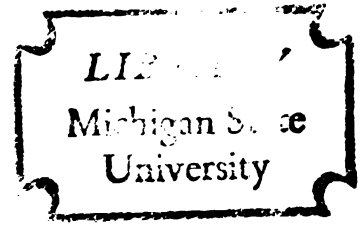


THE RELATION OF NERVES  
TO MULTIPLE REGENERATION  
IN A SINGLE NEWT LIMB

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## ABSTRACT

### THE RELATION OF NERVES TO MULTIPLE REGENERATION IN A SINGLE NEWT LIMB

By

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Early experiments in regeneration suggested that the establishment of three surfaces by constricting partially with a ligature, the forelimb of urodeles, results in the production of accessory structures on all three surfaces. Similar experiments conducted later indicate that regeneration occurs on the proximal side of the ligature.

Series 1 of this study was designed to re-investigate the distribution of growth of accessory structures on three surfaces of forelimbs in a large population of newts so that a range of variability of growth on the three surfaces might occur. It was found that accessory structures are not necessarily developed on all three surfaces of a single limb. Growth occurred on surfaces 1, 2, and 3; 1 and 2; 1 and 3; or 2 and 3. Surface 3 (the most proximal surface) produced more regenerates (76% of the cases) than the other surfaces.

When surface 3 (series 2) was blocked with whole skin the production of accessory structures on surfaces 1 and/or 2 was stimulated greatly.

In series 3, the distribution of accessory structures

on surfaces 1, 2, and 3 was correlated with the quantity of nerves on these surfaces during the early stages of regeneration. The mean number of nerve fibers on surface 3 at 5 days, 10 days, 15 days and 20 days is considerably higher than the nerve fiber counts of surfaces 1 and 2 during the same periods. Those limbs that possess a notch or digit on surface 3 and the absence of growth on surfaces 1 and 2 also show a decrease in nerve number of surfaces 1 and 2. An analysis of the mean number of nerve fibers on the blocked surface shows a noticeable decrease in comparison with level 3 of the normal limbs. Nerve fiber counts on surfaces 1 and 2 are markedly increased on those limbs where surface 3 was blocked with whole skin. Threshold experiments suggest that the irregular occurrence of accessory structures on surfaces 1 and 2 may be related to an insufficient number of nerve fibers on these surfaces. Similarly, a possible explanation for the regular occurrence of accessory structures on surface 3 is that the nerve fiber number on this surface is always above threshold.

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IN A SINGLE NEWT LIMB

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Lonnie C. Eiland

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## INTRODUCTION

Accessory limb formation has long been an intriguing aspect of amphibian limb regeneration. During the latter half of the 19th century, studies were devoted to comparing the external morphology of these structures that were produced in nature. Among the early studies, only a few involved experimental techniques that offered indications of tissue interactions that influenced the formation of accessory limbs. Thus laboratory observations with axolotls (Ambystoma mexicanum) and newts, (Triton cristatus) have revealed that accessory limbs can be produced from deep wounds resulting from bites among these animals when they are raised together (Przibram, 1921; Brunst, 1961). Experimentally, Della Valle (1913) produced accessory limbs in a newt (Triton cristatus) by fracturing the limb through the region of the elbow, and tying a ligature around the fracture to prevent the two surfaces from re-uniting. In order to maintain separate surfaces over a long period of time, the partially isolated distal segment of the limb was flexed to prevent its fusion with the proximal stump. Amputation occurred 30 days later through the wrist to form the third or most distal surface. This procedure eventually produced accessory limbs on all three surfaces. Della Valle reported

that regeneration from the most proximal surface was noticeably slower than from the middle and most distal surfaces.

There have been other experiments demonstrating the production of accessory limbs following the application of a ligature around the forelimbs of amphibians. Nasonov (1930) placed ligatures made from number 5 silk thread around the humerus and/or femur of large axolotls Ambystoma mexicanum without causing an external wound. This thread was tight enough to cause some compression of the soft tissue, but not enough to completely block the circulation of blood. He reported that, weeks later, tiny regeneration buds appeared proximal to the site of the constriction. Using Nasonov's methods, Kasanzeff (1930) found histologically that nerves split into bundles of different sizes proximal to the ligatures, but as nothing was then known of the quantitative effect of nerves in regeneration he explored this phenomenon no further.

Many studies have been done on the production of accessory limbs by (1) implanting extraneous substances near the site of contemplated accessory limb production; (2) irradiating the site; (3) deviating nerves; (4) or by transplantation methods. Collectively, those investigations involving implantation (Glick, 1931; Nasonov, 1936, 1937, 1938a, b, c; Fedatov, 1946; Breedis, 1952; Ruben, 1957, 1960; and Dent and Benson, 1966)

have employed over 200 types of implant material from many species. Of all the implant materials which have been used to stimulate accessory growth, tissues from Rana pipiens kidneys have proven to be the most effective (Breedis, 1952; Ruben, 1955; Ruben and Stevens, 1963; Carlson and Morgan, 1967).

There have been several reports of the production of accessory limbs as a result of transplantation of limb (Carpenter, 1932; Lecamp, 1935; Yntema, 1962; and Thornton and Tassava, 1969), of skin (Glade, 1957; Droin, 1959), and of epidermal caps (Thornton and Thornton, 1965). Carpenter (1932) demonstrated that developing limbs of Ambystoma punctatum larvae will give rise to accessory limbs upon transplantation to a heterotopic site. The limbs from larvae of stages 29-46 were transplanted to the flank and out of 68 operations, 22 yielded duplications. Limbs from stage 41 larvae, which are distinct appendages showing digits, produced seven of these duplicates. Out of 120 cases of limb transplantation from larval stages 45 up to metamorphosis, nine duplications occurred. The limbs at these stages were differentiated and functional. This experiment suggests that the frequency of accessory limb production is influenced by the degree of development in this species.

In the toad Alytes obstetricans, Lecamp (1935) grafted old limb buds and young limbs to a heterotopic,

non-limb field and obtained accessory limbs from the base of the graft. If a young limb is transplanted to a limb field, three limbs result. Yntema (1962) using the same species as Carpenter, transplanted aneurogenic limbs to normally innervated limb stumps of Ambystoma punctatum, and produced duplicates in 29 out of 397 cases. However, similar orthotopic transplants from aneurogenic to aneurogenic, normal to aneurogenic, and normal to normal resulted in no accessory structures. Heterotopic transplants of aneurogenic limbs to the flank of a normal host also produced accessory limbs in 20 out of 46 cases, whereas a reverse transplant of normal to aneurogenic produced none. Thornton and Thornton (1965) have reported the production of accessory limb outgrowths following the transplantation of epidermal caps to the basal areas of limb blastemata. In 27 out of 51 blastemata, both epidermal caps (the grafted and the regenerated) continued their individual growth following transplantation.

The production of accessory limbs of X-irradiation was first described by Brunst (1950a). Using localized X-irradiation of the lumbospinal region of the axolotl, this investigator has produced complete accessory hind limbs articulating with the pelvis. Brunst proposes that the stimulating effect may be produced only after a sufficient quantity of disintegration products have accumulated. The development of secondary tails by X-irradiation was also observed by Brunst (1950b). Brunst and Figge (1951)

extended this study in axolotls. They reported that the production of new structures depended upon the number of Roentgens in the exposure. The accessory tails were always in the dorsal, more proximal region of the original tails, while the growth of the original structure was always arrested. In a series of investigations on the effects of single doses of ultraviolet radiation on various aspects of regenerative activity (Butler and Blum, 1955; Blum et al., 1957) it was found that localized irradiation of the forelimbs of Ambystoma larvae may lead to two major results: (1) extensive regression of the limb; and (2) the induction in a high percentage of cases of accessory limbs at the site of irradiation. Butler and Blum (1963) also observed that accessory limbs were produced with the highest frequency at points of articulation of the skeletal structures following the sloughing off of epidermis damaged by irradiation. Although irradiation techniques elicit a regenerative response in a limb without the presence of surgical trauma (such as that which accompanies transplantation, implantation or nerve deviation), a wound epidermis apparently provides the necessary stimulus. Wallace (1972) suggests that migratory Schwann cells from a shielded part of the limb might serve as a source of mesenchymal cells following irradiation. Purdy (1967) demonstrated that accessory limb parts can be produced in the axolotl by surgical trauma alone, through the manipulation of limb tissues. Those tissues which appear to be the most influential in the production of accessory parts are



the skin and cartilage. The separation of cartilage produces a significant increase in the number of accessory limbs formed, particularly at that point where the cartilage protrudes from the limb at an angle. This area is analogous to an amputation surface.

