THE EFFECT OF NERVE AUGMENTATION ON THE CELLULAR ACTIVITY OF MESENCHYMATOUS CELLS WITHIN THE REGENERATION BLASTEMA OF THE HINDLIMB OF THE AXOLOTL, AMBYSTOMA MEXICANUM

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ABSTRACT

THE EFFECT OF NERVE AUGMENTATION ON THE CELLULAR ACTIVITY OF MESENCHYMATOUS CELLS WITHIN THE REGNERATION BLASTEMA OF THE HINDLIMB OF THE AXOLOTL, AMBYSTOMA MEXICANUM

By Nicholas C. Shuraleff, II

It has been demonstrated in this study that nerve augmentation of the regenerating axolotl hindlimb accelerates that rate of regeneration. Nerve augmentation significantly increased the mitotic rate during the growth phase of regeneration (9 to 15 days post-amputation). Using thymidine methyl-H^3, it has been shown that the mesenchymatous cell populations of augmented blastemata were more mature that those of normally innervated blastemata at 12 and 15 days post-amputation.

By systemmatically sampling the blastemata with an ocular grid, the mitotic figures of both augmented and normally innervated blastemata were randomly dispersed throughout the blastema, except immediately proximal to the apical wound epithelium where the mitotic index was significantly less throughout the growth phase. It has been shown that a dense core of cells exists in both augmented and normally innervated blastemata, at least 9 days prior to chondrogenesis. The amount of innervation had no

effect on the density of mesenchymatous cells within the blastema or on the distribution of those cells. Also, the amount of innervation had no effect on the rate at which mesenchymatous cells appeared in the blastema.

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INTRODUCTION

What does the nerve do during regeneration? Tweedy John Todd addressed himself to this question in 1823 and wrote, "If the sciatic nerve be intersected at the time of amputation, that part of the stump below the section mortifies... If the division be made after the healing of the stump, reproduction is either retarded or entirely prevented. And if the nerve be divided after reproduction has commenced or considerably advanced, the new growth either remains stationary, or it wastes, becomes shrivelled and shapeless, or entirely disappears. This derangement cannot, in my opinion, be fairly attributed to vascular derangement induced in the limb by the wound of the division, but must arise from something peculiar in the nerve." Since Todd's time, the influence of the nerve is still a major research topic in urodele limb regeneration studies (see reviews by Singer, '52, '59).

From the earliest stages of regeneration, the nerve is known to play an important role in the regeneration process. If the limb is denervated at the time of amputation, no regenerate forms. In the larva, an extensive histolysis of the limb tissues occurs which leads to complete limb resorption (Schotte & Butler, '41). Butler and Schotte ('41) noted that the regression process of dener-

vated limbs is similar to the dedifferentiation stage of regeneration, except that, in the denervated limb, the dedifferentiated cells (blastemal cells), henceforth called mesenchymatous cells (Singer, '59), fail to accumulate under the wound epidermis. If the nerves are allowed to re-enter the regressing limb, regression ceases, and a complete and normal regenerate develops. Schotte and Butler ('44) postulated that the nerve is required for the normal controlled formation of the blastema. On the other hand, denervation of the adult limb at the time of amputation results in the formation of a cicatrized region at the amputation surface without ensuing limb regression. Nonetheless, the newt limb does not regenerate without the presence of nerves.

Both in the adult and larval forms, denervation after the blastema has reached a sizable mound (see stages of Faber, '60; and Singer, '52) results in the cessation of growth and the precocious onset of morphogenesis. Such regenerates are small and morphologically normal, albeit malformations of limb structures may occur (Brunst, '27, '32).

Singer and Craven ('48) reported that complete denervation of newt blastemata causes an increase in blastemal length accompanied by a reduction in blastemal volume. They also reported that complete denervation during the stage of rapid blastemal growth causes a precipitous reduction in the number of mesenchymatous cells undergoing division

within the blastema. Although studies of mitotic activities have not been performed on denervated larval limbs, similarities in growth stoppage suggest that nerves may also affect cellular proliferation.

Reports on the effect of partial denervation on the initiation of regeneration and the rate of blastemal growth are quite conflicting. In the adult newt, Singer ('46b) found that a minimum number of nerve fibers is needed before regeneration commences. He stated that once the number of nerve fibers falls below a certain "threshold" quantity, no regeneration occurs. He concluded that the initiation of regeneration is an "all or none" phenomenon, that is, once the threshold level of innervation is reached, regeneration proceeds as if the entire complement of nerves is present. On the other hand, Karczmar ('46) found that the incidence of regeneration is linearly correlated to the amount of innervation in the larval limb. He stated that nerves are not only qualitatively needed for regeneration, but also that the effect of nerves is additive.

Partially denervated limbs are known to regenerate at a slower rate than normally innervated limbs, but whether the rate of regeneration can be correlated with the amount of innervation is not clear. Schotte ('23) and Weiss ('25) reported that there is an additive effect of the nerves on blastemal growth. In experiments on adult urodeles, they showed that a graded reduction in innervation causes a

graded decrease in the rate of regeneration. Karczmar ('46) also raised the point that the rate of regeneration in the limb is possibly correlated with the amount of innervation. Singer and Egloff ('49) found that partially denervated newt limbs do regenerate at a slower rate, but that the rate of regeneration is not a graded response to the amount of innervation. They went on to say that the slower rate of growth in partially denervated limbs falls well within the normal variation between growth rates of normally innervated limbs, thus totally discounting the concept that the influence of nerves on the incidence and rate of regeneration is additive.

Since partial and total denervation has been the only technique used to study the influence of nerves on the rate of urodele limb regeneration, the present investigation was undertaken to examine the effect of nerve augmentation on the rate of regeneration of normally regenerating urodele limbs and to test the hypothesis of Schotte ('23); Weiss ('25); and Karczmar ('46) that nerves have an additive effect on the rate of regeneration. Also the hypothesis of Overton ('50) that extra nerves increase the frequency of mitosis, was tested by calculating the mitotic indices at specific regions of normally innervated regenerates and regenerates whose nerve supply had been augmented.

MATERIALS AND METHODS

Animals and Their Maintenance

All animals used in the following experiments were *Ambystoma mexicanum* larvae and measured between 5.5 and 7.0 centimeters in snout-anal length. Their diet consisted of weekly feedings of beef liver. All larvae were fed approximately the same amount of liver at each feeding. Each animal was housed in a one quart squat fish bowl and was maintained at a temperature of $21 \pm 1^{\circ}$ C. Animals were exposed to daily fluctuations of natural light.

In each experiment, a different animal which had never been operated on before, was used. Animals were randomly selected from the colony without regard to any physical characteristics. Only animals in excellent physical condition were kept in the colony. Thus, experimental variability introduced by light, nutrition, and physical condition was reduced to a minimum.

Operative Techniques

In order to study the effect of additional nerves on regeneration, the nerve supply of the hindlimb was augmented by the following procedure. The larvae were anesthetized in a 1:1,000 solution of MS222 (Sandoz). Using

watchmaker's forceps and irridectomy scissors, the sciatic nerve of the left hindlimb was carefully dissected from the overlying musculature, from the point at which the sciatic nerve emerged from the pelvis, to the ankle. A sewing needle was inserted under the skin on the ventral side of the animal, slightly anterior to the cloaca, and pushed completely through the medila portion of the contralateral (right) limb, finally emerging at the ankle. The distal end of the dissected sciatic nerve was gently pulled through the eye of the sewing needle until as much of the nerve as physically possible was threaded. The sewing needle was then slowly pulled out of the limb, leaving the nerve behind. This technique insured that the sciatic nerve was pulled maximally distad into the contralateral limb without undue strain on the nerve fibers. The limb from which the nerve had been removed was amputated at the proximal end of the femur. Similarly, control animals had their hindlimbs amputated at the proximal end of the femur and had a sewing needle, minus the nerve, pulled through the contralateral limb at the same time experimentals were operated.

Tracing Techniques

To determine the effect of an augmented nerve supply (superinnervation) on the rate of blastemal growth, outline tracings of superinnervated blastemata were made at three day intervals from 9 to 30 days post-amputation. Animals were anesthetized in MS222 and placed on their backs

in a petri dish containing a moist gauze pad. To provide consistent limb orientation and to eliminate flexion of late stage regenerates, the limbs were horizontally positioned on a small glass bridge. Using a camera lucida attached to a dissecting microscope (7X), the ventral aspect of blastemata was drawn on graph paper (20 squares/inch) from 9 to 21 days post-amputation. Since the blastema underwent anterior-posterior compression during the formation of a palette, the anterior aspect of the blastema was traced from 24 to 30 days post-amputation, due to the fact that it provided the largest surface area.

Only one eye was used in viewing the limb, since the use of both eyes could shift the view of the regenerate, thus affecting the size of the trace. Since the limb stump was heavily pigmented compared to the sparsely pigmented blastema, the plane of amputation could easily be identified at the proximal border of the regenerate. The number of squares encompassed by the outline tracing of the blastema was subsequently counted and this number was used to represent blastemal volume. Also the day on which a palette stage blastema appeared was noted and used to denote the onset of morphogenesis. Similar tracings were made of normally innervated regenerating limbs which served as controls.

To determine whether redirected nerves by themselves could support regeneration, the indigenous nerve

supply (sciatic and femoral nerves) of one group of superinnervated limbs was transected at the proximal end of the femur 15 days after the limb had been superinnervated. The limb was simultaneously amputated midway along the tibia-fibula. Every seven days for a 21 day period the linear distance along the dorsal side of the limb between the proximal end of the femur and the distal tip of the blastema was measured with an ocular micrometer in the eyepiece of a dissecting microscope (7X). Morphological stages (see Faber, '60) of the blastema were recorded at each seven day interval. After each measurement interval, the indigenous nerve supply of the superinnervated limb was resectioned at the pelvis to insure that only the redirected nerves would influence regeneration at the forearm. Denervated amputated limbs, which had been normally innervated, served as controls.

