The effects of beryllium nitrate on tail regeneration in Rana Pipiens larvae

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THE EFFECTS OF BERYLLIUM NITRATE ON TAIL REGENERATION
IN RANA PIPIENS LARVAE

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ABSTRACT

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The Effects of Beryllium Nitrate on Tail Regeneration in Rana pipiens Larvae

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This study was undertaken to determine the effects of beryllium nitrate on regeneration in the tail of Rana pipiens larvae. The tails were amputated approximately 3 mm from the tips. Some were then treated with different concentrations of beryllium nitrate (0.5 N, 0.7 N, and 1.0 N). Normal regeneration of untreated amputated tails was fulfilled in an orderly manner. In the animals treated with beryllium nitrate, general tissue regeneration was inhibited. Considerable degeneration was observed in all tissues of the tail stumps. However, the elastic lamella of the notochordal sheath was stimulated to stretch, oftentimes breaking the continuity of the fibrous lamella. The elastic layer appeared highly ravelled within the degenerated tissues of the tail, and, in some cases, extended for 3 to 4 inches from the tail stump due to a break in the covering epithelium.
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CHAPTER I

INTRODUCTION

Regeneration of a tail or a limb of an amphibian is, in most cases, a direct development in situ of the portion lost. The cells of the regenerated tissues actually arise from the cells of old tissues and the regenerate thus develops in close relationship with the old tissues. It is this relationship between the old and the new that makes regeneration an interesting subject for experimental analysis. Regeneration, however, begins as a result of an initial stimulus (usually amputation), and is followed in sequential manner by: wound healing, dedifferentiation, blastema formation, differentiation and morphogenesis.

In recent years, experimental biologists have studied the effects of physical and chemical agents on regenerating systems. X-rays have been used to prevent regeneration and NaCl has been used to induce regeneration. The effects of colchicine and beryllium nitrate have been investigated recently.

The current investigation was designed to provide information on the effects of beryllium nitrate on tail regeneration in Rana pipiens larvae. Although an interpretation of the beryllium effect has not been provided, the results do indicate a "new" target for stimulatory action of beryllium.
CHAPTER

REVIEW OF LITERATURE

The complex processes of regeneration have been the subject of numerous investigations. Many of these studies have been directed to such phenomena as wound healing, source of "new" cells, tissue interactions, and factors contributing to the induction or inhibition of regeneration.

One of the first visible signs of regeneration, following, for example, amputation of a limb, is the closure of the wound. Several investigators have shown that wound healing was accomplished by migration of epidermal cells from areas surrounding the wound (see reviews of Needham, 1952, and Goss, 1961).

According to Rose (1948), the source of all the cells for a regenerating tissue was within a few millimeters of the surface of the amputated tissue. He stained the entire surface of a wound epithelium with 1% Nile blue sulfate and found that after a few days the wound epithelium at the periphery was almost colorless, whereas the central region was more darkly stained than at the beginning. He concluded that in the period before blastema formation, epidermal cells were continuously migrating from old epithelium toward the center of the wound epithelium.

Tissue interaction in regeneration has also received considerable attention. Butler (1931, 1933), Thornton (1938), Schotte (1940), and Forsyth (1946), reported that the process of regeneration in amphibians was dependent on the formation of regeneration cells from dedifferentiation of the local tissue of the amputated parts. Schotte indicated that failure of dedifferentiation of the tissues would result in the failure of
regeneration.

Thornton (1957) made some histological studies on the limbs of *Amblystoma* larvae and found that the regeneration blastema might be regarded as a composite structure, made up of dedifferentiated cells of injured tissues of the limb. Daily removal of the apical cap from the amputated limb tip of *Amblystoma punctatum* correlated with the failure of the establishment of a blastema and consequent inability of the limb to regenerate. He described an apical cap as a transitory structure of regenerates composed of epithelial cells which possessed a high degree of mitotic activity. It formed immediately after wound healing, and disappeared after the establishment of a proliferating blastema.

Steen and Thornton (1963) removed some skin from the stump of anurogenic limbs and replaced it with limb skin from innervated larvae. When the proximal two-thirds of the limb stump was covered by the skin from an innervated limb, while the distal third retained its own anurogenic skin, regeneration occurred. They concluded that limb skin was necessary for regeneration of anurogenic limbs of *Amblystoma* larvae.

