile, B: denervated profile.

Figure 15. Region 2 polysomal RNA profile of 21 day innervated and denervated newt forelimb regenerates labeled with P^{32} -NaPO₄ for 90 minutes. — optical density (254 nm), --- net counts/minute. Polysomes from innervated and denervated 21 day forelimb regenerates were prepared as described in Figure 8 except that 158 newts were used in this experiment with each newt receiving 58 μ Ci of P^{32} -NaPO₄ for 90 minutes. Instead of a TCA precipitation, region 2 (heavy polysomes) were collected from 2 control (innervated) gradients and pooled as were region 2 fractions from 2 denervated gradients. These fractions were then processed for polysomal RNA as described in Methods and Materials. A: innervated profile, B: denervated profile.

some profile compared to the control. However, analysis of the 28S and 18S peaks from Region 2 innervated (Graph A) and denervated (Graph B) optical density profiles revealed no differences in the amount of 28S and 18S ribosomal RNA present (Figure 15). This implied that there were as many polysomes in Region 2 of the denervated polysome profile as there were in Region 2 of the control profile.

All four scans (Figures 14 and 15) show little, if any radioactivity present in the gradients except for the 4-5S regions. An examination of the filters used in catching the polysomes revealed that the SDS-EDTA buffer had removed ribosomal and transfer RNA species, but not the labelled messenger RNAs. This view was supported by the work of Lee et al., (1971) in which labelled mRNAs containing adenylate-rich (poly-A) sequences selectively adhered to "Millipore" nitrocellulose filters while other types of RNA lacking poly (A) sequences were not bound. Lee et al., (1971a) were only able to remove these mRNAs rich in poly (A) by shaking the filters for one hour using a 0.5% SDS buffer at pH 9.0. This differed from the techniques used in this study in which the RNA was extracted from the filters by shaking in SDS-EDTA buffer at pH 7.8 for 30 minutes, 20°C. Difficulty in extracting labelled RNA from these filters using this technique was also reported by Heywood (personal communication). Previous success (Figure 12) in extracting mRNA from the filters

may have been due to the larger quantity of starting material initially used. Most of the poly (A) binding sites on the filter were probably already occupied and the wash extracted excess mRNA that was only poorly bound.

If the labelled filters were indicative of newly synthesized mRNA, then analysis of the filters could reveal much information about labelled messengers present in Region 1 and 2 polyribosomes (Table 1). Region 1 filters from the pooled denervated polysome gradients bound 63% less isotope than Region 1 control filters while Region 2 filters bound 53% less isotope than the Region 2 control filters. The average reduction in bound radioactivity was 58% in the filters from denervated polysomes compared to the control. These differences were not due to a difference in counts layered on the innervated and denervated polysome gradients because the variation in TCA precipitable counts was only 8%. There remains, however, the possibility that some of the radioactivity on the filters was due to labelled cosedimenting, nuclear RNA possessing poly (A) sequences. Evidence for the presence of poly (A) attached to nuclear RNA is contradictory at this time. For example, mouse plasmacytoma cells contain nuclear RNA with poly (A) sequences (Stevens and Williamson, 1972) while rat liver cells contain nuclear RNA without poly (A) (Hadjivassiliou and Brawerman, 1967). It remains to be determined whether poly (A) containing nuclear RNA is present in this system.

In order to circumvent the use of "Millipore" filters in this study, polysomes from P^{32} -6 hour labelled regenerates were sedimented through a 20% layer of sucrose in MSB (Figure 16). A long term incorporation period was used to label all types of polysomal RNA in this particular experiment. The pelleted polysomes were then resuspended and dissociated using the SDS-EDTA buffer. Polysomes prepared in this manner were free of monosomes and subunits (Lee et al., 1971b). The polysomal RNA was spun and the gradients fractionated as previously described. The optical density profile of the denervated polysomal RNA (Graph B) shows a 13% reduction in 28S and 18S rRNA compared to the control profile. This reduction was exactly equal to the decrease seen in polysomal RNA scans from 21 day control and denervated Region 1 polysomes. Additionally, there was a 27% decrease in incorporation of P^{32} into 28S and 18S ribosomal RNA in the innervated scan (Table 1). The 4-5S peak was decreased by 29% while other regions of the gradient were depressed by only 12%. In all, the denervated profile was depressed by 24% throughout the entire gradient. This amount of depression did not agree with short term (Figure 8-Table 1) or long term (Figure 5-Table 1) data which indicated a 46% reduction in precursor incorporation in denervated polysome profiles. It may be that some light weight polysomes which demonstrated greater incorporation differences (Figure 8) did not pellet at the bottom of the