Histological Techniques

Superinnervated and normally innervated limbs were fixed in Zenker's fixative in those series where the Feulgen reaction was used; otherwise, limbs were fixed in Bouin's fixative. All limbs were dehydrated in dioxana, embedded in paraffin, and sectioned at 10u (u = microns). Special attention was paid to the orientation of the limbs along the longitudinal and transverse axes during embedding and sectioning. However, limb orientation around the longi-

tudinal axis (anterior-posterior, dorsal-ventral) was randomized.

Since Litwiller ('40) reported that in the forelimb of the newt, Triturus pyrrhogaster, mitotic rates varied with the time of day, all limbs were fixed at 3:30 P.M. However, no correlation has been found between mitotic activity and the time of the day in Ambystoma larvae and Rana tadpoles (Cameron, '36) or in the axolotl (Hearson, '66).

Series I:

Analysis of Limb Innervation

In order to determine whether superinnervated limbs had in fact an increased nerve supply, superinnervated and normally innervated limbs, amputated midway along the femur 2, 4, and 6 days prior to fixation, were sectioned transversely from the proximal end of the femur distally to approximately 200u proximal to the base of the blastema. The sections were stained for nerves using the silver impregnation technique of Samuel ('53).

The cross sectional area of the axon bundles was determined under high power magnification (430X) of the most distal transverse section. The number of grid squares of an ocular grid superimposed over the axon bundle was counted and converted to square millimeters. Although the area of the axon bundles was not considered to be a precise measure-

ment of limb innervation, for not only was the axon included but also the surrounding perineurium, it was assumed to be an accurate measurement for comparing relative amounts of innervation since the same technique was used for both superinnervated and normally innervated limbs.

The cross sectional area of the limb, including cartilage and nerves, was calculated from the same section used in the determination of axon bundle cross sectional area. However, instead of using an ocular grid for area measurements, the section was projected from a triplex projector and the outer border of the section was traced on graph paper (20 squares/inch). The number of graph squares was then converted to square millimeters.

To standardize the amount of innervation, a ratio of axon bundle area to limb tissue area (nerve:tissue) was used to express the amount of relative innervation for both superinnervated and normally innervated limbs.

Series II:

Longitudinal Sections through Two to Six Day Regenerates

To determine whether the rate of appearance of mesenchymatous cells during the earliest stages of regeneration in superinnervated limbs was different from that of normally innervated limbs, counts of "blastemal cells" (explained below) were made of 2, 4, and 6 day regenerates.

The remaining unsectioned blastemata of limbs used in Series I were reembedded in paraffin. These blastemata were then sectioned along the longitudinal axis and stained with Regaud iron hematoxylin-Masson trichrome stain (Merchant, Kahn, and Murphy, '64) to emphasize general histological detail.

Since the earliest stages of regeneration are characterized by both the appearance of mesenchymatous cells from dedifferentiated internal tissues (Thornton, '38) and also by the invasion of lymphocytes into the wound area, and since lymphocytes cannot readily be histologically differentiated from mesenchymatous cells, no distinction was made between whether a cell was a lymphocyte or a mesenchymatous cell in scoring the cells. The term "blastemal cell" then referred to cells which although grossly appearing like mesenchymatous cells might also be lymphocytes.

Counts of blastemal cells were made under high power magnification (200X) using an ocular grid. At this power, the ocular grid encompassed $741000u^3$ of sectioned tissue. All blastemal cells within the boundary of the grid were counted. Cells which were not completely within the grid were excluded from the count.

To get a representative sample of the number of blastemal cells throughout the regenerate, five longitudinal sections through the regenerate were selected. Since at 2, 4, and 6 days post-amputation no definitive blastema had formed, no counts could be made distal to the amputated end

of the femur. Blastemal cell counts were made lateral to the cartilage by superimposing the distal border of the grid on an imaginary line connecting the amputated distal ends of the dermis (Figure 1). To further standardize sampling, the lateral border of the grid was also superimposed over the medial border of the dermis. Since anterior-posterior, dorsal-ventral axes were not predetermined during sectioning, only one sample site lateral to the cartilage was chosen for each section. The side of the section to be sampled was predetermined with the aid of random numbers.

Series III:

Longitudinal Sections through 9 to 15 Day Regenerates

Since denervation of the actively growing newt blastema caused a drop in mitotic activity, a series of experiments was designed to correlate the effect of superinnervation on the mitotic activity and density of mesenchymatous cells during those stages of regeneration characterized by rapid growth. All limbs were amputated midway along the femur. Regenerates of 9, 12, and 15 day superinnervated and normally innervated limbs were fixed six hours after they had been injected intraperitoneally with 3 microcuries of thymidine methyl-H^3 (Schwartz) in 100 lambda of distilled water. The sections were stained for DNA using the Feulgen reaction. No counterstain was used.

Since at 9, 12, and 15 days post-amputation a definitive blastema had formed at the wound surface and since few phagocytic cells were found in the blastema, all cells included in the results were considered to be true mesenchymatous cells. As in Series II, erythrocytes and leucocytes were excluded from the counts.

Cellular counts of this series and of the subsequent Series IV were obtained by counting under high power magnification (430X) all mesenchymatous cells completely within the area delineated by a square ocular grid superimposed over a predetermined site within the regeneration blastema (discussed below). At 430X, the grid encompassed $256000u^3$ of tissue. Nuclei within the grid were scored as either interphasic or mitotic nuclei. Only nuclei in late prophase, metaphase, anaphase, and early telophase were considered to be mitotic nuclei. Nuclei in early telophase were scored as a single mitotic nucleus. Also, the microscopic field of view was put slightly out of focus when the ocular grid was superimposed over the sample site to reduce possible bias.

The mitotic index of each sample site was determined by dividing the number of mitotic nuclei by the total number of nuclei (interphasic + mitotic) within the grid area and multiplying the result by 100. It should be noted that the term mitotic index as used in this study represents the percentage of cells in mitosis. This conforms to the present nomenclature used in other limb re-

generation studies (Chalkley, '54, Hearson, '66).

The cellular density was calculated by dividing the total number of nuclei within the grid by the area of the grid and multiplying the result by 10,000. Thus, cellular density was expressed as the number of mesenchymatous cells per 10,000u^3 of tissue.

Since counting all mesenchymatous cells within the blastema would have been a laborious task, a sampling scheme was designed which afforded not only an estimate of mitotic activity and cellular density throughout the entire blastema, but also within specific regions of the blastema. First, the width of the blastema was determined by counting the number of longitudinal sections which passed through the plane of amputation[1]. The number of blastemal sections was divided by six to obtain five sections passing through the blastema, each equidistant from the other along the longitudinal axis (Figure 2). Each of the five sections was numbered: Section III being the medial section, Sections II and II' being 33% of the limb radius from the medial section, and Sections I and I' being 66% of the limb radius from the medial section. Since rotation about the longitudinal axis was randomized, no distinctions were made between Sections II and II',

1. The plane of amputation was considered to be an imaginary line drawn transversely across a longitudinal section connecting the distal ends of the amputated dermis.

and I and I'. Either Section II or II'; and Section I or I', was selected with the aid of random numbers to be used for cell counts. Thus for each limb, three longitudinal sections were used, each section passing through a comparable portion along the transverse axis of the blastema for all limbs. Therefore, since each longitudinal section was in a plane passing edgewise through the transverse axis of the blastema, data obtained from longitudinal sections were considered to be the best estimate of mitotic indices and cellular densities along the transverse axis of the blastema.

Each section was in turn subdivided into 3 x 3 squares (Figure 3). The width and the height of the section were divided into thirds. Again since rotation around the longitudinal limb axis was random, only one of the two lateral subdivisions (Columns 1 or 1') was chosen for each height level (Rows A, B, and C). The lateral subdivisions to be sampled were predetermined with random numbers.

Due to the geometry of the blastema, which is best described as a paraboloid, the width of each section varied from section to section within the same limb. Likewise, the height of each section, not only varied from section to section, but also from day to day post-amputation. Also since the minimum area in which cell counts could be made was confined to the area of the grid, certain restrictions were placed on the selection of sample sites. If the width of the section was less than 2/3 the width

of the blastema, only the medial column (Column 2) was sampled. If the height of the section was less than two grid units (<320u), the proximal border of the grid was superimposed on the plane of amputation and only the most proximal level (Row A) was sampled. If the height of the section was less than three grid units (<480u), only the two most proximal levels (Rows A and B) were sampled. If the height of the section was more than three grid units (>480u), the height of the blastema was divided into three equal regions (Rows A, B, and C) from which the counts were made. Thus, only those areas were sampled which were large enough to completely fill the grid.

Series IV:

Transverse Sections through 9 to 15 Regenerates

Since Series III was designed to sample primarily along the transverse axis of the blastema, another group of 9, 12, and 15 day superinnervated and normally innervated regenerates were sectioned transversely to provide accurate sampling along the longitudinal axis. The same techniques and criteria used in Series III for amputation and cellular counts were also employed in this series, except for the fact that at 9:30 a.m. of the day the regenerates of Series IV were to be fixed, each larva was injected intraparatoneally with 12 microcuries of thymidine-methyl H^3 in 100 lambda of distilled water. Six hours later, the limbs were fixed.