Accessory structures produced as a result of deviating the sciatic nerve to the base of limbs, backs, and tails have been considered as a basis for implicating nerves with the induction of accessory limbs. Locatelli (1925, 1929) directed the femoral nerve to the surface of the body adjacent to the base of the hind limb. A blastema formed over the deviated nerve in its new location and eventually developed into a limb. Locatelli (1929) therefore suggested that the nerve possessed a specific morphogenetic stimulus. Guyénot and Schotté (1926) deviated the sciatic nerve to the surface of the middle of the back which resulted in the formation of a dorsal crest. However, when this nerve was diverted to the base of the tail, the resulting regenerate possessed characteristics of a tail. Thus they concluded that the nerves are important, but non-specific in stimulating morphogenesis. Singer (1946a, b; 1947a, b) has shown that the relation between nerves and the incidence of limb regeneration is a quantitative one. According to Singer, the ratio of number of nerve fibers to area of tissue at the amputation surface is of critical importance. Regeneration in the new limb only occurs when this nerve-tissue ratio is above a "threshold" or when at least one-third to one-half of the

normal number of nerve fibers is present at the amputation surface. Nerves exert their influence upon the tissues of the amputation surface and its corresponding mesenchymal cells during the early phases of regeneration (Kamrin and Singer, 1959). Moreover, when the limb of a newt is transplanted to the back, regeneration occurs at that site in the presence of a reduced number of nerves (Singer and Mutterperl, 1963). Thus, the threshold for a given limb level is not fixed but can be altered by experimental means, including transplantation trauma. Bodemer (1960) deviated limb nerves of different fiber content onto the surfaces of previously traumatized muscle at the base of the forelimb. There was a general correlation between the number of fibers comprising the deviated nerve and its capacity to induce a growth reaction. When fragments of newt liver were implanted at the base of the forelimb around the smallest deviated nerve, the incidence of growth reactions was approximately tripled. This again points to tissue variability in response to the nerve. Thus, the influence of nerve in the experimental duplication of limbs, whether by ligaturing, transplanting, implanting foreign tissues or traumatizing, must be carefully assessed.

Although the early experiments of Della Valle represent an important beginning in the study of multiple limb regeneration, it is unfortunate that a certain amount of doubt attaches to the validity of his data. Important among the uncertainties is the fact that the experimental

sample consisted of only one animal. Also the degree of injury sustained from the application of the ligature appears to be indeterminate because of the repeated trauma resulting from tightening and loosening the ligature during the course of the experiment. Amputation occurred 30 days following the application of the ligature which suggests that the stimulus for regeneration was delayed on the most distal surface. Yet, in spite of this delay and the fact that the most distal tissues were isolated except for a narrow strip of tissue (the isthmus), he reported that growth and development occurred at a faster rate distally. More recent experiments in regeneration have indicated that the wound epidermis and also the nerves, may elicit the stimulus for regeneration (Singer, 1952; Singer and Salpeter, 1961; Thornton, 1968, 1970 for reviews). Since the most proximal surface is exposed to more stump tissues, it appears that this surface should be dominant in accessory limb production and development, especially when it is compared with the more distal surfaces on the same limb. The present investigation was undertaken to: (1) re-examine the frequency of accessory limb formation on three amputation surfaces on the same limb, (2) test the effects of blocking surface 3 on accessory limb formation on surfaces 1 and 2 and (3) to correlate the number of nerves at each amputation surface with growth at that particular surface.

## MATERIALS, METHODS AND RESULTS

### GENERAL METHODS

Adult newts Notophthalmus (Triturus) viridescens viridescens (Rafinesque) used in the present experiments were purchased from William Lee, Oak Ridge, Tennessee. The average weight of these animals was 6.4gms while the average length from the snout to the tip of the tail was 120mm. All experimental animals were fed sliced beef heart three times per week and kept in one quart fish bowls prior to the operation at a laboratory temperature of  $20^{\circ}\pm 2^{\circ}\text{C}$ .

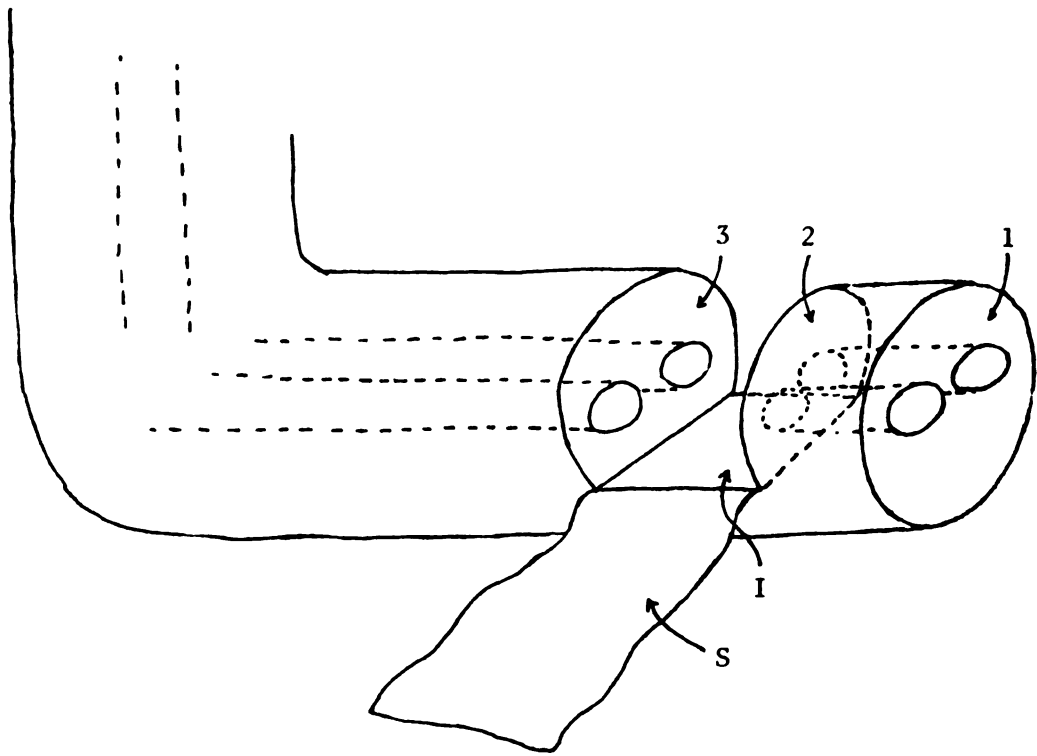
#### Operative Techniques

Following anesthesia in 1:1000 MS 222 (Sandoz), forelimbs of the experimental animals were amputated through their distal parts slightly proximal to where the radius and ulna articulate with the carpals. Particular attention was given to the original amputations to make sure that they were all at the same levels, since levels of amputation are of great concern in the present experiments. After all amputations, the cartilage was carefully trimmed with iridectomy scissors to insure wound closure in a reasonable amount of time (usually between 24 and 48 hours). All operations were started at approximately 72 hours