Recent studies of Niazi (1964) showed the effect of the destruction of the notochord in the stump on tail regeneration in the ammocoete larvae. After he amputated the tail, the stump notochord was destroyed with a needle for a considerable distance from the amputation surface. He observed that a tail-like posterior developed only in those which possessed a regenerating notochord. In the absence of the regenerating notochord, the regenerates were rounded in shape and were also relatively smaller and narrower. He suggested that the notochord influenced the migratory phase of the regeneration cells which were derived from the stump tissues, and that the notochord perhaps exerted this influence by keeping the stump firmly supported and by
stretching the regeneration posteriorly.

The discovery of certain physical and chemical agents which could induce or inhibit a particular regeneration process, has led, in recent years, to the importance of analyzing the factors regulating regeneration. Butler (1931, 1933) exposed amputated limbs of *Amblystoma* larvae to X-rays and observed that regeneration was inhibited; that the disappearance of the cartilaginous material of the limbs, which was due to a progressive dedifferentiation of the local cartilage, was induced by X-rays. He concluded that dedifferentiation was prevented through the action of X-rays and regeneration, therefore, could not take place. According to Goss (1957), irradiation of both skin and mesoderm of either the posterior or the anterior halves of the hind limbs of *Triturus viridescens*, followed by amputation, produced incomplete regenerates and even then only from the stumps which were shielded.

Rose (1945) induced regeneration of the limb of adult frogs by repeated irritation of the fresh amputated stump with hypertonic solutions of sodium chloride.

Thornton (1943) treated amputated forelimbs of a group of *Amblystoma* larvae with weak concentration of colchicine. No regeneration was observed after a period of time. In another group the larvae were allowed to regenerate for about 9 days, after which they were placed in colchicine. Further differentiation of the blastema was prevented. He concluded that colchicine inhibited regeneration, but the manner of its inhibition was not studied.

According to Singer, Flinker, and Sidman (1956), colchicine of higher concentrations, infused directly into early regenerate buds, effectively suppressed regeneration. They amputated the forelimbs of *Triturus viridescens* and allowed them to regenerate until about the 12th day after the amputation.
Colchicine was then infused directly into the blastema of this stage. Histological studies were made daily for 14 days after infusion. They found that the peripheral nerves were destroyed and limb regeneration was stopped. It was concluded that inhibition of regeneration was due to the destruction of nerves by colchicine.

Thornton (1949) suggested that beryllium salts, which inhibited a wound-factor in amphibians, also inhibited dedifferentiation, since the wound-factor initiated dedifferentiation. He argued that inhibition of dedifferentiation was due to a thickening of the lower layers of the dermis as a result of the beryllium treatment. In later investigations, Thornton (1950, 1951) showed that the effects of beryllium on limb regeneration in Amblystoma depended on the size of the larvae, the level of amputation, and the site of beryllium treatment. When a tip was severed from a beryllium inhibited limb stump several weeks after the original treatment, regeneration of that limb occurred. He concluded that beryllium reaction was localized within about 0.5 mm of the wound surface.

Schewing and Singer (1957) infused beryllium nitrate directly into both the early regenerate and non-amputated limbs of adult newts and found that beryllium destroyed the growth in regenerates in which growth was already underway, but such destructive consequences did not occur in non-amputated limbs. They suggested that the effects of beryllium nitrate might depend upon the pathological and reparative processes which had already started by the injury of amputation.
CHAPTER III

MATERIALS AND METHODS

*Rana pipiens* larvae were collected from a pond in Mozley Park, Atlanta, Georgia. The animals were kept in a large aquarium in the low temperature (15°C) laboratory in the Atlanta University Biology Research Laboratory until ready for use. All larvae were fed boiled lettuce once a week. At the time of experimentation, about 180 tadpoles, ranging from 20 to 25 mm in length, were selected. Throughout each experiment all larvae were kept in finger bowls which contained pond water; the water was changed twice a week.

Aqueous beryllium nitrate solutions (weight-volume), in concentrations of 0.5 \( \text{N} \), 0.7 \( \text{N} \) and 1.0 \( \text{N} \) were used in this investigation. (The salt was obtained from the Fisher Scientific Company, Manufacturing Chemists, Fair Lawn, New Jersey.) Special care was taken to maintain the strength of the solutions because beryllium nitrate, on exposure to air for long periods of time, reacts with carbon dioxide to form insoluble carbonates of beryllium, thus decreasing the concentration of beryllium ions in solution.