Sample sites along the longitudinal axis of the blastema were selected for each limb, dividing the total number of blastemal sections distal to the base of the blastema[2] by four (Figure 4). Thus three sample planes were created: one through the blastema 25% the total length of the blastema from the base of the blastema (Level D); one 50% of the blastemal length (Level C); one 75% of the blastemal length (Level B). A fourth level (Level A) was also created to sample mitotic activity 20u proximal to the base of the epidermal cap. Thus for each limb, four transverse sections passed through comparable portions along the longitudinal axis of the blastema for all limbs. Therefore, since each transverse section was essentially in a plane passing edgewise through the longitudinal axis of the blastema, data obtained from transverse sections were considered to be the best estimate of cellular activity along the longitudinal axis of the blastema.

In selecting sample sites along the transverse axis of the blastema, the width of the blastema was found by measuring in microns the diameter of the limb at the base of the blastema. The radius of the limb was then

2. The base of the blastema was considered to be that transverse section which had a continuous circular dermis. Although some limbs were slightly oblique to the transverse plane during sectioning, the appearance of a continuous dermis closely followed the first appearance of dermis as one viewed sections proximally through the blastema.

divided by three. Three imaginary concentric circles were drawn forming three rings around the longitudinal axis of the limb (Figure 5). By flipping a coin, sample sites either directly above or below the medial site (Ring I) were selected for Rings II and III. Since rotation around the longitudinal axis of the blastema was randomized during sectioning, there was no predetermined correlation between grid placement and the dorsal-ventral, anterior-posterior axes of the limb.

As in Series III, the geometry of the blastema and the size of the grid necessitated the use of certain restrictions on the sample sites. If the radius of the section was less than 5/6 the maximum radius of the blastema, only the two centermost rings were sampled. If the radius of the section was less than 1/2 the maximum radius of the blastema, only the most medial ring (Ring I) was sampled. It should be noted that at no time did the grid cover more than 1/3 the maximum radius of the blastema.

Audioradiographic Techniques

After the sections of Series IV had been stained for DNA and cellular counts (described above) completed, the coverslips were soaked off the slides in xylene. In complete darkness, the slides were dipped back to back into a solution (44°C) of Kodak NTB-3 photographic emulsion which was diluted 1:1 with water, and air dried at

room temperature for one hour in a vertical position. Slides were then placed, emulsion side down, in a slide box containing Drierite. Microslide boxes (Arthur Thomas 7059-B) were packed with 20 slides each. The style was found to be far superior to microslide boxes having clasps and hinges, since lids of this particular slide box fitted snugly over the base. The seams of the boxes were sealed with black electricians tape. Each box was inserted into a small insulated paper bag, containing Drierite, and stapled shut. Two of the small bags were inserted into a large insulated paper bag which also contained Drierite, and stapled. The sealed bags were then placed in a refrigerator at $5^{o}C$ and they were exposed for three weeks. After exposure, the slides were developed in complete darkness at room temperature in Kodak D-19 developed for 3 minutes, rinsed in water, fixed in Kodak fixer for 4 minutes, and washed in running water for one hour. This procedure produced a minimal amount of background.

After development, cellular incorporation of thymidine-H^3 by mesenchymatous cells was expressed as the number of labelled cells divided by the total number of nuclei within the grid. The sample sites used for counting unlabelled nuclei were also employed in counting labelled nuclei. Labelled nuclei were also scored as to whether they were interphasic or mitotic.

Before the cover slips were mounted on slides from Series III, they were dipped in photographic emulsion and dried like the slides in Series IV, except that a Wratten Series II darkroom light was used for illumination. The slides were then packed, emulsion side down, into large hinged microslide boxes containing Drierite and sealed with black electricians tape. The sealed boxes were placed in a refrigerator at 5°C and exposed from three to seven weeks. After exposure, the slides were developed in a room at 14°C, lit by a darkroom light using Kodak D-19 developer for five minutes, rinsed in water, fixed in Kodak fixer for ten minutes, and washed for one hour. However, even after seven weeks of exposure, none of the cells were distinguishable from the moderate background label. Thus, Series III was not used in autoradiographic studies.

The successfully developed slides of Series IV were treated differently from those of unsuccessful Series III in the following ways: (1) four times as much labelled thymidine was injected into Series IV animals; (2) Series IV was dipped and developed in complete darkness, Series III was not; (3) Series IV slides were placed in sealed insulated paper bags containing Drierite; (4) the microslide boxes of Series IV were small and had no hinges or latches; (5) Series IV was developed and fixed for shorter periods of time; and (6) Series IV was developed at comfortable room temperature instead of at 14°C. Any one or

all of the above changes could have been responsible for the success of Series IV and the failure of Series III.

Statistical Techniques

When the data met the tests of independence, variance homogeneity, and independence of variances and means, an analysis of variance was used to determine differences. All analyses of variance were calculated on a Control Data 3600 digital computer. The Duncan's New Multiple Range Test (Fryer, '66) was used exclusively to determine differences between means in the analyses of variance. The test was used only when significant differences were found in the analyses of variance; otherwise, the means were considered to be from the same population. In all statistical tests the 0.05 level of significance was used to determine differences between populations and means.

When the requirements for an analysis of variance were not met or when only two means were tested, the Student's "t" Test at the 0.05 level was used to determine differences between means. Where the nominal scale of measurement was used to describe the data, a chi square test was run on the data to determine differences.

In all statistical tests, the data used in the analyses were raw data, except for the analyses of mitotic index and cellular density in Series III and IV. In these series the average mitotic index or cellular density of

each sample region was used for each limb to expedite the analysis, since unequal replications of the raw data occurred due to blastemal geometry and to the criterian used for the selection of sample sites. For example, blastemata sectioned longitudinally were ideally divided into a 3 x 3 quadrant. If one wished to analyze cellular activity at three sample sites along one axis, there were ideally three sample replicates for each sample region. If, however, the blastema was so shaped that a 3 x 3 quadrant could not be constructed, one, two, or three sample replicates might occur at each sample region. To avoid an unwieldy statistical analysis, the sample replicates were averaged at each sample region for each limb, and this average was used as the basic element in the analysis of variance. This technique in no way impaired the sensitivity of the analyses (Peatman, '63).

RESULTS

Gross Morphological Changes of Superinnervated and Normally Innervated Blastemata Over Time

Changes in Blastemal Volume

To determine the influence of superinnervation on blastemal growth, outline traces of 49 superinnervated blastemata and 38 normally innervated blastemata show that superinnervated blastemata are significantly larger at three day intervals (Figure 6). However, superinnervated blastemata do not continue to increase in size at a faster rate than normals. From 9 to 12 days post-amputation, the percent proportional change $((\bar{X}e-\bar{X}c/\bar{X}e)x100)$ between superinnervated and normally innervated increases; however after 12 days post-amputation normally innervated blastemata approach the size of the superinnervated group (Figure 7). In fact, by 3 months post-amputation, both groups are the same size. Thus, the size of the final regenerate is not determined by the amount of innervation, even when the amount of innervation is augmented. Instead differences in blastemal size which are most pronounced at 12 days post-amputation appear to be due to differences in blastemal size occuring during the "growth stage" of regeneration. The size differential between the two groups leads to a precociousness in subsequent developmental

processes of superinnervated limbs, albeit the size differential continually decreases.

While the blastema is a symmetrical structure during 9-15 days post-amputation, the traced outline of the regenerate is related to the volume of the regenerate. A linear regression of the area of the largest longitudinal section for sections of Series III to the volume of the blastema shows that a correlation coefficient of 0.969 exists between traced area and volume with a slope of 817.663. The volume is calculated by multiplying the mean area of the 5 longitudinal sections used in Series III by the diameter of the blastema at the base. Thus, an outline trace of the blastema can be used to calculate the volume of the blastema.

After 15 days post-amputation the symmetry of the blastema changes as the regenerate undergoes dorsal-ventral flattening during palette formation. During this period of time, outline traces of the blastema are rough estimates of blastemal size and can not be used to accurately assess the volume of the regenerate. However, they are useful in determining gross differences in blastemal size.

Onset of Morphogenesis

Superinnervated blastemata not only regenerate at a faster rate during the stage of rapid cellular proliferation, but also reach a predetermined endpoint, the palette

stage, sooner than do normally innervated blastemata. A chi square test of the number of palette formations over time indicates that there are differences between the two groups ($p(X^2=16.207)<.01$). As seen in Figure 8, superinnervated blastemata precociously form a palette. In fact by 24 days post-amputation 72% of the superinnervated blastemata have developed a palette compared to only 38% of the normals, by 27 days 98% and 84% respectively. Thus, superinnervation is correlated not only to an increase in blastemal size, but also to the precocious onset of limb morphogenesis.

The criteria which are used to classify a palette stage regenerate are: the appearance of anterior-posterior flattening of the blastema, the formation of visible cartilaginous structures, and a slight indentation at the distal end of the regenerate which is an early sign of digit formation. These criteria are used because simple flattening of the blastema into a paddle shape is by itself highly subjective. Although chondrogenesis histologically commences at approximately 18 days post-amputation, the apparent delay in scoring palette stages is due solely to the criteria used.

Analysis of the Amount of Innervation

To assess the amount of extra innervation in superinnervated limbs, an examination was made of transverse sections (Series I) through the mid femur region approxi-

mately 200u proximal to the amputation plane. It reveals that superinnervated limbs have more innervation than do normally innervated limbs (Table A). The difference is not due to a difference in limb size, for both groups have the same cross-sectional area (tissue area), but instead to a significant difference ($p(t)<.01$) in the absolute amount of axon bundle area. This absolute differential in absolute innervation is reflected in a 28% proportional increase in the nerve:tissue ratios between the two groups.