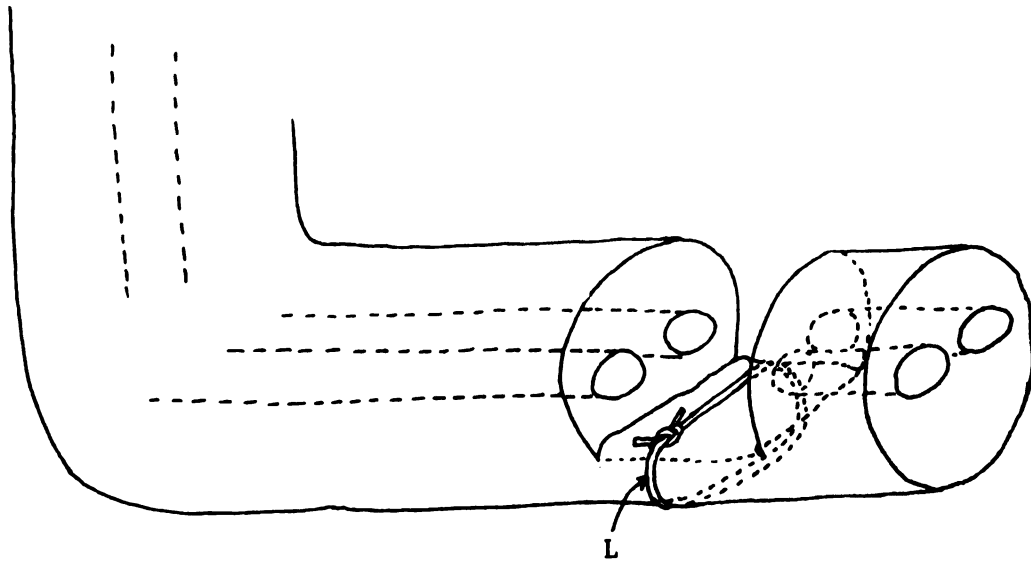
post-amputation because it was necessary that the regeneration processes at the three surfaces be initiated as near together as possible. Prior to the operation, the experimental animals were re-anesthetized and placed on sterile gauze moistened with sterile Steinberg's solution. Using sterile watchmakers forceps and iridectomy scissors, an incision of approximately 0.5mm in length was made on the dorsal surface of the mid forearm, and the skin carefully separated from the underlying musculature. A segment of tissues containing muscle, cartilage, blood vessels, nerves, and part of the separated skin was removed, thus creating a narrow ventral strip of tissues (henceforth called the isthmus) approximately 0.5mm in length, to support the distal segment of the limb, and also to establish three amputation surfaces that were arbitrarily designated (and henceforth referred to) as surfaces 1, 2, and 3. The most distal surface was designated as surface 1, the middle surface 2 and the proximal surface 3 (Fig. 1a). A sufficient number of nerves and blood vessels was left intact so that circulation and tissue maintenance in the distal segment could be retained. A skin flap (Fig. 1b) from the incision border was wrapped around the isthmus which also contained a very limited amount of muscle for support. Two ligatures made from no. 5 silk thread were tied around the isthmus with enough pressure to support the skin, but not to interfere with the circulation in the distal segment (referred to as the "isolate"). The animals were placed in a recovery

Figure 1a. A diagrammatic drawing of the lower arm of the forelimb of the newt Notophthalmus showing the isthmus (I) and wound surfaces 1, 2, and 3 (arrows).

Figure 1b. A diagrammatic drawing of the lower arm of the forelimb of the newt showing a flap of skin from the incision border being held in place around the isthmus by a ligature (L).



1a



1b

chamber at  $12^{\circ}\pm 2^{\circ}\text{C}$  on moist sterile gauze, allowed 24 hours to recover, and then placed individually on gauze in small squat fish bowls containing 100ml of sterile Steinberg's solution. After the three surfaces were covered by a wound epithelium, the animals were transferred to aerated water, where they were observed throughout the duration of the experiments. Similarly, normal limbs were amputated at the same levels and observed until the notch stage of blastemal development appeared. Representatives of the types of accessory structures formed on each surface were photographed at different times prior to fixation.

#### Histological and Nerve Quantitative Techniques

Experimental limbs and normal limbs were fixed in Bouin's fixative, embedded in paraplast, sectioned at  $8\mu$ , and stained with Masson's Trichrome stain for connective tissues, or Harris hematoxylin and eosin. Proper care was taken to orient the three surfaces in the same plane with the isthmus during embedding.

Tissues utilized for quantitating the nerve fibers were sectioned transversely and stained with protargol according to the method of Bodian (1936). The nerve fibers were counted according to the methods of Singer (1946a, b; 1947a, b). For area determinations, all sections were magnified 59 times and projected on paper with a drawing tube attached to a Wild microscope and subsequently traced with a Gelman planimeter. To increase the accuracy of the



planimeter, the outline of the cross section was traced twice and the readings were averaged. In order to make sure that the nerve counts were accurate, practice counts of nerve fibers were made on cross sections selected at random from normal limbs until an acceptable degree of reliability was established (coefficient of variability = 6.13 in five normal limbs). The method of converting planimeter readings from  $\text{cm}^2$  to  $(100\mu)^2$  as given by Singer (1947b) and Van Stone (1955), was used and can be written as follows:

$$\frac{\text{Planimeter readings}}{(\text{magnification})^2} \times 10^4 = \text{area in } (100\mu)^2$$

A threshold range of nerve fiber counts was also established. The procedures used in establishing this range were similar to the method of Singer (1947b). The fiber contents were arranged in a decreasing order, beginning with the normal limb and extending to the denervation procedure that yielded the lowest number of fibers.

Series 1  
The Distribution of Accessory Structures on Surfaces 1, 2 and 3

The results of the original ligature experiments (Della Valle, 1913) suggest that accessory structures may occur on three surfaces on a single limb consistently (Fig. 2). Similar experiments conducted later by Nassonov (1930) and Kasanzeff (1930) indicate that a relatively few

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Figure 2. A photograph of the limb of a newt showing the appearance of accessory structures on surfaces 1 (2 digits), 2 (3 digits) and 3 (4 digits) at 85 days post-operation. Accessory structures were formed on all three surfaces in only eight cases out of 126 limbs. (15x)



of the total regenerates occur on the proximal side of the ligature. It has also been observed that the formation of accessory structures depends upon the fracture of cartilage (Kasenzeff, 1930; Purdy, 1967). Moreover, the latter investigator proposes that the skin must also be injured to provide a wound epithelium otherwise induction will not occur at all. Purdy (1967) also suggests that the maintenance of separate amputation surfaces may also be important in accessory limb production. In order to keep the wound surfaces separated over a period of time, a rectangular block of tissue was removed from the mid lower arm region, and a ligature placed around the resulting isthmus. Preliminary experiments using large axolotls, Ambystoma mexicanum, and newts revealed that some variability existed in the occurrence of accessory structures on each surface. It was also observed that the limb skin of the newt is stronger in proportion to the size of the limb than that of the axolotl and offers a greater degree of support to the isolated segment of the limb. This series was designed to investigate the distribution of growth on three surfaces in a large population of animals, which permits study of the degree of variability occurring on the three surfaces.

### Procedure

One hundred and ninety six adult newts, Notophthalmus (Triturus) viridescens viridescens survived out of a total

of 200 operated animals. These animals were observed for 120 days post amputation. The morphological development of the regenerating surfaces was determined on a comparative basis by gross observations of the first appearances of a notch, digit, or spike as criteria for the regenerative rate. Since the animals used in this experiment were exceptionally large in comparison to other animals of the same species (Bishop, 1943), 30 animals amputated through the forelimbs at levels equivalent to the surfaces of the experimental animals were used as a comparison of the rates of development to the notch stage.

### Results

Within the first five weeks, low mounds and blisters simulating blastemata often developed and receded, thus making an accurate gross morphological staging of the early developing regenerate unreliable; however, the more striking histological features of each surface are similar to those reported by Kasanzeff (1930) and Bodemer (1958). It is of interest that the accessory structures stimulated by forming three surfaces on a single limb do not differ fundamentally from nerve-induced supernumeraries or normal limb regeneration.