Figure 16. Polysomal RNA profile of 21 day, innervated and denervated forelimb regenerates labeled with P^{32} - $NaPO_4$ for 6 hours. ————— optical density (254 nm), — — — — net counts/minute. Newts (140) were denervated on day 19 post-amputation and 2 days later were injected intraperitoneally with 75 μ Ci each of P^{32} - $NaPO_4$ for 6 hours. Following incorporation, the innervated and denervated regenerates were collected separately and homogenized in MSB-bentonite and DEP. The 2 homogenates were centrifuged at $17K \times g$, 10 minutes, $0^\circ C$ and the supernatants were layered on 7 ml of 20% sucrose-MSB in a cellulose-nitrate ($5/8" \times 4"$) tube. The homogenates were centrifuged in a Beckman L2-65B ultracentrifuge using an SW 27.1 rotor at 26,000 r.p.m., 1 hour, $0^\circ C$ thus pelleting the polysomes. The pellets were resuspended in 0.8 ml of SDS-EDTA buffer for 30 minutes. The 2 buffer fractions were then layered on a 10-30% sucrose gradient in SDS-EDTA buffer and spun at 27,000 r.p.m., $16\frac{1}{2}$ hours, $20^\circ C$ using an SW 27.1 rotor with $5/8" \times 4"$ buckets. The gradients were then fractionated and processed for TCA precipitable counts as previously described in the Methods and Materials section. A: innervated (control) profile, B: denervated profile.

tube during centrifugation. If this is true, Figure 16 represents the contribution of the heavier polysomes which show less of a difference in incorporation between denervated and control.

It was also noted that the amount of 28S and 18S RNA recovered in these gradients was considerably less than the amount recovered on "Millipore" filters (Figures 14 and 15). The 6 hour labelling period made it difficult to calculate the type and character of the mRNA in the gradients because of the labelled 28S, 18S and 4-5S peaks. A longer centrifugation time and a short term label would significantly increase the amount of data that could be derived using this technique.

5. The incorporation of amino acid precursors into newt polyribosomes of innervated and denervated regenerates

The reduction of incorporation of RNA precursors into messenger and ribosomal RNA prompted an investigation to determine the extent that protein synthesis was depressed after two days of denervation. Accordingly, 21 day innervated and denervated regenerates were labelled with an H^3 -amino acid mixture and polysome gradients were run (Figure 17). The polysome fractions were subjected to TCA precipitation and counted. Graph A shows the incorporation of labelled precursors into nascent peptides attached to polysomes from innervated regenerates while Graph B shows the pattern obtained from denervated regenerates. In both

Figure 17. Polysome profiles of innervated and denervated 21 day newt forelimb regenerates labeled with H^3 -amino acids for 1 hour. — optical density (254 nm), --- net counts/minute. Right forelimbs of 80 newts were denervated 19 days post-amputation while left limbs served as controls. On day 21, each newt was intraperitoneally injected with $25\mu Ci$ of a H^3 -amino acid mixture for 1 hour. Following incorporation, the regenerates were collected and processed for TCA precipitable counts in the polysome profile. The optical density of the 2 profiles and the net counts/minute were adjusted to a standard of 15 mg of protein (supernatant) for a basis of comparison. A: innervated (control) profile, B: denervated profile.

cases, the light polysomes incorporated the most isotope thus indicating either that there are more polysomes present in this region or that they are more actively engaged in protein synthesis compared to Graph A (control)(Table 1). This depression in protein synthesis is very uniform throughout all regions of the polysome profile.

While TCA supernatant counts did not indicate precursor pool sizes, it did indicate that differences seen in the gradients were not due to variation in the amount of supernatant counts and in this case the denervated supernatant counts were actually 19% greater than those from the control (Table 1).

DISCUSSION

Since the homogenization conditions used have been shown to inactivate ribonuclease and minimize mechanical breakage, it is plausible to assume that the polysomes obtained from newt forelimb regenerates were undegraded. The fact that polysomes were present in the profiles was confirmed by both the ability to extract 28 and 18S RNA plus 4-5S RNA from 2 separate regions of the polysome gradient and the ability of the polysomes to incorporate labelled amino acids into nascent peptides.