Analysis of Amputated Superinnervated and Normally Innervated Limbs whose Normal Nerve Supply is Severed

Changes in Morphological Stages

The ability of the redirected nerve alone to support regeneration was tested in this experiment. Using morphological stages of regeneration as a criterion, limbs whose only nerve supply is that of the redirected sciatic nerve from the contralateral limb (partially innervated) are able to support regeneration to a greater degree than totally denervated limbs (Figure 9). By 21 days post-amputation, 92% of the partially innervated group show signs of regeneration compared to only 50% of the totally denervated group. Thus, the redirected sciatic nerve of superinnervated limbs can support regeneration.

Although the mound stage is used to score regenerative ability, the use of this stage may be dubious.

Table A

Amount of Innervation at Mid Femoral Level of Superinnervated and Normally Innervated Limbs

Group	Cases	Mean Tissue Area (mm^2)	Mean Axon Area (mm^2)	Nerve:Tissue Ratio
Super	21	30.859 (5.043)*	0.624 (0.0021)	0.0201
Normal	17	30.279 (1.993)	0.440 (0.0008)	0.0144

* Standard Error

During the dedifferentiative stage of regeneration, swelling at the distal end of the amputated stump may occur due to edema. This swelling in many ways appears like that of a mound stage regenerate when viewed in vivo. Indeed, those blastemata which are scored as mound stage regenerates may be non-regenerating limbs with a swollen wound area. This error certainly exists. If, however, only the cone and palette stages are used to score regenerative ability, 42% of the limbs with a redirected sciatic nerve show signs of regeneration compared to none of the totally denervated group.

Changes in Limb Length

When the effect of partial innervation (the redirected sciatic nerve alone) on changes in total limb length is measured, no significant differences are found between

the limb lengths of the 12 partially innervated and 12 totally denervated limbs amputated midway along the tibia-fibula (Figure 10).

Since accurate measurements of total limb length are difficult, the lack of significance between the two groups is probably due to the high degree of variability in the measuring technique itself. Also since the data are of changes in total limb length, not blastemal length, linear measurements need not be correlated with blastemal morphogenetic development. Although partially innervated limbs undergo some regression by 21 days post-amputation, 42% of that group develop into either a cone or palette stage.

Since complete denervation can not be claimed for the entire hindlimb with certainty due to the difficulty in severing the fine branches of the lumbar plexus which innervate the thigh muscles, the term "total denervation" refers to the absence of functional nerves at the forearm level, the level of amputation. The lack of response to pricking the forearm with a sharp pin is considered to indicate that denervation is complete. Superinnervated limbs whose normal nerve supply is severed (partially innervated) do respond when the forearm is pricked; totally denervated limbs do not respond. Both partially innervated and totally denervated limbs respond to pin pricking at the base of the hindlimb.

Analysis of the Appearance of Mesenchymatous Cells During the Earliest Stages of Superinnervated and Normally Innervated Regenerates

This experiment is designed to test whether the appearance of mesenchymatous cells can be influenced by increasing the amount of innervation. Using the average number of mesenchymatous-like cells from 5 random longitudinal sections taken from each 36 limbs, a two-way analysis of variance of the data (Table B) shows that there is no significant difference in the number of "blastemal cells" between superinnervated (33.2) and normally innervated limbs (29.4). Thus, superinnervation does not alter the rate at which mesenchymatous cells first appear at the wound area.

Although no difference in the number of blastemal cells exists between the two groups, there is a significant difference in the number of blastemal cells from one day to the next. However, only at 2 days post-amputation is the number of mesenchymatous-like cells different from that at 4 and 6 days post-amputation, which are not different from each other. Possibly, the high number of blastemal cells at 2 days is a reflection of the mass invasion of lymphocytes into the wound area and is not due to the appearance of true mesenchymatous cells. As Thornton ('38) has shown, dedifferentiation does not begin until after 2 days post-amputation. At 4 days post-amputation, the number of mesenchymatous-like cells drops appreciably. This decrease

is probably due to a reduction in the lymphocyte population, followed by a further reduction in lymphocytes at 6 days post-amputation with a concomitant increase in the mesenchymatous cell population.

Since the number of lymphocytes is considered to be a constant for both superinnervated and normally innervated limbs, the number of blastemal cells is taken to be an estimate of the relative number of true mesenchymatous cells between the two groups. Also it should be noted that the sampling technique is designed to provide an estimate of the number of blastemal cells throughout the entire wound area. This does not eliminate the possibility that within a specific region of the limb superinnervation may alter the rate of mesenchymatous cell appearance. Until a method can be devised to differentiate mesenchymatous cells from lymphocytes, the possibility that regional differences occur does exist; however, it appears unlikely.

Analysis of Cellular Activity of Superinnervated and Normally Innervated Regenerates

The analysis of cellular activity, not only between superinnervated and normally innervated blastemata, but also for specific regions of the blastema, is a complicated one. For clarity, the analysis of cellular proliferation will be separated from the analysis of cellular activity. The relationship between cellular proliferation and cellular density will be studied as a separate topic later in

Table B

Two Way Analysis of Variance of Mesenchymatous-Like Cells of Superinnervated and Normally Innervated Limbs from Two to Six Days Post-Amputation

Source	DF	SS	MSS	F	p
Innervation (A)	1	87.111	87.111	1.097	>0.10
Time (B)	2	2031.647	1015.824	12.792	<0.0005
A x B	2	263.615	131.808	1.660	>0.10
Error	30	2382.373	79.412		
Total	35	4764.746			

Days Post-Amputation	2	4	6
$\bar{X}$'s	42.1	28.1	24.7*

* A continuous line beneath means indicates a lack of significance at the 0.05 level between underlined means

Legend: DF - Degrees of Freedom
SS - Sums of Squares
MSS - Mean Sums of Squares
F - F Value
p - Probability

this report. Also since each method of sectioning is designed to present an accurate estimate of cellular activity along only one plane of the blastema, different analyses will be used for each sectioning method.

Cellular Activity along the Transverse Axis of the Blastema

Longitudinal Sections (Series III)

As mentioned earlier, longitudinal sections through the blastema are considered to be the best estimate of cellular activity along its transverse axis of the blastema. Each longitudinal section can be visualized as a plane perpendicular to and intersecting an imaginary transverse plane through the blastema, resulting in a straight longitudinal line at the point of intersection. There are three longitudinal sections from each blastema, each a predetermined distance from the other. Thus, longitudinal sections best describe cellular activity along the center line of the blastema, parallel to and 33% of the limb radius from the center line, and parallel to and 67% the limb radius from the center line.

The results of a three way analysis of variance of mitotic indices between 14 superinnervated and 14 normally innervated blastemata (Table C) show that the mitotic index of superinnervated blastemata is significantly greater than that of normally innervated blastemata. The increased level of mitotic activity of the superinnervated blastemata coupled with the lack of significance between the interaction terms (AxB, AxC, AxBxC) indicates that superinnervation is correlated with a higher mitotic index at 9, 12, and 15 days post-amputation and at all sample

Table C

Three Way Analysis of Variance of Mitotic Indices between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Transverse Axis of Longitudinally Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	18.442	18.442	6.106	0.016
Time (B)	2	11.065	5.532	1.831	0.168
Sites (C)	2	3.248	1.624	0.537	0.587
A x B	2	1.646	.823	0.272	0.762
A x C	2	10.677	5.338	1.767	0.179
B x C	4	10.302	2.575	0.852	0.497
A x B x C	4	15.115	3.778	1.251	0.298
Error	67	202.365	3.020		
Total	84	281.351			

Innervation	Super	Normal
$\bar{X}$'s	2.49	1.48

sites along the transverse axis of the blastema. Thus, the influence of the extra nerves is not restricted to any specific region along the transverse axis of the blastema, but is a constant effect throughout the entire blastema, affecting it uniformly.

Although the amount of innervation alters the level of mitotic activity, there are no significant differences

in mitotic index at either 9 days (1.48), 12 days (2.26), or 15 days post-amputation (2.28), or between sample sites along the transverse axis: at the center line (1.68), at 33% of the limb radius lateral to the center line (1.96), or at 66% of the limb radius lateral to the center line (2.31).

While the mitotic indices of superinnervated blastemata are greater than those of normally innervated blastemata, an analysis of cellular density between the two groups (Table D) indicates that the amount of innervation does not affect the density of mesenchymatous cells within the blastema, nor does the amount of innervation alter the density of cells over time or along the transverse axis of the blastema. Indeed, the density of cells within the blastema is independent of nerves.

The cellular density of both superinnervated and normally innervated blastemata does significantly vary over time and between sample sites along the transverse axis. At 9 days post-amputation, the cellular density is significantly less than at 12 days which is significantly less than at 15 days post-amputation. Along the transverse axis, the cellular density at the medial sample site (at the center line) is not different from that at the paramedial site (33% the limb radius lateral to the centerline), but both the medial and paramedial sites are significantly more dense than the lateral site (66% the limb radius

lateral to the centerline). Thus, within the blastema a dense cylinder of cells occurs in both superinnervated and normally innervated blastemata and is present throughout the entire proliferative stage of regeneration. Indeed, the blastema is not a homogeneous mass of cells during the proliferative stages, but instead exhibits an organization of mesenchymatous cells well before the onset of chondrogenesis.

Transverse Sections (Series IV)

As a rough double check of cellular activity along the transverse axis as ascertained from longitudinally sectioned blastemata, a similar analysis of mitotic indices of 14 superinnervated and 14 normally innervated blastemata sectioned transversely (Table E) shows that the mitotic indices of superinnervated blastemata are also significantly greater than those of normally innervated blastemata. Also like the previous experiment, mitotic indices do not differ from each other along the transverse axis, that is the medial region (1.97) does not differ from that at the paramedial region (2.40), both of which do not differ from the lateral region (1.80). Unlike the analysis of longitudinally sectioned blastemata, mitotic indices are different over time. The mitotic index at 9 days post-amputation is different from that at 12 days, but not different from the mitotic index at 15 days post-amputation.