Table 1 summarizes the positive cases of regeneration on the three surfaces at 120 days. It should be noted that, contrary to the report of Della Valle (1913), the

Table 1

- a. The positive cases of regeneration on three surfaces of the forelimb of the newt through 120 days post amputation. Growth occurred on individual surfaces and different combinations of the three surfaces involved. The frequency of growth is greater on surface 3 than the growth on surfaces 1, 2, 3; 2 and 3; or 1 and 2.
- b. The mean time (days) of development to a notch, spike, or digits on the three surfaces and also the final accessory structures formed at 120 days. The accessory structures on surface 3 are formed earlier and are completely developed to the 3-digit or 4-digit stage. The final accessory structures formed on these surfaces include a spike, 1 digit, 2 digits, 3 digits, a hand and double hands.

Table 1a

Positive Cases of Regeneration on Three Surfaces of the Forelimb on Notopthalmus viridescens

Surface and Surface Combinations	Number of Cases	Percentage
1 2 3	8	6.3%
2 and 3	19	15.1%
1 and 2	3	2.4%
3	96	76.2%
Total	126	100.0%

Table 1b

Mean Time (days) for the Appearance of a Notch, Spike, or Digits on the Surfaces and Final Accessory Structures Formed at 120 Days

Surface	Mean Time of Development to a Notch, Spike or Digit (days)	Final Accessory Structure Observed at 120 Days		
		Spike-2 digits	3 digits-Hand	Double Hand
1	70	10	0	1
2	70	21	8	0
3	55	22	101	0



most proximal surface (#3) regenerates most frequently (97%) and the most distal surface (#1), least frequently (8.7%). Furthermore, Figure 3 clearly demonstrates that accessory structures are not developed on all three surfaces on a single limb in each case as is suggested by Della Valle's report. The formation of accessory limbs on all three surfaces was experienced in only eight cases. Surface 3 was the only individual surface to produce a high proportion of regenerates (76% of the cases) while all the remaining surface combinations exhibit positive responses in a smaller percentage (23.8%) of the cases. It should be noted also that accessory structures are formed earlier on surface 3 than on the distal surfaces (Table 1b). Surface 3 displays more completely developed structures (hands with three or four digits) than surfaces 1 or 2. A spike is formed terminally in only six cases on surface 3.

Table 2 shows the normal regeneration to notch formation at three levels on the lower arm of the newt following amputation. These levels are comparable with the levels of the three surfaces on the experimental animals. It has also been reported by Goodwin (1946) and Manner, Zapisek and Vallee (1960) that in red-spotted newts no significant differences in the rate of regeneration occur among adults of different sizes or ages. Recent experiments on size and regeneration rate in the newt (Pritchett and Dent, 1972) are interpreted as indicating that larger limbs regenerate more slowly than smaller limbs because

Figure 3

- a. A photograph of an operated newt limb showing the absence of growth on surface 1, a notch on surface 2, and 2 digits on surface 3 at 65 days post operation. (7x)
  
- b. A photograph of an operated newt limb showing 1 digit on surface 3, 3 digits on surface 2 and the absence of growth on surface 1 at 71 days post operation. (7x)
  
- c. A photograph of an operated newt limb showing 4 digits on surface 3 and the absence of growth on surfaces 1 and 2 at 85 days post operation. (7x)
  
- d. A photograph of an operated newt limb showing a notch on surface 3, a spike on surface 2 and the absence of growth on surface 1 at 78 days post operation. (7x)

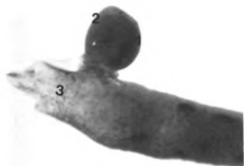
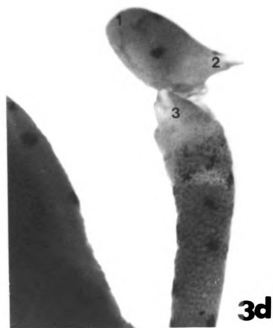
**3a****3b****3c****3d**

Table 2. The normal regeneration to notch formation at three amputation levels on the lower arm following amputation at these levels. This table shows the mean time of normal regeneration to the notch stage on each level of the forelimb of the large newt. It is also important to note that in the normal limb no significant difference exists in the amount of time to reach the notch stage of development at three levels of the forelimb similar to the three surfaces on the experimental limbs.

Table 2  
 Normal Regeneration to Notch Formation at Three Levels on  
 the Lower Arm of the Newt Following Amputation

	Number of Animals					N	Mean No. of Days	
Level I	1	6	1	2	2	10	37.0 ± 1.53	
Level II	1	4	2	3	3	10	38.5 ± 1.67	
Level III		2	5	3	3	10	42.0 ± 1.019	
	20	25	30	35	40	45	50	39.1
	<u>Days of Regeneration</u>							<u>Mean Number of Days</u>

as the limb grows the increase in amount of neuroplasm does not keep pace with the increase in mass of the other limb tissues. The animals used in the present experiments are extremely large when compared with those used by Pritchett and Dent (1972). Our newts weigh an average of 6.4 gms while those of Pritchett and Dent weighed 3.40 to 4.41 gms. It is clear that regeneration of forelimbs of the large newts in the present study was slower than regeneration in the small newts reported by Goss (1969). Thus our newts reached the notch stage of forelimb regeneration at an average time of 39 days (Table 2), while Goss reports that his small newts attained the notch stage at 28 days.

Series 2  
The Effect of Blocking Surface 3 with Whole Skin

Series 1 indicated clearly that surface 3 preceded surfaces 1 and 2 to the notch stage. This observation suggests the possibility that surface 3, in some way, may be partially inhibiting growth at the two distal surfaces. To test this possibility, advantage was taken of the findings of Tornier (1906), Schaxel (1921), and Godlewski (1928) that when the amputation surface is covered by whole skin (dermis and epidermis), regeneration on that surface is inhibited. Thus, this series of animals underwent blocking of surface 3 with whole limb skin in order to see if there would then be an increase in the production of accessory structures on surfaces 1 and 2.

### Procedure

A flap of skin from the dorsal part of the lower arm on one forelimb of each of 40 animals was used to block surface 3. Surgical sutures were used to maintain the skin flap in close contact with the open surface. The sutures were applied as firmly as possible immediately after the operation to prevent the migration of a wound epithelium over the amputation surface. The operated animals were kept in a recovery chamber at  $15^{\circ} \pm 2^{\circ}\text{C}$  for 24 hours, and observed for 120 days.

### Results

If properly oriented, a flap of whole skin sutured over amputation surface 3 will suppress regeneration on this surface. Table 3 shows the resulting accessory structures on surfaces 1 and 2 following the blocking of surface 3 with whole skin. In 22 cases regeneration was completely absent on surface 3 (Fig. 4), but surface 1 regenerated in 63.6% of the cases (as contrasted with 8.7% of cases with surface 3 not blocked). Regeneration at surface 2 also increased from 23.8% (Table 1a) to 54.6% (Table 3).

Table 3. The frequency of accessory structures forming on surfaces 1 and/or 2 following the blocking of surface 3 with whole skin during the operation. Surface 3 was successfully blocked in 22 cases out of 40 animals. Growth occurred on the two distal surfaces in 100% of the cases prior to 120 days post amputation.



Table 3

Frequency of Development at Surfaces 1 and/or 2 Following  
the Blocking of Surface 3 with Whole Skin  
(See Text for Description of Procedure)  
(N) = 22

Responding Surfaces	Frequency of Positive Regeneration	Percentage	Type of Accessory Structure Developed
1	10	45.4%	5-2 digits 3-3 digits 2 hands
2	8	36.4%	4-2 digits 3-3 digits 3 hands
1 and 2	4	18.2%	2-3 digits 2-2 digits 2-3 digits 2 hands
Total	22	100.0%	

Figure 4. A photograph of a limb exhibiting the growth of two complete digits on surfaces 1 and 2 at 85 days post amputation. The absence of growth on surface 3 results from blocking surface 3 with whole skin. (7x)



Series 3  
An Analysis of Nerves on Surfaces 1, 2 and 3

The results of series 3 show a remarkable increase in the regenerative response of distal surfaces 1 and 2 when surface 3 is blocked. It has been well established that a threshold number of nerve fibers is needed for limb regeneration in the newt Triturus (Singer, 1963; Thornton 1970 for reviews). The effects of nerves on regeneration have been shown to be most important in the early phases of regeneration during which time section of the nerves completely interrupts growth (Schotté and Butler, 1941a, 1941b; Singer and Craven, 1948). But if the regenerate reaches a critical size or stage in its development, it can differentiate in the absence of the neural stimulus. In the newt Triturus regeneration is suppressed when denervation occurs before 13 days post amputation. However, if denervation is delayed until 17 days or later, a time when mitotic proliferation has been initiated, regeneration proceeds, but at a slower rate. The questions now raised are: are nerves unevenly distributed to the three surfaces? If so, are there fewer nerves found at surfaces 1 and 2? Finally, does blocking regeneration at surface 3 result in an increase in nerve numbers at surfaces 1 and 2? The experiments of series 4 were designed with these questions in mind.