In this system, ribonuclease degradation was controlled by a combination of gentle homogenization with a Dounce style homogenizer (loose-fitting), cold temperatures, pH and the inhibitors bentonite and diethylpyrocarbonate (DEP). Increased concentrations of DEP could be used with success if correspondingly higher concentrations of tris were used to prevent acidification of the homogenate. Care must be taken during homogenization so that mechanical breakage of polysomes does not occur through over homogenization.

Compared to an equal starting amount of chick embryo leg muscle, the yield of newt non-membrane bound polysomes was comparatively low as was the 75S monosome peak. This

result may be common for cells which are taken from starving animals (Lee et al., 1971). A greater yield of polysomes could be obtained by using detergents such as sodium deoxycholate or Triton X-100 to liberate membrane bound polysomes which apparently exist in large numbers in blastema cells (Bryant et al., 1971; Popeila, 1972). However, the use of detergents would probably increase the quantity of rapidly labelled cosedimenting material seen in these gradients which is thought to be nuclear RNA (Plagemann, 1969; Baltimore and Huang, 1968; Olsnes, 1970). This might not prove to be too great a problem if EDTA treated polysome gradients are run concurrently with experimental gradients (figure 13). Once counts present in EDTA treated profiles are deducted from counts detected in polysome gradients, the remaining radioactivity represents the amount of precursor incorporation into polyribosomes. The obvious disadvantage of this technique is that it requires twice the amount of starting material which presents severe problems in this system since new regenerates are very small.

Denervation causes a decrease in the amount of RNA precursor (H^3 -Uridine, P^{32} - $NaPO_4$) that is incorporated into the ribosomal RNA of monosomes and polyribosomes extracted from 21, 28, and 35 day regenerates. The amount of decrease in incorporation in denervated 21 day regenerates labelled with H^3 -Uridine for six hours was 49% in the

monosome region (75S) and 46% in the polysome region of the gradient. This data is in good agreement with that obtained by Morzlock and Stocum (1972). In that study, 21 day innervated regenerates and regenerates which had been denervated for two days were labelled with H^3 -Uridine for seven hours and whole cell RNA extraction was performed by the phenol extraction technique. Disc gel electrophoresis was used in separating the various RNA components. It was determined that there was a 47% decrease in the amount of 28S rRNA present in denervated regenerates compared to controls and a 38% decrease in 18S rRNA. Dresden (1969) reported approximately a 50% decrease in RNA precursor incorporation into total cellular RNA in denervated 19 day regenerates with respect to controls.

These data support the view that there is a definite neurotrophic influence on the amount of newly made ribosomal RNA present during limb regeneration. However, the decrease in H^3 -Uridine incorporation into ribosomal RNA does not seem to be a limiting factor in term of protein synthesis since there was an ample monosome (75S) peak present in the denervated polysome profiles of 21, 28, and 35 day regenerates (Figures 5, 6 and 7).

The incorporation of labelled messenger RNA into polyribosomes also seems to be under neurotrophic control. In the short term labelling experiments of the present study, ribosomal RNA was not labelled although 4-5S RNA was label-

led to an extent (Figures 14 and 15). About 82% of the counts present in the polysome gradients from regenerates labelled for 90 minutes with P^{32} was due to labelled messenger RNA, the remainder of the counts were probably due to labelled cosedimenting material (Figure 13). Further proof that messenger RNA was present in short labelling period gradients was the selective nature of the binding of radioactive material onto "Millipore" filters which is characteristic of mRNA containing poly (A) sequences (Lee et al., 1971a) (Figures 14 and 15, Table 1). In the one experiment using P^{32} labelled 35 day regenerates (Figures 11 and 12), labelled RNA was extracted off the filters and it was shown that the label was not due to labelled ribosomal RNA selectively adhering to the filters for there was no label in rRNA regions of the profile (Figure 12). Success in getting labelled material off the filters in this case, may have been due to the fact that more starting material was used. The binding sites on the filters were fully occupied and excess labelled messenger RNA could then have been extracted by the SDS-EDTA buffer.

It is important to add that differences in incorporation between denervated and innervated profiles were not due to a difference in the number of polysomes present. Figures 14, 15 and 16 indicate almost equal quantities of 28 and 18S rRNA suggesting that the number of polysomes present in innervated and denervated profiles were

approximately the same.