Table D

Three Way Analysis of Variance of Cellular Densities Between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Transverse Axis of Longitudinally Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	0.137	0.137	00.257	0.614
Time (B)	2	71.303	35.651	66.489	<0.0005
Sites (C)	2	9.687	4.844	9.033	<0.0005
A x B	2	0.783	0.391	0.730	0.486
A x C	2	0.294	0.147	0.274	0.761
B x C	4	3.176	0.794	1.481	0.218
A x B x C	4	2.265	0.566	1.056	0.385
Error	67	35.925	0.536		
Total	84	120.598			

Days Post-Amputation	9	12	15
$\bar{X}$'s	0.90	2.44	3.12
Sample Sites	Medial	Paramedial	Lateral
$\bar{X}$'s	2.33	2.25	1.66

Whereas the change in mitotic indices over time of the transversely sectioned series (Series IV) shows a distinct parabolic trend (index at day 9<12<15), this trend is not observed in the longitudinally sectioned series (Series III) where the index at day 9<12=15. These dif-

Table E

Three Way Analysis of Variance of Mitotic Indices between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Transverse Axis of Transversely Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	24.146	24.146	9.090	0.004
Time (B)	2	33.898	16.949	6.380	0.003
Sites (C)	2	5.092	2.546	0.958	0.389
A x B	2	3.600	1.800	0.678	0.511
A x C	2	5.811	2.905	1.094	0.341
B x C	4	7.507	1.876	0.707	0.590
A x B x C	4	4.230	1.058	0.398	0.809
Error	66	175.324	2.656		
Total	83	258.963			

Innervation	Super	Normal	
$\bar{X}$'s	2.59	1.52	
Days Post-Amputation	9	15	12
$\bar{X}$'s	1.45	1.70	2.90

ferences tend to indicate that in Series III the growth phase of regeneration may be slightly longer than in Series IV. Two primary differences in technique may account for the differences in mitotic indices over time. (1) Since Series III was fixed in April whereas the limbs of Series

IV were fixed in August, differences in the hours of daylight and the time of the year may have affected the mitotic cycle. Interestingly, the series fixed in April appears to have a longer growth phase of regeneration than the one fixed in August. (2) Since larvae of Series III were injected with 4 microcuries of thymidine whereas those of Series IV were injected with 12 microcuries of thymidine, possibly the growth rate of Series IV may have been accelerated. Greulich, Cameron, and Thrasher ('61) and Barr ('63) show a direct correlation exists between mitotic activity and the amount of exogenous thymidine in mouse duodenal epithelium and HeLa cells, respectively. However in this report, differences in mitotic indices of urodele regenerates over time between the two different series do not alter the fact that within each series superinnervated blastemata have a greater mitotic index than do normally innervated blastemata.

Analysis of cellular density along the transverse axis of transversely sectioned blastemata (Table F) shows that like cellular density along the transverse axis of longitudinally sectioned blastemata, differences occur over time and along the transverse axis, but not between superinnervated and normally innervated blastemata. The cellular density at 9 days post-amputation differs from that at 15 days which in turn differs from that at 12 days post-amputation.

Table F

Three Way Analysis of Variance of Cellular Densities between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Transverse Axis of Transversely Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	1.902	1.902	3.171	0.080
Time (B)	2	89.048	44.524	74.236	<0.0005
Sites (C)	2	7.532	3.766	6.279	0.003
A x B	2	6.020	3.010	5.184	0.009
A x C	2	0.937	0.469	0.781	0.462
B x C	4	4.017	1.004	1.674	0.166
A x B x C	4	0.836	0.209	0.348	0.844
Error	66	39.584	0.600		
Total	83	151.690			

Days Post-Amputation	9	15	12
$\bar{X}$'s	1.24	3.23	3.71
Sample Sites	Lateral	Medial	Paramedial
$\bar{X}$'s	2.38	3.04	3.08

Along the transverse axis the cellular density of the medial site does not differ from that of the paramedial site, but both of the more medial sites have a higher cellular density than the lateral site. Although transverse sections are not considered to be the best estimate of

cellular activity along the transverse axis, transverse sections do substantiate the idea that a dense cylinder of cells exists within the blastema throughout the proliferative stage, irrespective of the amount of innervation.

Cellular Activity along the Longitudinal Axis of the Blastema

Transverse Sections (Series IV)

As previously stated, transverse sections are considered to be the best estimate of cellular activity along the longitudinal axis of the blastema. Each transverse section can be visualized as a plane perpendicular to and intersecting an imaginary longitudinal plane through the blastema, resulting in a straight transverse line at the point of intersection. Since there are four transverse sections for each blastema in the sample, each a predetermined distance from the other, transverse sections best describe cellular activity: i.e., immediately beneath the apical wound epithelium, 75% the total length of the blastema from the base of the blastema, 50% the length of the blastema, and 25% the length of the blastema.

Consistent with the other sampling methods, an analysis of mitotic indices shows that the mitotic indices of superinnervated blastemata are significantly greater than those of normally innervated blastemata (Table G).

Table G

Three Way Analysis of Variance of Mitotic Indices between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Longitudinal Axis of Transversely Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	18.363	18.442	8.559	0.005
Time (B)	2	35.096	17.548	8.168	0.001
Sites (C)	3	31.896	10.632	4.949	0.003
A x B	2	3.052	1.526	0.710	0.494
A x C	3	13.953	4.651	2.165	0.098
B x C	6	15.047	2.508	1.167	0.332
A x B x C	6	10.765	1.794	0.835	0.542
Error	83	178.309	2.148		
Total	106	306.481			

Innervation	Super	Normal
$\bar{X}$'s	2.36	1.63

Days Post-Amputation	9	15	12
$\bar{X}$'s	1.40	1.64	2.78

Sample Sites (% length of blastema)	100	50	75	25
$\bar{X}$'s	0.97	2.50	2.20	2.13

Furthermore, the increased level of mitotic activity of superinnervated blastemata is maintained over time and along the longitudinal axis. Also consistent with the mitotic indices of transversely sectioned blastemata which are used as a double check of cellular activity along the transverse axis, the mitotic index at 9 days post-amputation is similar to that at 15 days, both of which are different from the mitotic index at 12 days post-amputation.

Along the transverse axis of the blastema, the mitotic index at the sample site 20u proximal to the apical wound epithelium is significantly less than at 75% the length of the blastema, 50%, and at 25% the length of the blastema. However, the mitotic indices at 75%, 50%, and 25% the length of the blastema are not significantly different from each other. This pattern is found in both superinnervated and normally innervated blastemata and over time, albeit the mitotic activity of superinnervated blastemata is generally greater along the longitudinal axis.

As in the other sampling methods, there is no difference in cellular density between superinnervated and normally innervated blastemata; however, cellular density does change over time (Table H). Unlike differences in cellular density along the transverse axis, there are no differences in cellular density along the longitudinal axis.

Only the three proximal sample sites were used in

Table H

Three Way Analysis of Variance of Cellular Densities between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Longitudinal Axis of Transversely Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	0.480	0.480	0.759	0.387
Time (B)	2	79.087	39.544	62.536	<0.0005
Sites (C)	2	2.728	1.364	2.157	0.124
A x B	2	3.647	1.824	2.884	0.063
A x C	2	0.562	0.281	0.445	0.643
B x C	4	2.208	0.552	0.873	0.483
A x B x C	4	1.818	0.455	0.719	0.582
Error	64	40.470	0.632		
Total	81	131.730			

Days Post-Amputation	9	15	12
$\bar{X}$'s	1.49	3.54	3.84

assessing cellular density along the longitudinal axis. Since the site at 20u proximal to the apical wound epithelium is not large enough to make accurate density determinations, this site is not included; however, rough estimates of cellular density at the distal most region indicate that it does not differ from more proximal regions.

Longitudinal Sections (Series III)

Due to the fact that no blastema is longer than 480u at 9 days post-amputation and due to the restrictions placed on the selection of sample sites (see Materials and Methods), no counts were made in the distal-most sample site. Therefore, a three way analysis of variance was not used. Instead, a series of one way analyses of variance was used to assess cellular activity. Since counts taken from longitudinally sectioned blastemata are not considered to be the best estimate for cellular activity along the longitudinal axis but as a rough double check of transversely sectioned blastemata, the alterations in the analysis were not deemed critical.

As with the analysis of mitotic indices from transversely sectioned blastemata, the mitotic indices of superinnervated blastemata are greater than that of normally innervated blastemata (Table I). Unlike the transversely sectioned series, there are no differences between days post-amputation (Table J), although the mitotic index at 9 days post-amputation (1.31) tends to be less than at 12 days (2.18) or at 15 days post-amputation (2.53).

Mitotic indices along the longitudinal axis (Table K) were not significantly different between the distal 1/3 of the blastema (2.17), the middle 1/3 (1.98), and the proximal 1/3 of the blastema (1.98). Although an examination of just the means suggests a distal-proximal gradient

Table I

One Way Analysis of Variance of Mitotic Indices between Superinnervated and Normally Innervated Blastemata Sectioned along the Longitudinal Axis

Source	DF	SS	MSS	F	p
Innervation	1	7.562	7.562	4.187	0.045
Error	67	121.007	1.806		
Total	68	128.567			

Innervation	Super	Normal
$\bar{X}$'s	2.35	1.69

Table J

One Way Analysis of Variance of Mitotic Indices between Days Post-Amputation of Longitudinally Sectioned Superinnervated and Normally Innervated Blastemata

Source	DF	SS	MSS	F	p
Time	2	9.172	4.586	2.535	0.087
Error	66	119.397	1.809		
Total	68	128.569			

Table K

One Way Analysis of Variance of Mitotic Indices between Sample Sites along the Longitudinal Axis of Longitudinally Sectioned Superinnervated and Normally Innervated Blastemata

Source	DF	SS	MSS	F	p
Sites	2	0.438	0.219	0.113	0.893
Error	66	128.131	1.941		
Total	68	128.569			

of mitotic indices, the trend is not verified by the analysis.