## Procedure

All nerve counts were made following the methods outlined by Singer (1947a, b). Newts were divided into five groups of experiments as follows:

- (1) Nerve counts were made at amputation surfaces equivalent to surfaces 1, 2, and 3 previously described. Surface 2 is at the mid forearm level; corresponding to Singer's (1947a) mid-forearm level; surface 1 and surface 3 are slightly distal and proximal respectively to the mid forearm. There were 10 cases at each level.
- (2) A second group underwent operations as described in preceding pages so that a proximal stump amputation surface (3) and two surfaces of the isolate (1 and 2) were produced. These limbs were fixed for nerve counts at 5, 10, 15, and 20 days of regeneration. There were 10 cases of each at these fixation times. By 20 days regenerates in these cases are only at late bud or early mound stages.
- (3) A further group of operated limbs in which surfaces 1 and 2 of the isolate showed no signs of regeneration at 60 days were assayed for nerve fibers. In these 40 cases surface 3 regenerated.
- (4) A group of 20 cases in which surface 3 was blocked with whole skin grafts, as previously described,

and in which surfaces 1 and 2 of the isolate regenerated, were assayed for nerves on surfaces 1 and 2 at 20 days (n=10) and surface 3 at 60 days (n=10).

- (5) A final "threshold" group of newts underwent nerve counts at the mid forearm level using the methods of Singer (1946a). In all cases amputation of the limb was made at mid forearm and variable numbers of nerve fibers sectioned to discover the threshold number needed for regeneration in these very large newts. Four groups of 10 newts were used as follows: (a) limbs containing spinal nerves 3 and 4; (b) 4 and 5, (c) 3 and 5, and (d) 3 alone. Subsequent nerve sections were made at 10 day intervals. Amputations of the limb were accomplished simultaneously with the initial denervations and the cartilage was carefully trimmed to insure proper wound closure. Only one limb on each animal was partially denervated since it has been reported that linear regeneration rate is significantly slower if a contralateral limb is removed (Tweedle, 1971). Observations were made on the limbs periodically beginning at approximately 10 days following the denervations. Regeneration was considered positive if a regenerate appeared as late as 20 days following the time required for regeneration in the normal limb. The methods used for quantitating the nerve fibers are the same

as has been described in this paper.

## Results

The results of the denervation, or "threshold," group are shown in Tables 4 a, b, c, and d. From the 33 surviving partially denervated animals, regeneration was observed in 16 cases, which is approximately one-half of the total number of cases observed. In Table 8 the limbs are arranged in a decreasing order by nerve fiber content beginning with the normal limb and extending down to the limb with the lowest number of nerves, a procedure adopted from Singer (1946a, 1947a). In confirmation of Singer (1947a), a threshold range of nerve fibers was found, above which regeneration always occurred and below which it was always absent. The upper limits of the threshold lie between 1440 and 1511 nerve fibers; and the lower limit between 780 and 842 fibers, as is indicated by the cross line in Table 5. These threshold values differ only slightly from those found by Singer (792-1470) in the smaller newts which he used. Singer (1947b) found that the fiber requirement at the amputation surface could be expressed in a ratio of nerve fibers to amputation surface area. Thus at the mid-lower arm level he found that a range of 8.6-11.2 fibers for every  $(100\mu)^2$  of surface area was needed for regeneration. Following Singer's (1947b) method, nerve fiber numbers in the entire threshold range were plotted against percentage of





Table 4

- a. The results of transecting Brachial nerves 4 and 5 in the mid lower arm during the denervation process in the newt. The number of fibers remaining, nerves per  $(100\mu)^2$  and regeneration (positive or negative) is emphasized for each case.
  
- b. The results of transecting Brachial nerves 3 and 5 in the mid lower arm during the denervation process in the newt. The number of fibers remaining, nerves per  $(100\mu)^2$  and regeneration (positive or negative) is emphasized for each case.
  
- c. The results of transecting Brachial nerve 3 in the mid lower arm during the denervation process in the newt. The number of fibers remaining, nerves per  $(100\mu)^2$  and regeneration (positive or negative) is emphasized for each case.
  
- d. The results of transecting Brachial nerves 3 and 4 in the mid lower arm during the denervation process in the newt. The number of fibers remaining, nerves per  $(100\mu)^2$  and the regeneration (positive or negative) is emphasized in each case.

Table 4a

Regeneration Following the Transection  
of Brachial Nerve 4 and 5

Fiber Number	Fibers per (100 $\mu$ ) <sup>2</sup>	Regeneration	Number of Cases
780	4.5	-	1
765	6.1	-	1
1251	4.8	+	1
595	3.8	-	1
894	3.3	-	1
1116	5.7	-	1
1151	5.8	-	1
1050	4.4	+	1
993	4.9	-	1
749	3.9	-	1

Mean  
934  $\pm$  66.72

Mean  
4.68  $\pm$  0.32

Table 4b

Regeneration Following the Transection  
of Brachial Nerves 3 and 5

Fiber Number	Fibers per (100 $\mu$ ) <sup>2</sup>	Regeneration	Number of Cases
1340	8.9	+	1
1125	7.1	+	1
1713	7.9	+	1
1608	8.1	+	1
1187	7.0	-	1
1220	8.3	-	1
842	5.6	+	1
883	5.2	-	1
1070	6.52	+	1

Mean 1220.89  $\pm$  98.39      Mean 7.19  $\pm$  0.42

Table 4c

Regeneration Following the Transection  
of Brachial Nerve 3

Fiber Number	Fibers per (100 $\mu$ ) <sup>2</sup>	Regeneration	Number of Cases
1511	7.05	+	1
1413	10.03	+	1
2111	10.04	+	1
1440	7.71	-	1
2161	9.1	+	1
1205	5.49	-	1
1000	5.32	+	1
902	3.57	+	1
1394	5.56	+	1
1562	8.19	+	1

Mean  
 1469.9  $\pm$  130.05      Mean  
 Mean  
 7.13  $\pm$  0.68

Table 4d

Regeneration Following the Transection  
of Brachial Nerves 3 and 4

Fiber Number	Fibers per (100 $\mu$ ) <sup>2</sup>	Regeneration	Number of Cases
556	6.00	-	1
499	3.61	-	1
295	1.12	-	1
205	1.70	-	1
417	2.56	-	1
432	2.27	-	1

Mean 400.67  $\pm$  53.07      Mean 2.88  $\pm$  0.71

Table 5. A threshold range established in the partially denervated series by arranging in a descending order, the fiber content of each denervated limb. Also shown is regeneration (positive or negative) and the Brachial nerve(s) operated.