As in the case of the long incorporation period experiments, the greatest difference seen between denervated and control profiles was in the 21 day short term experiment (Figure 8- Table 1). The 35 day experiment (Figure 10) indicated the least difference seen between denervated and innervated profiles. This lessening of the neurotrophic influence on mRNA incorporation into polysomes may, in part, explain why regenerating limbs become nerve-independent. A nerve-independent regenerate is capable of forming a well-structured limb, but the limb is smaller in size (Singer and Craven, 1948; Powell, 1969). Denervation of late stage (35 day) regenerates has less of an effect on the ultimate size attained by the regenerate. Ostensibly, mRNA incorporation into polyribosomes may become more and more nerve-independent as time progresses during regeneration. This would allow post-denervation protein synthesis and morphogenesis to continue.

Probably one of the most interesting results of this study is the depression of labelled messenger RNA incorporation throughout the denervated profiles of 21, 28, and 35 day regenerates compared to the controls. This suggests that virtually all mRNAs are affected by denervation to some extent. Changes that occur in denervated regenerates must surely require protein synthesis since 10-12 day blastemata that are denervated acquire a basement membrane

underneath the epidermis and produce more connective tissue matrix (Bryant et al., 1971). Perhaps this process occurs to such a limited extent that differences in labelled mRNA incorporation cannot be detected by this technique.

It is dangerous to view the results of this study solely in terms of messenger RNA synthesis since a great deal of control can be exerted by cells between the time the messenger RNA is transcribed in the nucleus to the time it is translated in the polysome (Lehninger, 1970). Although variations in precursor pool sizes do not seem to make a difference in this system (Singer and Caston, 1972), the possibility of mRNA turnover in the nucleus has not been explored. Until these and other mechanisms of control are studied further in the newt forelimb regeneration system, it is not possible to say that the neurotrophic action is one of direct genetic control on the level of the gene to regulate messenger RNA synthesis. However, the depression of labelled mRNA and rRNA incorporation into polyribosomes due to denervation, as determined by this study, is consistent with this hypothesis.

A possible mechanism of action for the neurotrophic effect might involve control of RNA polymerase activity in the nucleus. This means of control has been shown to exist in other eukaryotic systems (Griswold and Cohen, 1972). Evidence presented in this study which is consistent with this theory is the reduction of precursor incorporation into

both messenger and ribosomal RNA. Other studies have also indicated that transfer RNA is also affected by denervation (Morzlock and Stocum, 1972). This line of research should be explored more fully in future experiments.

Finally, although it was determined that in 21 day denervated regenerates there was about a 50% decrease in mRNA incorporation into polysomes, there was only a 23% decrease in the level of protein synthesis (Table 1-Figures 8, 14 and 15). It would normally be expected that a 50% decrease in mRNA incorporation would soon result in a 50% reduction of protein synthesis if the mRNA population was turning over rapidly. However, if some of the messenger RNAs were stable (long-lived), protein synthesis would decrease at a slower rate (Spirin, 1966). In the H^3 -amino acid incorporation experiment (Figure 17-Table 1), it was ascertained that there was a uniform decrease in the amount of amino acid incorporation due to denervation in all fractions of the gradient which indicated that all polysomes were equally affected. This data can be correlated with the fact that the 28S and 18S peaks are equivalent between denervated and control polysomal RNA profiles of Region 2 polysomes (figure 15) while there is a decrease in the amount of 28S and 18S rRNA in the denervated polysomal RNA profile of Region 1 polysomes. This implies that there is little, if any, decrease in the number of polysomes present in Region 2 due to denervation while Region 1 polysomes are

decreased due to nerve deprivation. Since there is no lack of monosomes in denervated regenerates, it must be that there are fewer mRNAs in the Region 1 polysomes of the denervated regenerates. It is therefore suggested that Region 2 messenger RNAs are somewhat more stable while Region 1 messengers are not as stable.

The development of this system, which allows the study of labelled mRNA incorporation into polysomes of regenerating forelimbs, opens up many possibilities for future research. Paramount to this line of research is the need to definitely prove that the 90 minute labelled RNA is indeed messenger RNA and this can only be done by showing that it can direct protein synthesis in vitro. The examination of denervation effects on specific messenger RNAs such as myosin mRNA or histone mRNAs would then be possible.

Also, experiment could be performed to ascertain whether or not differences seen in mRNA uptake into polysomes are a direct reflection of control of mRNA synthesis (transcriptional control). If so, then the neurotrophic influence might properly be called one of transcriptional control at the level of the gene. Lastly, the evidence that there might be a population of stable mRNAs, especially in Region 2 polysomes, should prompt an investigation into the validity of this result. Does the existence of stable mRNAs explain the phenomenon of the transition stage between nerve-dependence and nerve-independence? During the nerve

transition period, an accumulation of a stable population of mRNAs might occur which can function in morphogenesis independent of neurotrophic control. These and other problems can now be further explored using this system.