The present investigation consisted of three experimental and one control series:

**Control Series.** The tail of each larva was amputated about 3 mm from the distal end. The tail tips were re-amputated at selected intervals of days until the 35th day after the original amputation. Forty-five animals were used in this series.

**Experimental Series I.** After a 3 mm sector of the tail from each of 45 larvae was removed, the amputated surfaces were immediately treated with 0.5 \( \text{N} \) beryllium nitrate for 15 seconds. The tail tips were re-amputated at selected intervals of days until the 35th day post-amputation.
Experimental Series II. Forty-five animals were used in this series. Basically, the same operation was performed on these animals as on those of the previous series, except that the wound surface was treated with 0.7 M beryllium nitrate. The tips were re-amputated at selected intervals of days until the 35th day after the original operation.

Experimental Series III. In the 45 animals used in this series, the operation was essentially the same as that of the previous series, except that 1.0 M beryllium nitrate was used to treat the wound surface. Re-amputation was performed at selected intervals of days until the 35th day after the original operation.

All tissues were fixed either in Bouin's or Carnoy's fluid, embedded in paraffin, and stained by the hematoxylin-eosin method of Guyer (1953), or the Mallory's triple procedure outlined by Humason (1962). Photomicrographs were taken of selected sections.
CHAPTER IV

OBSERVATIONS

Data obtained in this study have been arranged to include both external and internal features of regeneration in normal untreated and in beryllium treated amputated tails of *Rana pipiens* larvae.

**External Features**

Normal regeneration of the tail in *Rana pipiens* larvae was fulfilled in an orderly manner. Epidermal wound healing took place immediately after amputation when epithelial cells bridged across the wound (Fig. 1). The amputated tail stump thickened and a bud was evident on the 7th day post-amputation (Fig. 2). The bud enlarged rapidly to form a cone 10 days after amputation, as shown in Fig. 3. Regeneration was allowed to continue until the 35th day after amputation. Such a regenerate was characterized by the reformation of a definitive tail as the cone tapered and flattened (Fig. 4).

The animals treated with 1.0 N, 0.7 N, and 0.5 N beryllium nitrate showed almost the same features. Immediately after the larvae received the beryllium treatment and were returned to pond water, they wiggled for about a minute or two and then became stationary and quiet for about an hour. Five of the 1.0 N treated animals were dead within 24 hours. The wound surface was darkened after one day and later had the appearance of a closed wound. Between the 5th and 7th day after amputation, varying shapes of tail stumps were observed (Figs. 5 - 7). On the 14th and 15th day after amputation a thread-like material extended from the notochord region of the amputated tail in 5 of the 0.5 N treated animals. This material increased in length and when measured was about 3 to 4 inches on the
Fig. 1. Appearance of the tail stump shortly after amputation. The amputated surface has healed.

Fig. 2. Presence of a bud at the tip of the tail stump 7 days after amputation.
Fig. 3. Enlargement of the bud to form a cone 10 days post-amputation.

Fig. 4. The formation of a definitive tail as the cone tapered and flattened 35 days after amputation.
Fig. 5. The shape of a 1.0 N beryllium nitrate treated tail stump 7 days post-amputation.

Fig. 6. Form of a 0.7 N beryllium nitrate treated tail stump on the 6th day after amputation.
Fig. 7. The shape of a 0.5 N beryllium nitrate treated tail stump 5 days post-amputation.

Fig. 8. The thread-like extension from the tail stump seen on the 30th day post-amputation in 0.5 N beryllium nitrate treated larvae.
30th day after amputation (Fig. 8).

**Histological Observations**

Epidermal healing in untreated larvae was accomplished by migration of epidermal cells from the areas surrounding the wound surface. This phase, referred to as wound healing, was followed by a dedifferentiation of intact tail tissues. While this event was occurring a regeneration blastema was forming. Figures 9 and 10, which represent sections through a normal regenerating tail stump, 21 days after amputation, clearly illustrate the formation of the blastema. Some of the cells at this period began to differentiate into muscles (m, Figs. 11 a, b) and notochord (n, Fig. 12). The notochordal sheath was prominent in many of the sections (ns, Fig. 13). Morphogenesis of the tissues continued so that by the 35th day after amputation normal tail histology had been restored.