Table 5

A Threshold Range Established for Regeneration  
in the Lower Arm of the Newt

Nerve Number	Regeneration	Brachial Nerves Operated	Brachial Nerves Unoperated
2650	+	---	3,4,5
2116	+	3	4,5
1713	+	3,5	4
1608	+	3,5	4
1562	+	3	4,5
1511	+	3	4,5
1440	-	3	4,5
1413	+	3	4,5
1394	+	3	4,5
1340	+	3,5	4
1251	+	4,5	3
1205	-	3	4,5
1187	-	3,5	4
1151	-	4,5	3
1125	+	3,5	4
1120	-	3,5	4
1116	-	4,5	3
1050	+	4,5	3
1000	+	3	4,5
993	-	4,5	3
902	+	3	4,5
894	-	4,5	3
883	-	3,5	4
842	+	3,5	4
780	-	4,5	3
765	-	4,5	3
749	-	4,5	3
595	-	4,5	3
556	-	3,4	5
499	-	3,4	5
417	-	3,4	5
295	-	3,4	5
205	-	3,4	5



regenerates obtained. A median fiber number was obtained representing the number of nerve fibers required to induce regeneration in 50% of the animals. This value was computed as approximately 1100 nerve fibers at level 2 (surface 2). It is interesting that this value compares closely with Singer's median value for the lower arm (1085) as does the range (920-1310 in this study and 940-1230 in Singer's). However, in the small newts, Singer found a median value of 9.9 fibers per  $(100\mu)^2$  of amputation surface while the much larger newts of the present study have a value at level 2 (mid lower arm) of 3.4 fibers per  $(100\mu)^2$ . The much lower nerve:surface area ratio needed for regeneration in the larger newts of this study is unexplained, and further work on this problem is needed. It is clear, however, that our threshold value expressed as number of nerve fibers per limb needed for regeneration is comparable with similar threshold values obtained by Singer.

The mean number and range of nerve fibers counted in transverse sections of surfaces both in the normal limb (group 1) and in the isolate at 5 day intervals from day 5 through day 20 are shown in Table 6. The average number of nerve fibers on surface 2 (2648) is slightly higher than Singer (1947a) found for his small newts (2125 for the lower arm). The mean number of nerve fibers at the three surfaces of the operated limbs show a remarkable and unexplained decrease from the normal (2488-level 1; 2648.5-level 2 and 3034-level 3) after amputation. Since surfaces

Table 6. Mean nerve number, and range of nerve numbers in normal limb levels 1, 2, and 3; and experimental surfaces 1, 2, and 3 for 5, 10, 15 and 20 days.

Table 6

Nerve Numbers in Normal Limb Levels 1, 2, and 3 and  
Experimental Surfaces 1, 2, and 3  
for 5, 10, 15 and 20 Days

Days of Regeneration	No. Newts	No. Limbs	Mean No. of Nerves	Range of Nerve Numbers
			<u>SURFACE 1</u>	
Normal	10	10	2488.50 $\pm$ 143.99	1757-3025
5	10	10	209.50 $\pm$ 56.70	71- 610
10	10	10	250.90 $\pm$ 48.03	70- 516
15	10	10	324.63 $\pm$ 53.79	99.0 - 509
20	10	10	600.60 $\pm$ 89.83	268-1195
			<u>SURFACE 2</u>	
Normal	10	10	2648.5 $\pm$ 153.75	2047-3399
5	10	10	330.40 $\pm$ 67.89	110- 612
10	10	10	302.20 $\pm$ 43.32	148- 624
15	10	10	615.00 $\pm$ 120.47	268-1260
20	10	10	1393.90 $\pm$ 133.60	589-2104
			<u>SURFACE 3</u>	
Normal	10	10	3034 $\pm$ 74.70	2653-3402
5	10	10	1051.50 $\pm$ 84.99	706-1402
10	10	10	1070.30 $\pm$ 150.03	594-1844
15	10	10	1737.75 $\pm$ 145.48	784-2154
20	10	10	2015.90 $\pm$ 88.24	1400-2346

1 and 2 show significantly sharper decreases in nerve fibers after operations than does surface 3 on the proximal limb stump, possibly the removal of tissues at the site of the ligature includes significant nerve fibers. This would also correlate with the lower percentage of regenerates at these surfaces as compared with that at surface 3. As Figure 5 shows, the mean number of nerves at surface 3 surpasses the lower threshold value at all times of regeneration while for the two distal surfaces the nerve counts indicate that lower levels of threshold are attained only at 20 days (surface 2) and perhaps later for surface 1. It is, however, important to note that at 20 days 10% of the surface 1 cases have nerve fiber counts within the threshold. This compares with 8.7% regeneration from surface 1 in this study.

An assay of the nerve fibers in transverse sections of 10 limbs carefully selected from a group of 40 animals in which surface 3 had regenerated to at least notch stage but in which no growth had occurred on surfaces 1 and 2 at 60 days reveals that the mean nerve fiber numbers on surfaces 1 (396 fibers) and 2 (551 fibers) are decreased to a noticeable extent when compared with surface 1 (2488 fibers) and 2 (2648 fibers) on the normal limbs (Fig. 6). It is clear from these data that nerve counts for surfaces 1 and 2 in this group are well below threshold for regeneration, and no regeneration did occur. It should be noted that the upper limits of the range of nerve fiber numbers

Figure 5. A comparison of the mean numbers of nerves per surface at three levels in the normal limb with surfaces 1, 2, and 3 in the operated limb at 5, 10, 15, and 20 days post amputation. Surface 3 maintains a higher number of nerves from day 5 through day 20. Surface 1 remains below the lower limits of the threshold (horizontal line) established by denervation through 20 days.

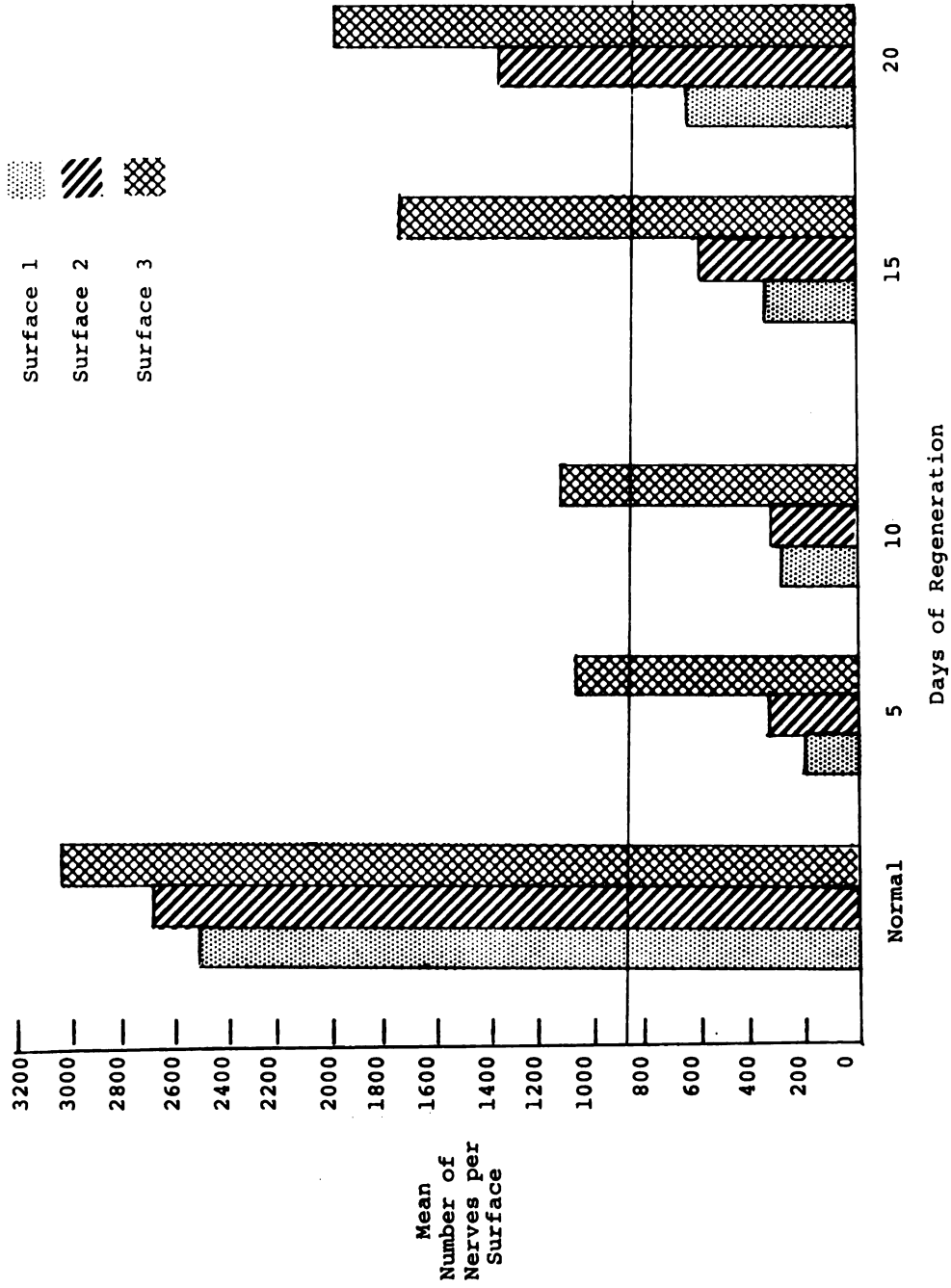


Figure 5

Figure 6. A comparison of the mean number of nerves per surface on limbs exhibiting the absence of regeneration on surfaces 1 and 2 at 60 days post amputation with levels 1 and 2 on the normal limbs. The mean number of nerves on surfaces 1 and 2 of the operated animals is reduced in comparison with the normal limbs. The mean numbers of nerves on these surfaces are below the lower limits of the threshold range (horizontal lines) established by the denervations.