In summary, short and long term labelled polysomes can be extracted from innervated and denervated newt forelimb regenerates without degradation. It has been shown that a two day denervation period adversely affects both ribosomal and messenger RNA incorporation into non-membrane bound polyribosomes of 21, 28, and 35 day regenerates. The least affected of these was the 35 day stage of regeneration. Denervation also has an adverse effect on protein synthesis in 21 day regenerates but this effect was not as great as the effect on RNA incorporation.

APPENDIX A

TABLE 1. Comparison of results from experiments on 21 day, innervated and denervated newt forelimb regenerates.¹

EXPERIMENT	INNERVATED total net counts	DENERVATED total net counts	% DECREASE as seen in dener- vated compared to innervated
6 hour, label into polysomes (Fig. 5)			
monosome	25955	13126	49%
polysome	20026	8713	56%
90 minute, P ³² label in polysomes (Fig. 8)			
polysome	1498	684	46%
90 minute, P ³² label in polysomal RNA on millipore filters (Figs. 14 & 15)			
TCA supernatant	11,558	10,649	8%
region 1 filter	873	325	63%
region 2 filter	734	344	53%
region 1 & 2 fil- ters combined	1607	669	58%
6 hour, label into polysomal RNA (Fig. 16)			
TCA supernatant	62,073	52,651	15%
28S ribosomal RNA	524	378	28%
18S ribosomal RNA	464	342	26%
4-5 S RNA	459	327	29%
other regions	667	586	12%
entire gradient	2140	1633	24%
1 hour, H ³ -amino acid label into nascent peptides (Fig. 17)			
TCA supernatant	718,823	890,968	+19% ²
polysome	21,786	16,856	23%

1. All denervations carried out on day 19 post-amputation.
2. denervated counts show a 19% increase in activity over controls in this particular case.

BIBLIOGRAPHY

- Anton, H. J.. 1965. In: Regeneration in Animals. (V. Kiortsis and H.A.L. Traumpusch, eds.), North-Holland Publ. Amsterdam.
- Baltimore, D. and Huang, A.S.. 1968. Isopycnic separation of subcellular components from poliovirus-infected and normal HeLa cells. *Science* 162:572.
- Bischler, V.. 1926. L'influence du squelette dans la regeneration et les potentialities des divers territoires du membre chez Triton cristatus. *Rev. Suisse Zool.* 33:431.
- Bodemer, C.W. and Everett, A.B.. 1959. Localization of newly synthesized proteins in regenerating newt limbs as determined by radioautographic localization of injected methionine-S35. *Develop. Biol.* 1:327.
- Bromley, N. and Orechowitch, W.N.. 1934. Ueber die proteolyse in den regenrierenden gewebe. II. Mitteilung: Die aktivität der gewebe-protease in verschiedenen gebieten des regenerats. *Biochem Z.* 272:324.
- Bryant, S.V., Fyfe, D. and Singer, M.. 1971. The effect of denervation on the ultrastructure of young limb regenerates in the newt, Triturus. *Develop. Biol.* 24:577.
- Burnett, A.L., Kary, C.E. and Lagorio, A.M.. 1971. Induction of growth in newt and frog limbs after perfusion with extracts from newt blastemas. *Nature* 234:98.
- Butler, E.G.. 1935. Studies on limb regeneration in x-rayed Ambystoma larvae. *Anat. Record* 62:295.
- Butler, E.G. and O'Brien, J.P.. 1942. Effects of localized X-radiation of the urodele limb. *Anat Record* 84:407.
- Butler, E.G. and Puckett, W.O.. 1940. Studies on cellular interaction during limb regeneration in Amblystoma. *J. Exptl. Zool.* 84:223.
- Chalkley, D.T.. 1954. A quantitative histological analysis of forelimb regeneration in Triturus viridescens. *J. Morph.* 94:21.