The pattern of regeneration just described failed to occur in animals treated with beryllium nitrate. Preliminary histological observations on the 14th and 21st day after amputation revealed characteristic tissue destruction (Fig. 14). It was most striking in the subepidermal loose connective tissue and the muscles where degenerative changes were rapid. Other connective tissues were also found to be degraded. The blastema failed to form and an orderly dedifferentiation was not evident. A thick covering, apparently epithelial in nature, bordered the amputated surface (Fig. 15).

Sections from tails of beryllium treated animals, 35 days after amputation, revealed similar characteristics. Distal tissues appeared to have degenerated and masses of debris, mingled with extravasated blood, spread around the notochord, which failed to regenerate (Fig. 16). In
a number of 0.5 N treated animals, degeneration and retraction of the tissues around the notochord was observed (Figs. 17, 18 a, b). A careful examination disclosed that a delicate covering surrounded the exposed side of the notochord (c, Fig. 18 a).

A highly ravelled thread-like material (Figs. 19-21) was observed in the mass of degenerated tissue between the outer epithelial covering of the amputated surface and the notochord. Upon closer inspection this thread was found to be the elastic lamella of the bilaminar notochordal sheath. Throughout the tail, both in the stump and toward the body (el, Fig. 22), it was in folds, oftentimes penetrating the notochord and carrying with it segments of the broken fibrous lamella of the sheath (f, Fig. 23). This folded and ravelled elastic lamella resembled the thread-like material (see Fig. 8) seen extending from the tails of animals treated with the 0.5 N beryllium nitrate. An isolated piece of this thread is shown in Fig. 24.
Fig. 9. Blastema formation in normal regenerating tail stump 21 days after amputation. Tangential section also shows the notochord (n) directly below the thickened blastema (b). Hematoxylin-Eosin. 45X.

Fig. 10. Longitudinal section through a 21-day regenerate. The blastema (b) and notochord (n) are quite evident. Hematoxylin-Eosin. 10X.
Fig. 11 a. Differentiation in a 21-day regenerate: muscles (m). Hematoxylin-Eosin. 10X.

Fig. 11 b. Higher magnification of the blastema (b) and differentiating muscles (m) in a 21-day regenerate. Hematoxylin-Eosin. 45X.
Fig. 12. Notochord (n) differentiation in a 21-day regenerate. Hematoxylin-Eosin. 45X.

Fig. 13. Formation of the notochordal sheath during differentiation in a 21-day regenerate. Hematoxylin-Eosin. 10X.
Fig. 14. Beryllium-treated tail stump on 21st day after amputation. Note general tissue destruction and absence of a blastema. The notochord (n) is the most prominent structure. Hematoxylin-Eosin. 10X.

Fig. 15. Beryllium-treated tail stump showing a thick epithelial-like covering (e) over wounded surface. General tissue destruction is evident. Hematoxylin-Eosin. 10X.
Fig. 16. A beryllium-treated tail stump 35 days after amputation. Note the general tissue destruction, especially near the tip. The notochord itself may be seen near the arrows. Hematoxylin-Eosin. 10X.

Fig. 17. Part of a beryllium-treated tail stump showing tissue retraction around the notochord (n). A distortion in the form of the notochord itself may be seen near the arrows. Hematoxylin-Eosin. 10X.
Fig. 18 a. A higher magnification of the left side of the tail tip seen in Fig. 17. Note the appearance of the notochordal (n) cells and the thin covering (c) around them. Part of the fibrous (f) and elastic (el) lamellae of the notochordal sheath are visible. Hematoxylin-Eosin. 45X.

Fig. 19 b. A higher magnification of the right side of the tail tip seen in Fig. 17. Note the degenerative tissue (dt) on the side of the notochord (n). The folded appearance of the elastic lamella (el) is quite evident. Hematoxylin-Eosin. 45X.
Fig. 19. A beryllium-treated tail stump showing the extreme ravelling of the elastic lamella of the notochordal sheath (el). The lamella occupies the area between the degenerative stump covering (dc) and the notochord (n). A part of the fibrous lamella (f) is shown at the tip of the notochord. Hematoxylin-Eosin. 45X.