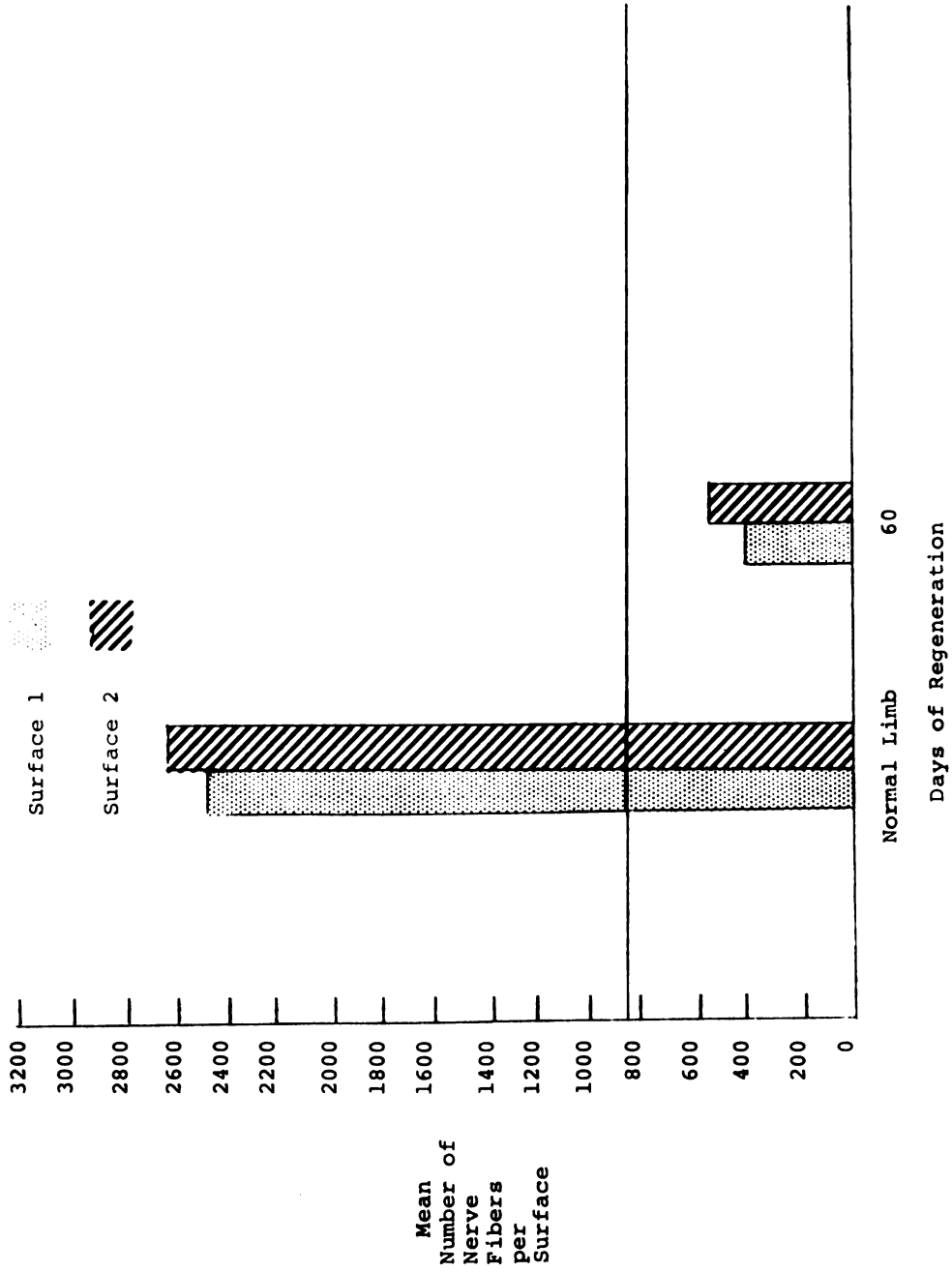
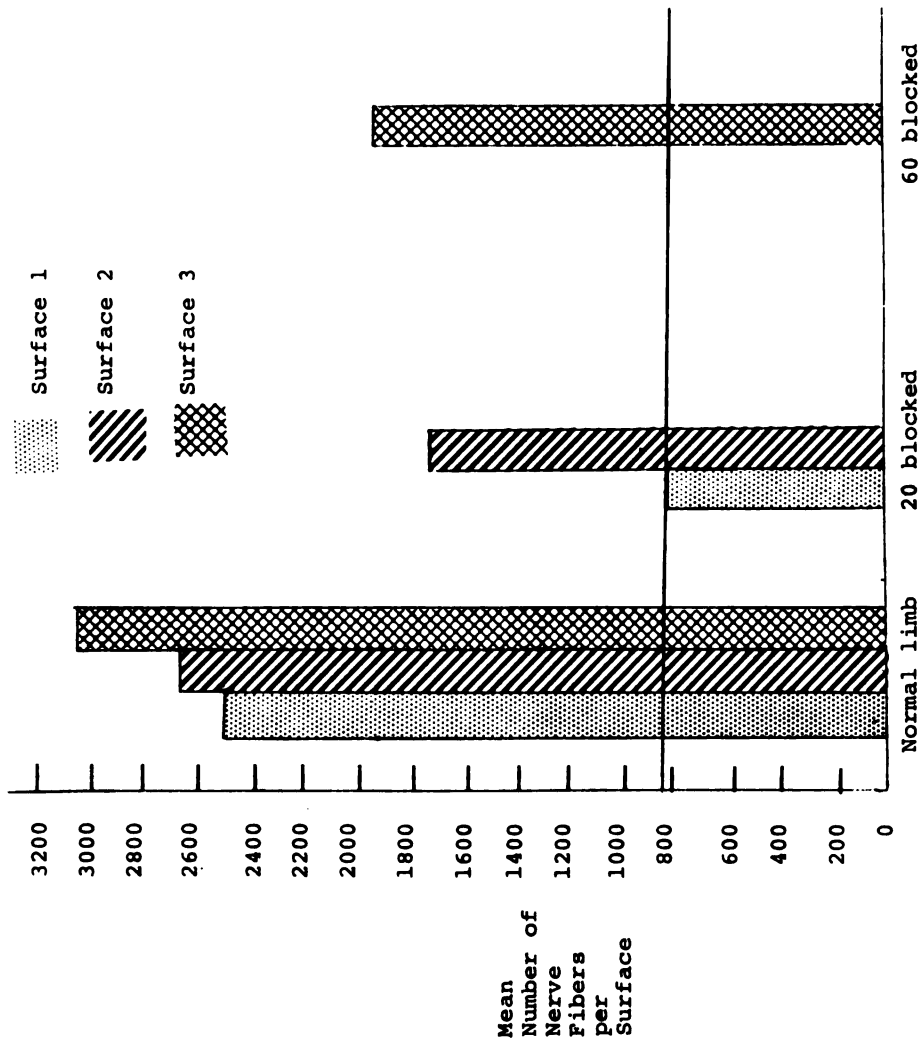


Figure 6



in these distal surfaces, failed also to reach threshold. However in 10 cases in which surface 3 was prevented from regenerating by a skin graft (p. 33), the mean nerve fiber number at 20 days reveals an increase in fibers on surfaces 1 (816 fibers) and 2 (1738 fibers) when compared with surface 1 (600 fibers) and 2 (1393 fibers) where all three surfaces remained open for the same length of time (Fig. 7). Thus both surface 1 and 2 exceed or are within the threshold range for regeneration when surface 3 is blocked. This correlates with the increased percentage of regeneration of these two surfaces (Table 3). In the remaining ten cases of this group fixed at 60 days, regeneration on surfaces 1 and 2 was too far advanced to yield an accurate nerve count for them but, as Fig. 7 shows, the mean number of nerve fibers (1941 fibers) on surface 3 shows a noticeable decrease in comparison with level 3 of the normal limb.