- Chalkley, D.T.. 1959. The cellular basis of limb regeneration. In: Regeneration in Vertebrates. (C.S. Thornton, ed.), p 34. Univ. of Chicago Press, Chicago, Ill.
- Deck, J.D. and Futch, C.B.. 1969. The effects of infused materials upon the regeneration of newt limbs. I. Blastemal extracts in denervated limb stumps. *Develop. Biol.* 20:332.
- Dresden, M.H.. 1969. Denervation effects on newt limb regeneration: DNA, RNA, and protein synthesis. *Develop. Biol.* 19:311.
- Egar, M. and Singer, M.. 1971. A quantitative electron microscope analysis of peripheral nerves in the urodele amphibian in relation to limb regenerative capacity. *J. Morph.* 133:387.
- Griswold, M.D. and Cohen, P.P.. 1972. Alteration of Deoxyribonucleic acid-dependent ribonucleic acid polymerase activities in amphibian liver nuclei during thyroxine-induced metamorphosis. *The Journal of Biological Chemistry* 247(2):353.
- Guth, L.. 1968. "Trophic" influences of nerve on muscle. *Physiological Reviews* 48:645.
- Guth, L., Dempsey, P.J. and Cooper, T.. 1971. Maintenance of neurotrophically regulated proteins in denervated skeletal and cardiac muscle. *Experimental Neurology* 32:478.
- Hay, E.D. and Fischman, D.A.. 1961. Origin of the blastema in regenerating limbs of the newt Triturus viridescens. *Develop. Biol.* 3:26.
- Hay, E.D.. 1966. Regeneration. Holt, Reinhart and Winston, N.Y., N.Y..
- Heywood, S.M., Dowben, R.M. and Rich, A.. 1967. The identification of polyribosomes synthesizing myosin. *P.N.A.S. (USA)* 57(4):1002.
- Heywood, S.M. and Rich, A.. 1968. In vitro synthesis of native myosin, actin, and tropomyosin from embryonic chick polyribosomes. *P.N.A.S. (USA)* 59:590.
- Heywood, S.M. and Nwagwu, M.. 1968. De novo synthesis of myosin in a cell-free system. *P.N.A.S. (USA)* 60:229.

- Heywood, S.M., Dowben, R.M. and Rich, A.. 1968. A study of muscle polyribosomes and the coprecipitation of polyribosomes with myosin. *Biochemistry* 7(9):3289.
- Heywood, S.M.. 1969. Synthesis of myosin on heterologous ribosomes. *Sympos. Quant. Biol.* 34:799.
- Heywood, S.M. and Nwagwu, M.. 1969. Partial characterization of presumptive myosin messenger ribonucleic acid. *Biochemistry* 8(9):3839.
- Jacoli, G.G.. 1968. Mechanism of inhibition of ribonuclease by bentonite. *Can. J. Biochem.* 46:1237.
- Johnson, E.A. and Singer, M.. 1964. A histochemical study of succinic and lactic dehydrogenases in the regenerating forelimb of the adult newt, Triturus. *Proc. Soc. Exptl. Biol. Med.* 117:27.
- Karmen, A. and Singer, M.. 1959. The growth influence of spinal ganglia implanted into the denervated forelimb regenerate of the newt, Triturus. *J. Morph.* 104:415.
- Kelly, D.J. and Tassava, R.A.. 1973. Cell division and ribonucleic acid synthesis during the initiation of limb regeneration in larval axolotls (Ambystoma mexicanum). *J. Exptl. Zool.*- in press.
- Laufer, H.. 1959. Immunochemical studies of muscle proteins in mature and regenerating limbs of the adult newt, Triturus viridescens. *J. Embryol. Exp. Morph.* 7:431.
- Lee, S.Y. and Brawerman, G., 1971. Pulse labeled ribonucleic acid complexes released by dissociation of rat liver polyosomes. *Biochemistry* 10(3):510.
- Lee, S.Y., Mendecki, J. and Brawerman, G.. 1971. A polynucleotide segment rich in adenylic acid in the rapidly-labeled polyribosomal RNA component of mouse sarcoma 180 ascites cells. *P.N.A.S. (USA)* 68:1331.
- Lee, S.Y., Krsmanovic, V. and Brawerman, G.. 1971a. Initiation of polysome formation in mouse sarcoma 180 ascites cells. Utilization of cytoplasmic messenger ribonucleic acid. *Biochemistry* 10(5):895.
- Lehninger, A.I.. 1970. Biochemistry. Worth Publ. Inc. N.Y., N.Y..
- Lentz, T.. 1969. Development of the neuromuscular junction. I. Cytological and cytochemical studies on the neuro-