Comparing mitotic indices along the longitudinal axis of transversely sectioned blastemata to those of longitudinally sectioned blastemata, the mitotic indices of the two sampling methods are in accord, for there are no differences for either technique along the longitudinal axis for comparable sample sites: the upper 1/3 site being comparable to the site at 75% the length of the blastema of transversely sectioned blastemata, the middle 1/3 site being comparable to the site at 50% the length of the blastema, and the proximal 1/3 site being comparable to the site at 25% the blastemal length. There is no site in the longitudinally sectioned series comparable to the site 20u proximal to the apical wound epithelium of the transversely sectioned series.

As with the other sampling techniques, superinnervation does not alter the density of mesenchymatous cells within the blastema (Table L). Also the cellular density significantly increases with time in both groups (Table M). Although there are no significant differences in cellular density along the longitudinal axis (Table N), mesenchymatous cells tend to be more dense distally than proximally. Indeed, the analysis of cellular density using longitudinally sectioned blastemata is consistent with that using transversely sectioned blastemata.

An Analysis of Mitotic Index vs Cellular Density

In order to determine whether the frequency of mitosis is related to the substructure of mesenchymatous cell masses within the blastema, a regression analysis was used to correlate mitotic index with cellular density at each sample site. The analysis reveals that there is no correlation between mitotic index and cellular density, be the blastema either superinnervated or normally innervated (Figure 11). Even though the number of mitotic figures may be greater in denser masses of cells, the mitotic index is generally the same as in less dense areas. Indeed, changes in mitotic indices are a function of the amount of innervation, not of the density of the cellular masses.

Table L

One Way Analysis of Variance of Cellular Densities between Superinnervated and Normally Innervated Blastemata Sectioned along the Longitudinal Axis

Source	DF	SS	MSS	F	p
Innervation	1	0.542	0.542	0.466	0.497
Error	67	77.884	1.162		
Total	68	78.426			

Table M

One Way Analysis of Variance of Cellular Densities between Days Post-Amputation of Longitudinally Sectioned Superinnervated and Normally Innervated Blastemata

Source					
Time	2	46.110	23.055	47.087	<0.0005
Error	66	32.315	0.490		
Total	68	78.426			

Days Post-Amputation	9	12	15
$\bar{X}$'s	0.99	2.48	3.29

Table N

One Way Analysis of Variance of Cellular Densities between Sample Sites along the Longitudinal Axis of Longitudinally Sectioned Superinnervated and Normally Innervated Blastemata

Source	DF	SS	MSS	F	p
Sites	2	4.456	2.228	1.988	0.145
Error	66	73.969	1.121		
Total	68	78.426			

Incorporation of Thymidine Methyl-H^3

Along the Longitudinal Axis

This study was designed to determine whether thymidine incorporation is proportionately different between superinnervated and normally innervated blastemata, between specific regions of the blastema, and over time of transversely sectioned blastemata (Series IV). Focusing on thymidine incorporation along the longitudinal axis, an analysis of the percent labelled nuclei shows that there is no significant difference between superinnervated and normally innervated blastemata or over time, but there are differences between sample sites along the longitudinal axis (Table O). Indeed, a gradient of thymidine incorporation exists along the longitudinal axis with a lower percentage of the cells being labelled distally than proximally, irrespective of the amount of innervation.

Table 0

Three Way Analysis of Variance of Percent Labelled Nuclei between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Longitudinal Axis of Transversely Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	35.444	35.444	0.928	0.347
Time (B)	1	51.772	51.772	1.356	0.258
Sites (C)	3	791.113	263.704	6.907	0.002
A x B	1	181.576	181.576	4.756	0.041
A x C	3	153.099	51.033	1.337	0.291
B x C	3	285.551	95.184	2.493	0.089
A x B x C	3	120.823	40.274	1.0548	0.390
Error	20	763.609	38.180		
Total	35	2831.829			

Sites (% length of blastema)	100	75	50	25
$\bar{X}$'s	2.91	10.43	12.66	18.40

Comparing the proportion of cells incorporating thymidine to mitotic indices and cellular densities along the longitudinal axis reveals an interesting relationship. Whereas a proportionately greater number of cells are labelled proximally than distally, a greater density of cells exists distally than proximally. Indeed, an inverse

relationship exists between the propensity of cells to incorporate thymidine and the density of cells. Interestingly, however, is the fact that no such relationship exists between mitotic index and thymidine incorporation, except at 20u proximal to the apical wound epithelium where the mitotic index and thymidine incorporation are significantly less than at more proximal levels.

Along the Transverse Axis

Although transverse sections are not considered to be the best estimate of cellular activity along the transverse axis, the data collected from transversely sectioned blastemata correlates with mitotic indices and cellular densities calculated from longitudinal sections. Therefore, the use of transverse sections is considered to be a good, although not optimum, method for studying cellular activity along the transverse axis.

An analysis of the percent labelled nuclei shows that there are differences over time and between sample sites along the transverse axis, but none between superinnervated and normally innervated blastemata (Table P). At 12 days post-amputation a greater percentage of the cells incorporate thymidine than an 15 days.

As along the longitudinal axis of the blastema, there is a greater propensity for cells to be labelled in less dense regions along the transverse axis. The incor-

Table P

Three Way Analysis of Variance of Percent Labelled Nuclei between Superinnervated and Normally Innervated Blastemata, between Days Post-Amputation, and between Sample Sites along the Transverse Axis of Transversely Sectioned Blastemata

Source	DF	SS	MSS	F	p
Innervation (A)	1	104.027	104.027	2.224	0.154
Time (B)	1	277.566	277.566	5.934	0.026
Sites (C)	2	358.835	179.418	3.836	0.042
A x B	1	388.713	388.713	8.310	0.010
A x C	2	48.463	24.232	0.518	0.605
B x C	2	40.172	20.086	0.429	0.658
A x B x C	2	70.633	35.316	0.755	0.485
Error	17	795.183	46.775		
Total	28	2630.289			

Days Post-Amputation	12	15
$\bar{X}$'s	18.54	10.74

Sites	Medial	Paramedial	Lateral
$\bar{X}$'s	11.51	14.03	20.95

poration of label is proportionately greater at the lateral (less dense) sample sites than at the more medial ones, irrespective of the amount of innervation. Indeed, a lateral-medial gradient of labelled cells exists along the transverse axis.

Relationships between Mitotic Indices and Percent Labelled Cells

To determine the relative stage of blastemal growth between superinnervated and normally innervated blastemata at 12 and 15 days post-amputation, ratios (S/M)[3] were calculated by dividing the mitotic index into the percent labelled cells for each labelled blastema. Analyses of variance between the S/M ratios of superinnervated and normally innervated blastemata show that the S/M ratios of superinnervated blastemata are significantly lower than those of normally innervated blastemata, for samples taken along both the transverse axis (Table Q) and along the longitudinal axis (Table R) of transversely sectioned blastemata. Since the smaller the S/M ratio gets, the closer the cellular population is to the end of the growth phase (Fabrikant, '67; Goss, '67) the cellular populations of superinnervated blastemata are more advanced in development than those of normally innervated blastemata. No differences exist between S/M ratios at 12 and 15 days for samples along the transverse axis (Table S) and the longitudinal axis (Table T).

3. Since the duration of both DNA synthesis (S phase) and the mitotic phase (M phase) of the cell cycle are not known for mesenchymatous cells of the blastema, the S/M ratios used in this study show only the relative differences in the stage of growth between groups and do not demonstrate whether the cellular populations are before, at, or after the logarithmic growth stage.

Table Q

One Way Analysis of Variance of S/M Ratios between Super-innervated and Normally Innervated Blastemata Sampled along the Transverse Axis

Source	DF	SS	MSS	F	p
Among	1	8.849	8.849	6.454	<.05
Within	8	10.973	1.371		
Total	9	19.822			

Innervation	Super	Normal
$\bar{X}$'s	1.40	3.32

Table R

One Way Analysis of Variance of S/M Ratios between Super-innervated and Normally Innervated Blastemata Sampled along the Longitudinal Axis

Source	DF	SS	MSS	F	p
Among	1	2.848	2.848	9.307	<.025
Within	8	2.449	0.306		
Total	9	5.297			

Innervation	Super	Normal
$\bar{X}$'s	1.08	2.17

Table S

One Way Analysis of Variance of S/M Ratios between Days Post-Amputation of Superinnervated and Normally Innervated Blastemata Sampled along the Transverse Axis

Source	DF	SS	MSS	F	p
Among	1	1.345	1.345	0.582	>>.25
Within	8	18.477	2.309		
Total	9	19.822			

Table T

One Way Analysis of Variance of S/M Ratios between Days Post-Amputation of Superinnervated and Normally Innervated Blastemata Sampled along the Longitudinal Axis

Source	DF	SS	MSS	F	p
Among	1	1.512	1.512	3.180	>.10
Within	8	3.814	0.476		
Total	9	5.326			

DISCUSSION

Discussion of Results

In the past the basic technique used to determine the influence of nerves on urodele limb regeneration has been that of total or partial limb denervation. In an exhaustive series of experiments, Singer (46a, 46b, 47a, and 47b) showed that a minimal number of nerve fibers is required before regeneration commences. Yet the results of these experiments lead one to question whether the use of denervation is the best method for determining the quantitative relationships between the amount of innervation and the rate of blastemal growth. The number of nerve fibers is not a constant but is highly variable from limb to limb. Also limbs with comparable subnormal nerve supplies do not necessarily begin to regenerate at the same time. Furthermore, excessive branching of regenerating nerve fibers makes the estimation of the number of nerve fibers based on the number of parent axons difficult. These variables suggest the difficulty in standardizing the amount of innervation needed to support regeneration.