Fig. 20. Similar to Fig.19. Note the accentuated ravelling of the elastic lamella (el) and degenerative tissues around it. Hematoxylin-Eosin. 45X.
Fig. 21. A higher magnification to capture the extent of raveling of the elastic lamella (see arrows). Hematoxylin-Eosin. 97X.

Fig. 22. A beryllium-treated tail region removed from the most distal end. Note the notochord (n) and the folded elastic lamella (el). General tissue destruction is also prominent in this region. Hematoxylin-Eosin. 10X.
Fig. 23. Similar to Fig. 22. Note folded elastic lamella (el) where it has penetrated the notochord (n), carrying with it pieces of the broken fibrous lamella (f). Hematoxylin-Eosin. 10X.

Fig. 24. An isolated piece of the thread-like extension seen in tails treated with 0.5 N beryllium-nitrate. Non-stained. 45X.
CHAPTER V

DISCUSSION

The essential features of regeneration of amphibian appendages have been summarized by Goss (1961) and Hay (1962). The results of the present study corroborated the previous findings. There was initial wound healing (epidermal), followed by dedifferentiation. The latter process led into a proliferative phase, resulting in the establishment of a blastema. Differentiation ensued and a definitive tail was evident by the 35th day after amputation.

The failure of amputated tail stumps of *Rana pipiens* larvae to regenerate following treatment with beryllium nitrate is in agreement with an earlier observation by Needham (1941). Generally, however, while factors contributing to the loss of regenerative ability by beryllium have provided the basis for numerous investigations, no tangible explanation of its action has been offered. Some proposals have included inhibition of a "wound factor" (Thornton, 1949), development of an overly thickened epithelium of the wound surface (Thornton, 1950), failure of tissue dedifferentiation and inhibition of proteins required for differentiation (Needham, 1952). In an *in vitro* study, Chevremont and Firket (1951) reported that inhibition of growth and mitotic abnormalities were produced by beryllium ions. They suggested that the effect of these ions was to inhibit the enzymes concerned in phosphate and nucleoprotein metabolism. Needham (1952) proposed that alkaline phosphatase activity was inhibited.

While the findings here do not offer an explanation, they do support some of the previous observations on tissue alterations by beryllium treat-

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covered with darkened thick epithelium shortly after amputation; (2) de-differentiation of the treated tails was not too evident in histological features when compared with control sections. In keeping with an argument put forward by Thornton (1949), it would appear that beryllium nitrate inhibited dedifferentiation by causing a thickening of the covering epithelium to the extent that the necessary interaction between epidermis and sub-dermal tissues was prevented (see discussion on information transfer during limb regeneration by Rose, 1962).

The present observations constitute, according to our knowledge, the first report of a direct action of beryllium nitrate on the elastic lamella of the notochordal sheath. Thornton (unpublished comment) looked upon the thread-like outgrowth as notochordal. To him, using Rana sylvatica tadpoles, the beryllium inhibited the regeneration of all tail tissues except the notochord. Results described in this thesis provide clear evidence that notochord regeneration did not occur; that the extensive thread-like outgrowth was, in fact, the elastic lamella of the notochordal sheath. This lamella appeared highly ravelled in the area of the tail stump just beneath the epithelial covering. During retraction and degeneration of stump tissue, a split occurred in the covering, allowing the unravelling and, hence, extension of the elastic lamella.

The precise nature of the lamella is not known. It gives the usual staining reaction for elastic tissue and is non-fibrillar in high resolution electron micrographs (Hunter, 1962). Recently, Delaney (1966) has shown that it is not hydrolyzed by elastase, trypsin, or collagenase.
CHAPTER VI

SUMMARY AND CONCLUSION

1. Regeneration of the tail in normal untreated Rana pipiens occurred within 35 days after amputation.

2. Treatment of the wound surface of an amputated tail with beryllium nitrate resulted in an inhibition of regeneration. Wound epithelium rapidly closed the wound surface of beryllium nitrate treated larvae and no blastema was formed.

3. Beryllium nitrate stimulated the elastic lamella of the notochordal sheath to elongate excessively.

4. This lamella usually appeared highly coiled and ravelled within the degenerated tissues of the tail stump. However, in some cases it extended for 3 to 4 inches from the tail stump due to a break in the covering epithelium.
LITERATURE CITED


Schotte, O.E. 1940. The origin and morphogenetic potencies of regenerates. Growth, 3(Suppl.): 59-76.