- muscular junction in the regenerating limb of the newt, Triturus. J. Cell. Biol. 42:431.
- Liebowitz, P. and Singer, M.. 1970. Neurotrophic control of protein synthesis in the regenerating limb of the newt, Triturus. Nature 225:824.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J.. 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193:265.
- Mizell, M.. 1968. Limb regeneration: induction in the newborn opossum. Science 161:283.
- Mizell, M. and Isaacs, J.J.. 1970. Induced regeneration of hindlimbs in the newborn opossum. Am. Zool. 10:141.
- Morzlock, F.V. and Stocum, D.L.. 1971. Patterns of RNA synthesis in regenerating limbs of the adult newt, Triturus viridescens. Develop. Biol. 24:106.
- Morzlock, F.V. and Stocum, D.L.. 1972. Neural control of RNA synthesis in regenerating limbs of the adult newt, Triturus viridescens. Wilhelm Roux Archiv. 171:170.
- Needham, A.E.. 1941. Some experimental biological uses of the element beryllium (glucinum). Proc. Zool. Soc., A. 111:59.
- Norman, W.P. and Schmidt, A.J.. 1967. The fine structure of tissues in the amputated-regenerating limb of the adult newt Diemictylus viridescens. J. Morphol. 123:271.
- Olsnes, S.. 1970. The isolation of polysomes from rat liver. Contamination of polysomes by ribonucleoprotein of nuclear origin. Biochim. Biophys. Acta. 213:149.
- Peadar, A.M. and Singer, M.. 1965. A quantitative study of forelimb innervation in relation to regenerative capacity in the larval, land stage, and adult forms of Triturus viridescens. J. Exptl. Zool. 159:337.
- Plagemann, P.G.. 1969. RNA synthesis in exponentially growing rat hepatoma cells. I. A caution in equating pulse-labeled polyribosomal RNA with messenger RNA. Biochimica et Biophysica Acta. 182:46.
- Popeila, H.. 1972. Ultrastructural studies of denervation-induced regression of limb and blastema tissues in larval axolotls, Ambystoma mexicanum. M.S. Thesis, Department of Zoology, Ohio State University.

- Powell, J.A..1969. Analysis of histogenesis and regenerative ability of denervated forelimb regenerates of Triturus viridescens. J. Exptl. Zool. 170:125.
- Rich, A.J., Warner, J.R. and Goodman, H.M.. 1963. In: Cold Spring Harbor Symposia on Quantitative Biology 28:269.
- Rose, F.C. and Rose, S.M.. 1965. The role of normal epidermis in recovery of regenerative ability in X-rayed limbs of Triturus. Growth 29:361.
- Rzehak, K. and Singer, M.. 1966. Limb regeneration and nerve fiber number in Rana sylvatica and Xenopus laevis. J. Exptl. Zool. 162:15.
- Salpeter, M.M. and Singer, M.. 1960. The fine structure of mesenchymatous cells in the regenerating forelimb of the adult newt, Triturus. Develop. Biol. 2:516.
- Samaha, F.J., Guth, L. and Albers, R.W.. 1970. The neural regulation of gene expression in the muscle cell. Experimental Neurology 27:276.
- Schmidt, A.J.. 1960. Preblastemic changes of intramuscular glycogen in forelimb regeneration of the adult newt, Triturus viridescens. J. Exptl. Zool. 145:43.
- Schotté, O.E., Butler, E.G. and Hood, R.T.. 1941. Effects of transplanted blastemas on amputated nerveless limbs of urodele larvae. Proc. Soc. Exptl. Biol. Med. 48:500.
- Singer, M.. 1946a. The nervous system and regeneration of the forelimb of adult Triturus: IV. The stimulating action of a regenerating motor supply. J. Exptl. Zool. 101:221.
- Singer, M.. 1946b. The nervous system and regeneration of the forelimb of adult Triturus: V. The influence of number of nerve fibers, including a quantitative study of limb innervation. J. Exptl. Zool. 101:299.
- Singer, M.. 1947a. The nervous system and regeneration of the forelimb of adult Triturus: VI. A further study of the importance of nerve number including quantitative measurement of limb regeneration. J. Exptl. Zool. 104:223.
- Singer, M.. 1947b. The nervous system and regeneration of the forelimb of adult Triturus: VII. The relation between number of nerve fibers and surface area of amputation. J. Exptl. Zool. 104:251.
- Singer, M. and Craven, L.. 1948. The growth and morpho-