As a result of denervation's inherent variables, the question of whether nerves have a quantitative graded effect on the rate of regeneration is much confused.

Weiss ('25) and Schotte ('26) felt that the nerves do have a graded influence on the regeneration rate. Using partially denervated forelimbs of the adult Triton, they reported that the greater the degree of denervation, the slower the regeneration rate and the rate of limb morphogenesis. While Singer and Egloff ('49) reported that partially denervated newt limbs regenerate at a slower rate, they proposed that no correlation exists between the degree of denervation and the regeneration rate.

Singer and Egloff's ('49) negation of the graded response of regeneration rate to nerves was based on extrapolations of nerve fiber number from previous partial denervation experiments (Singer, '46b). Only the average fiber number was used for each group. Indeed, for different states of partial denervation, not only does the nerve fiber number at the amputation surface vary greatly from limb to limb, but also differences in regeneration rates are highly variable. Thus, correlations between nerve fiber number and regeneration rates are difficult to interpret, particularly when the disparity between the average fiber number is small between the different groups. It is interesting that those limbs with the highest average nerve number regenerate the fastest while those limbs with the lowest average nerve fiber number regenerate the slowest. Possibly, correlations between nerve fiber number and regeneration rates are more meaningful at the extreme ends of the partial denervation spectrum.

In the partial denervation experiments, the growth rates were determined by comparing either the morphological stage or the size (length and volume) of late stage regenerates. No accurate measurements were made of changes in blastemal size prior to limb morphogenesis. Thus differences in the size or stage of late stage regenerates may have been due to an actual difference in growth rates and/or to differences in the onset of regeneration with limbs actually having the same growth rate. If growth rate differences do occur, comparisons of only late stage regenerates would fail to reveal at what period during regeneration the differences first occur.

Superinnervation as a technique is designed to circumvent some of the problems arising from denervation. Whereas the possibility exists that partially denervated limbs either will not regenerate or will commence regeneration later than normally innervated limbs, these variables play no role in the study of superinnervated limbs. The problem of nerve branching during regeneration is a factor indigenous to both superinnervated and partially denervated limbs. However, in the superinnervated state, nerve branching does not influence the regenerative ability of the limb.

In this report, the augmentation of the normal nerve supply results in the acceleration of regeneration. However as changes in blastemal size illustrate, super-

innervated blastemata do not constantly have greater growth rates than normally innervated blastemata. The only difference in growth rates occurs during those stages associated with rapid cellular proliferation. After the proliferative stage, superinnervated regenerates continue to be advanced in development, although their growth rate is the same as that of normally innervated regenerates. It can not be stated catagorically that a direct correlation exists between the amount of innervation and the regeneration rate, but there seems little doubt that superinnervation and partial denervation increase and decrease regeneration rates, respectively.

Konieczna-Marczynska and Skowron-Cendrzak ('58) reported that nerve augmentation of the postmetamorphic Xenopus laevis hindlimb results in a reduced rate of regeneration. Their findings are in direct contradiction to those of this study. Although they stated that superinnervated limbs regenerate at a slower rate than normally innervated limbs, no references were made to numbers, sizes, or stages of the regenerates over time. On the other hand, they reported that final limb morphogenesis of superinnervated limbs was more advanced than that of limbs with lesser innervation. Again pertinent data were omitted. Whether variation in regeneration rates and limb morphogenesis is due to the treatment or to normal variations in growth can not be determined from the data. Indeed,

the highly variable nature of Xenopus regeneration reduces the accuracy of quantitative comparisons.

As mentioned previously, the influence of the nerves is most pronounced during the stages of rapid cellular proliferation. Indeed the results of this report demonstrate that superinnervation is correlated with an increased rate of cellular proliferation. Other studies also correlate the rate of proliferation with the level of innervation. Singer and Craven ('48) observed that in the newt total denervation results in a reduction of mitotic activity. Overton ('50) showed that explanted spinal cord segments can increase mitosis in Xenopus epidermis.

Singer, Ray, and Peadon ('64) inferred from dye displacement studies within the newt blastema that blastemal growth is greater in those blastemal regions adjacent to areas of high innervation. However in the axolotl, no relationship is found between innervation and growth rates for specific blastemal regions. In the axolotl, the greater mitotic activity of superinnervated blastemata is not restricted to areas of high innervation, but is due to a general increase in mitotic activity throughout the blastema. Although there is a tendency towards greater mitotic activity in the peripheral regions of the regenerate, the regions of high innervation do not have mitotic indices significantly different from those of low innervation. Hearson ('66) also found no correlation between

mitotic activity and regions of high innervation. Thus the movement of dye in the peripheral-ventral regions of the newt blastema, may be due to a movement of cells in that region, not to increased cellular proliferation.

Recent observations have tended to dispel the notion that the blastema is a homogeneous mass of cells during the stages of cellular proliferation. Faber ('60) found that carbon particles inserted into the mesenchyme at or near the tip of the mound blastema generally tend to remain the same distance from the amputation surface, but that some of the particles are displaced distally. Faber attributed the relative displacement of carbon markings to an "apical proliferation centre", that is, in the early stages, growth is considerably more pronounced in the distal regions of the blastema than in the proximal regions. He went on to say that "the greater density of distal mesenchyme is a consequence of apical proliferation".

Using longitudinal sections to determine mitotic indices along the longitudinal axis, Hearson ('66) corroborated the existence of Faber's apical proliferation centre. He found that a gradient of mitosis exists in the axolotl blastema, the distal most region having a significantly greater mitotic index than the more proximal regions. The greatest disparity between distal and proximal regions occurs at 10 days post-amputation. The gradient disappears by 19 days post-amputation, the beginning of digit formation.

On the other hand, Litwiller ('39) showed that the base of the blastema has a higher mitotic index at the early cone stage, but at later stages mitotic rates are predominantly greater in the distal two-thirds of the blastema. The distal tip has a relatively low value, albeit his results are vague. Using transverse sections to determine mitotic activity along the longitudinal axis of the adult newt blastema, Chalkley ('54) did not find a distal-proximal gradient of mitotic indices. In fact, the mitotic index of the distal most section sampled is the lowest of all the blastemal sections. However, Chalkley ('54) was not clear as to whether the distal most section is immediately beneath the apical wound epithelium or up to 160u proximal from it. Nonetheless his work corroborated that of Litwiller ('39).

Seemingly contradictory reports on mitotic regionalization within the regeneration blastema are probably due to the varying methods of sampling the blastema. Before any sampling scheme can be undertaken, several variables must be taken into consideration: (1) variations in mitotic indices; (2) differences in time; and (3) proper selection of a sampling method. To overcome the variability in mitosis, a number of blastemata must be used to get a representative average. Likewise over time, a number of different blastemata should be used at each point in time. Also a sampling method must be conceived which actually samples those regions of the blastema from which

conclusions are drawn. Finally in order to unbiasly determine whether differences do exist, the appropriate statistical analysis must be used.

By the above criteria, each of the previous experiments has been found wanting. In Faber's ('60) work no attempt was made to describe the mitotic patterns within the blastema or to quantitize either the regionalization or density of his carbon markings. Both Litwiller ('39) and Chalkley ('54) used transverse sections to determine mitotic indices along the longitudinal axis, considered to be the most accurate method, but neither worker accounted for variations in mitotic indices between animals and sample sites. Thus whether differences truly exist is not known.

Although Hearson considered variability of mitotic indices, the results are based on longitudinal sections. His sampling technique has several drawbacks in determining mitotic indices along the longitudinal axis. (1) Since the grid used for sampling was superimposed along the longitudinal axis of the blastema, each sample incorporated a number of different longitudinal levels. Thus each sample site was not indicative of a specific level along the longitudinal axis. At best his sampling method yielded gross approximations of mitotic indices over large portions of the longitudinal axis. (2) Due to the irregular shape of the blastema, certain alterations were made

in sampling which vary from blastema to blastema, namely that if the grid was larger than the sample site, additional counts were taken from another section. This was especially true of small blastemata. Thus some counts were made near the apical wound epithelium while others included cells as far as 160u proximal to the apical wound epithelium. (3) The distal most and proximal most sample sites were situated along the center line of the section while the intermediate sample sites were located at the periphery of the section. The counts were in reality estimates of mitotic indices not along the center line of the blastema, but skewed towards the periphery at the middle levels of the blastema. No correction was made for this skewness in sample sites transversely across the section in the analysis. If a distal-proximal gradient of mitotic indices exists along the longitudinal axis, particularly along the center line of the blastema, the sampling method was not designed to test for it.

The results of this report demonstrate that no regionalization of mitoses occurs within the blastema from 9 to 15 days post-amputation. The one exception is immediately proximal to the apical epithelium where the mitotic index is significantly lower than at any other region within the blastema. This pattern of mitotic activity is fairly constant in both superinnervated and normally innervated blastemata. The low mitotic index at the distal tip of the

axolotl blastema is consistent with the observations of Chalkley ('54) in the newt.

Although casual reference has been made to the arrangement of mesenchymatous cells within the blastema, no attempt has been made to assess the regionalization of mesenchymatous cells within the blastema during the proliferative stages. Horn ('42) showed that over time the cellular density of the Ambystoma larval blastema continues to increase but made no attempt to ascertain density changes within different regions of the blastema. Chalkley ('54) counted the total number of mesenchymatous cells per section, but made no reference to cellular density. Litwiller ('29) pointed out that the distal half of the blastema is more dense than the proximal half and that cellular density tends to increase with time. However, differences in the size of animals and the use of only one animal at each time period, makes a meaningful conclusion questionable.