Figure 7. A comparison of the mean number of nerves per surface on surfaces 1 and 2 at 20 days and surface 3 at 60 days on limbs where surface 3 was blocked with whole skin. A noticeable decrease in nerve number was observed on surface 3 of the blocked series, but there are significant increases of nerves at surfaces 1 and 2.



Days of Regeneration

Figure 7

## DISCUSSION

The experiments described in foregoing pages provide data which corroborate and extend the works of Della Valle (1913) who found that a newt limb provided experimentally with three wound surfaces later exhibited regenerates on all three of them. The large number of cases studied in the present work, however, provided clear evidence that regeneration need not occur on all three surfaces as Della Valle had concluded. Indeed, the most common pattern of regeneration proved to be outgrowth from surface 3 alone (76%). Nevertheless, regenerative outgrowth occurred from all three wound surfaces in 6.3% of the cases; from wound surfaces 1 and 2 in 2.4% of the cases; and from 2 and 3 in 15.1% of the cases.

It is clear also that not only did the most proximal wound surface (3) regenerate with greater frequency than did the distal ones (1 and 2), but it also formed regenerates earlier than did the distal wound surfaces and these regenerates differentiated more completely. A disto-proximal gradient of regenerative activity has been described many times. Rose (1957), for example, has reported that in hydroids, hydranths inhibit regeneration of similar organs when they are in direct axial alignment. In the

absence of hydranths, stem sections of Tubularia will regenerate new hydranths. Tucker (1959) reported that extracts of Lineus heads would inhibit head formation in posterior segments of the worm. A clear cut example of a disto-proximal inhibitory phenomenon has not been found in amphibian limbs. The provocative experiments of Monroy (1941) and Goss (1956), however, should be mentioned here. They have shown that the angle of fusion of two newt limb stumps influences the occurrence of regeneration which decreases inversely with increase of angle of fusion. Oberprillar (1968) studying the regenerates produced by similarly fusing X-irradiated newt limb stumps to unirradiated ones, reported that irradiation eliminated the morphogenetic field of that limb so that no inhibition occurred in her experiments. She interprets her results as conforming to Rose's theory of disto-proximal inhibition.

The supernumerary regenerates obtained by Della Valle (1913), Nassonov (1930) and Kasanzeff (1930) after applying ligatures would seem to illustrate further the phenomenon of distal inhibition.

As Rose (1964) points out, the ligature might well isolate the proximal stump (wound surface 3 of the present work) from inhibitory influences originating in the distal limb regenerate, thus regenerates, as these authors have found, could develop on the proximal wound surface. However, no such barrier exists between wound surfaces 2 and 1 in the present work, yet surface 2 regenerated more frequently

(23.8%) than did surface 1 (8.7%). This, as well as the high percentage of regenerates from surface 3, is not explained by the distal-dominance theory. A further difficulty for this theory is the greatly increased regeneration from wound surfaces 1 and 2 when wound surface 3 is inhibited by a skin graft. Here, it would seem, is evidence that an inhibitory influence might be flowing from the most proximal surface to the distal ones, quite the reverse of expectation if the distal inhibition theory is operating.

The influence of quantity of nerves in limb regeneration is well documented (Singer, 1952 for review). The question, therefore, arises as to the quantitative relation of nerves to the regeneration at the three amputation surfaces of the limbs described in foregoing pages. When nerve counts were made, they strongly suggested that the number of fibers and the nerve-tissue ratio on surface 3 were consistently higher than those on surfaces 1 and 2, for the first 20 days of accessory limb development.

The mean number of nerve fibers at surface 3 is always above 1511, even in the blocked surface. The range of nerve fibers at surface 2 at 20 days is from 589-2104 while for surface 1 at 20 days it is from 268-1195. It has been found in the present study that regeneration always occurs above a threshold of 1440 nerve fibers and never occurs at 780 nerve fibers or below, and in between these values regeneration is variable. Thus it can be seen that the number of fibers at surface 3 is always above threshold.

This correlates well with percent regeneration from this proximal surface (97.6%). However, at surfaces 1 and 2, fiber numbers are much more variable, as is regeneration from these surfaces. Thus at surface 2, 40% of the cases have fiber numbers above threshold range (842-1440). This compares with 23.8% of regeneration from this surface. Similarly, 90% of the cases of amputation surface 1 have below 842 nerve fibers and only 8.7% regeneration is obtained from this surface. Particularly significant is the fact that when the proximal wound surface 3 is blocked by a skin graft, nerve fiber numbers markedly increase at surfaces 1 and 2. Thus for surface 1, 50% of the cases have counts above 842 and regeneration occurred from this amputation surface in 63.60% of the cases. In 80% of cases surface 2 had 842 or more nerve fibers and 54.2% regeneration. These data clearly point to a neural mechanism controlling regeneration at these three surfaces, rather than a hypothetical flow of inhibitory substance.

An unexpected finding was the low nerve-stump tissue ratio required for regeneration. Singer (1947b), using New England newts (Triturus viridescens viridescens) found that a nerve-tissue ratio of 9.9 (expressed as a single median value) was needed for regeneration for the lower arm, while we find a median threshold value of 3.4. However, the correspondence between the range of nerve fibers needed for regeneration in the lower arm as reported by Singer and that found here is remarkably close--792 to

1470 (Singer; 1947b) and 842 to 1440 (present study). We can not explain the lack of correspondence between Singer's nerve-tissue ratio and ours. It is, however, well known that tissue conditions may alter nerve requirements for regeneration. Thus, Singer and Mutterperl (1963) obtained regeneration from limb segments grafted to the back in the newt. Nerve numbers, in these experiments, were one-third those normally needed for regeneration (nerve:surface area ratio of 2.9). These authors concluded that extensive trauma of grafting may have in some way reduced tissue requirements for nerves. Similarly, Thornton and Tassava (1969) found that transplanted Ambystoma larval limbs could regenerate without nerves. Perhaps the extensive trauma of ligaturing the limb, in the present reports, partially explains our results; nevertheless, limbs of these large newts undergoing simple amputation regenerate very well. This relationship needs further study.



## SUMMARY

This investigation has demonstrated that the establishment of three surfaces on a single limb of the newt Notophthalmus viridescens is not consistently followed by the regeneration of accessory structures on all three surfaces. Surface 3 exhibits noticeably more accessory structures than either surface 1 or 2. Nerve counts on the three surfaces suggests that neural influences may be related to growth on these surfaces. Evidence from these counts shows that the number of fibers are consistently higher on surface 3 than on surfaces 1 and 2. Additional support for a relationship between nerves and growth on these surfaces was shown on limbs where surface 3 was blocked. Surfaces 1 and 2 on these limbs have an increased number of nerve fibers, while the fiber number on surface 3 is significantly reduced. The results of denervation experiments suggest that the failure of a significant number of accessory structures to appear on surface 1 and 2 may be related to an insufficient number of nerve fibers on these surfaces. Similarly, a possible explanation for the regular occurrence of accessory structures on surface 3 is that the nerve fiber number of this surface is above the threshold range, while those values on surfaces 2 and 1 occur within and below the threshold respectively.

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