- genesis of the regenerating forelimb of adult Triturus following denervation at various stages of development. J. Exptl. Zool. 108:279.
- Singer, M.. 1950. Induction of the regeneration of the limb of the adult frog by augmentation of the nerve supply. Anat. Record 108:518.
- Singer, M.. 1951. Induction of regeneration of forelimb of the frog by augmentation of the nerve supply. Proc. Soc. Exp. Biol. Med. 76:413.
- Singer, M.. 1952. The influence of the nerve in regeneration of the amphibian extremity. Quart. Rev. of Biology 27: 169.
- Singer, M.. 1954. Induction of regeneration of the forelimb of the post-metamorphic frog by augmentation of the nerve supply. J. Exptl. Zool. 126:419.
- Singer, M.. 1960. In: Developing Cell Systems and Their Control. (D. Rudnick, ed.), p 115. Ronald Press, N.Y., N.Y..
- Singer, M. and Caston, J.D.. 1972. Neurotrophic dependence of macromolecular synthesis in the early limb regenerate of the newt, Triturus. J. Embryol. Exp. Morph. 28:1.
- Sperry, D.G.. 1971. The effect of denervation on nucleic acid synthesis and mitotic activity in nerve-dependent and nerve-independent blastemas of the axolotl, Ambystoma mexicanum. M.S. Thesis, Department of Zoology, The Ohio State University.
- Spirin, A.S.. 1966. On "masked" forms of messenger RNA in early embryogenesis and in other differentiating systems. In: Current Topics in Developmental Biology. 1:1. (A. Monroy and A.H. Moscona, eds.) Academic Press, N.Y., N.Y..
- Steen, T.P.. 1968. Stability of chondrocyte differentiation and contribution of muscle to cartilage during limb regeneration in the axolotl (Siredon mexicanum). J. Exptl. Zool. 154:207.
- Stevens, R.H. and Williamson, A.R.. 1972. Specific IgG mRNA molecules from myeloma cells in heterogeneous nuclear and cytoplasmic RNA containing Poly-A. Nature 239:143.
- Taban. G.. 1955. Quelques problemes de regeneration chez les urodeles. Rev. Suisse Zool. 62:387.

- Tassava, R.A.. 1969. Hormonal and nutritional requirements for limb regeneration and survival of adult newts. J. Exptl. Zool. 170:33.
- Thornton, C.S.. 1938a. The histogenesis of muscle in the regenerating forelimb of larval Amblystoma punctatum. J. Morph. 62:17.
- Thornton, C.S.. 1938b. The histogenesis of the regenerating forelimb of larval Amblystoma after exarticulation of the humerus. J. Morph. 62:219.
- Thornton, C.S.. 1949. Beryllium inhibition of regeneration on amputated forelimbs of larval Amblystoma. J. Morph. 84:459
- Thornton, C.S.. 1950. Beryllium inhibition of regeneration II. Localization of the Beryllium effect in amputated limbs of larval Amblystoma. J. Exptl. Zool. 114:305.
- Thornton, C.S.. 1951. Beryllium inhibition of regeneration III. Histological effects of beryllium on the amputated forelimbs of Amblystoma larvae. J. Exptl. Zool. 118:467.
- Thornton, C.S.. 1956. The relation of epidermal innervation to the regeneration of limb deplants in Ambystoma larvae. J. Exptl. Zool. 133:281.
- Thornton, C.S.. 1957. The effect of apical cap removal on limb regeneration in Ambystoma larvae. J. Exptl. Zool. 134:357.
- Thornton, C.S.. 1958. The inhibition of limb regeneration in urodele larvae by localized irradiation with ultra-violet light. J. Exptl. Zool. 137:153.
- Thornton, C.S.. 1962. The influence of head skin on limb regeneration in urodele amphibians. J. Exptl. Zool. 150:5.
- Thornton, C.S.. 1968. Amphibian limb regeneration. Advances in Morphogenesis 7:205.
- Thornton, C.S.. 1970. Amphibian limb regeneration and its relation to nerves. Am. Zoologist 10:113.
- Todd, J.T.. 1823. On the process of reproduction of the members of the aquatic salamander. Quart. J. Sci. Lit., Arts 16:84.

- Triplett, E.L., Herzog, R. and Russell, L.P.. 1970. Isolation and partial characterization of amphibian tyrosine oxidase polysomes. J. Embryol. Exp. Morph. 24(1):109.
- Venkatesan, N. and Steele, W.J.. 1972. Effects of diethyl pyrocarbonate on the isolation and recovery of bound polysomes from a particulate fraction of rat liver. F.E.B.S. letters 24(2):215.
- Weiss, C. and Rosenbaum, R.M.. 1967. Histochemical study on cell death and histolysis during regeneration. I. Distribution of acid phosphomonoesterase activity in the normal, the regenerating and the resorbing forelimb of the larval spotted salamander. J. Morph. 122:203.