Usually the density of mesenchymatous cells is thought to be homogeneous during the early stages of rapid proliferation, followed by an increase in density at the distal half. Prior to chondrogenesis axial condensation appears which subsequently develops into cartilagenous structures. Faber ('60) felt that the increase in cellular density in the distal regions is due to the distal "proliferation centre". Essentially, the cellular substructure

of the blastema is thought to arise in lieu of chondrogenesis and/or as a de facto result of cellular proliferation.

The results of this report clearly show that a definite substructure exists within the blastema during the entire proliferative stage. From 9 to 15 days post-amputation sampling along the transverse axis shows that the medial 2/3 of the blastema is significantly more dense than at the periphery. The amount of innervation has no influence on either the degree of cellular density or the configuration of the cellular substructure. As the blastema continues to grow, the overall density increases, but the increases are proportional for all regions of the blastema. Thus even during the earliest stages of cellular proliferation, the blastema is structured. The axial condensations of later stage blastemata do not appear just prior to chondrogenesis, but are probably the result of the intensification of mesenchymatous cells around a preexisting template.

Horn ('42), Litwiller ('39) and Chalkley ('54) reported that a temporal relationship exists between mitotic activity and the number of mesenchymatous cells in the blastema. The mitotic index is greatest during the "cone" stage but then decreases as limb morphogenesis begins. The cellular density of the blastema continues to gradually increase throughout the proliferative stages in a more or less linear pattern. Thus for the entire blastema, mitosis leads to an increase in cell number and cellular density.

Faber ('60) and Hearson ('66) stated that there also exists a spatial correlation between the density of mesenchymatous cells and mitotic activity. They inferred that since certain regions of the blastema, namely the distal portion, appear to be more dense than other areas, mitotic activity must ipso facto be greater. However, neither worker demonstrated that in fact cellular density is correlated with mitotic activity.

In the present study, no correlation was found between the density of mesenchymatous cells and mitotic index at the various sample sites throughout the blastema. Thus mitosis is not influenced by the density of the surrounding cells, at least not prior to chondrogenesis. The only variable which influences the rate of mitosis is the amount of innervation. The spatial arrangement of the blastemal substructure then is not a direct function of mitotic activity. More will be said about this point shortly.

Although in the axolotl mitosis is not a function of cellular density, the incorporation of thymidine methyl-H^3 by mesenchymatous cells does correlate with the cellular density. In the blastema a greater percentage of mesenchymatous cells incorporated thymidine in areas of low cellular density, e.i. in the proximal and lateral regions of the blastema. Conversely, thymidine incorporation was less frequent in the distal and medial regions. This corroborates the observations of thymidine incorporation of Hay

and Fischman ('61). Although they did not correlate their data to cellular density, they reported that there is a greater propensity for labelled mesenchymatous cells in the proximal regions of the blastema. No statement was made regarding thymidine incorporation along the transverse axis.

To state that an unqualified correlation exists between thymidine incorporation and cellular density would be erroneous. A prime consideration in any labelling experiment is the availability of the label to those cells incorporating the label. Peadon and Singer ('66) showed that the proximal regions of the blastema are more vascularized than the distal regions. Since the label is transported to the blastema via the blood stream, this factor must be considered. Also since the less dense areas are labelled proportionately more, cells in these regions may have easier access to the label (see Feinendegen, '67, for review).

The rate of cellular proliferation has yet to be established for regenerating limbs. Hay and Fischman ('61) observed that in the new blastema labelled mitoses first appear on one day post-injection and a major dilution of the label is evident by three days post-injection. They estimated that the full mitotic cycle takes about three days. In the proximal regions of the regenerate, they noted that heavily labelled cells are still present four to five days post-injection and infer that proliferation

may be more rapid distally. However, until accurate techniques can be devised to measure the duration of the mesenchymatous cell cycle and the possible distal movement of labelled cells from the stump to the blastema, their results remain purely inferential.

The results of this study show that in the axolotl no labelled mitoses are found six hours post-injection, suggesting that the G_2 phase of mitosis is at least that long. Since all limbs are fixed six hours post-injection, no label dilution effects are reported.

Although mitotic indices of superinnervated blastemata are significantly greater than those of normally innervated blastemata, both groups have the same percentage of labelled cells at 12 and 15 days post-amputation. Since, however, mitotic indices and percent labelled cells are not recorded over the entire regeneration period, but are limited to the phase of very active growth, mitotic indices may differ from the percent labelled cells at a given time during growth. For example, during the early stages of growth, proportionately more cells synthesize DNA than divide. As growth progresses, the relationship changes, so that as the population of cells nears maturation or the end of the growth phase, proportionately more mitotic cells than cells synthesizing DNA are found.

In this study, one can not determine exactly how close a given population of cells is to maturation, since

the duration of DNA synthesis and the mitotic phase are not known. However, comparisons can be made between cellular populations of superinnervated and normally innervated blastemata. The cellular populations of superinnervated blastemata are significantly closer to the end of the growth phase than those of normally innervated blastemata as determined by S/M ratios. The evidence that the cellular populations of superinnervated blastemata are more advanced, is corroborated by their advanced blastemal development during the proliferative stages and also during limb morphogenesis.

The results of this study demonstrate that the effect of increasing the nerve supply above the normal level of innervation causes an increase in the rate of regeneration. This increased rate is not due to an increase in the appearance of mesenchymatous cells in the blastemata, but instead to an acceleration of mitotic activity within the blastema. Whether duration of the entire cell cycle is decreased by nerve augmentation, or specific phases of the cycles is not known.

Theoretical Implications

Blastemal Morphogenesis

Many studies have dealt with the origin of blastemal cells. Thornton ('38), Manner ('58), Chalkley ('54), Hay and Fischman ('61), and Steen ('68) have shown that the cells which comprise the blastema are derived from the dedifferen-

tiation of previously differentiated tissues of the amputated limb stump. The process of dedifferentiation is completed approximately one week after amputation in the larva and about two weeks in the newt. Chalkley ('54) calculated that dedifferentiated cells are continuously being added to the blastemal cell population up to the three digit stage, but that most cells enter the blastema early in its development.

To date the most widely held hypothesis on the behaviour of dedifferentiated cells within the blastema itself is that of Thornton ('57), Faber ('60), Thornton and Steen ('62) and Hearson ('66). They felt that the apical wound epithelium, particularly the apical cap, mediates the accumulation and proliferation of mesenchymatous cells in the blastema. Their conclusions were based mainly on the correlation between the appearance of the apical cap and the accumulative and proliferative stages of regeneration and on the carbon marking experiments of Faber mentioned earlier. However, the correlation of two events need not imply a cause and effect relationship, and the displacement of carbon particles within the blastema may be explained in another way. As reported on Page 64, the existence of an apical proliferation centre is not verified by the results of this study or by the experiments of other workers. What then are the morphogenetic events within the blastema which influence the morphology of the blastema and the displacement of carbon particles?

Figure 12 is a diagrammatic model based on the results of Chalkley ('54), Hay and Fischman ('61), and of this report which may possibly serve to better explain the initial accumulation and growth of the blastema.

Stage I: The first group (Group I of mesenchymatous cells appears in the limb stump.

Stage II: A second group (II) of mesenchymatous cells appears in the stump due to the dedifferentiation of stump tissues. Concomitant with the appearance of Group II cells, Group I cells start to undergo mitosis in the stump.

Stage III: Cells from Groups I and II continue to undergo mitosis while additional dedifferentiated cells appear in the limb stump (Group III). The cells from Group I have now entered the blastema and are beneath the wound epithelium.

Stage IV: The dedifferentiation process has now ceased or decreased in intensity. Since the mitotic rate is the same throughout the blastema, cells from all groups undergo additional mitoses. The cells from Group I are displaced distally by the influx of cells from

the stump and the proliferation of more proximal cells.

Stage V: Essentially most of the dedifferentiated stump tissues have entered the blastema. Proliferation continues at the same rate throughout the blastema but is now restricted to cells within the blastema. The pattern established in the earlier stages is amplified with the continued distal displacement of Group I cells.

Although the model portrays waves of cellular activity, the process of mesenchymatous cell migration and proliferation should be visualized as a continuous process. For simplification of the model, absolute numbers of dedifferentiating and proliferating cells are not represented. Nonetheless, the basic pattern of cellular dedifferentiation, proliferation, and distal displacement are proposed to be that of the model.

As this study shows, the mitotic index is relatively uniform throughout the blastema, albeit the mitotic index immediately beneath the apical wound epithelium is low while the lateral regions of the blastema tend to have a higher mitotic index. The distal portion of the blastema contributes proportionately more blastemal cells, as the above model proposes, not because their mitotic rates are

faster, but because they have been present in the blastema the longest. All cells in the distal regions may not be members of the first cellular group, for surely some mixing of cells takes place. If carbon particles were to be inserted into the blastema after the major influx of dedifferentiated cells into the blastema has subsided, then the majority of the particles would be expected to remain relatively stationary, although some particles would be displaced distally. Indeed, Faber's ('60) carbon marking experiments support this contention, for Chalkley ('54) and Hay and Fischman ('61) showed that the major influx of dedifferentiated cells into the blastema occurs during or before the stage at which Faber placed carbon particles into the blastema. If no "proliferation centre" exists, but instead growth results from a uniform displacement of cells, then carbon particles inserted into later stage blastemata (cone) would not be expected to be displaced as much. Faber's experiments also support this contention.

Interestingly, the propensity to incorporate tritiated thymidine is greater in the lateral regions of the blastema, and, conversely, the density of mesenchymatous cells in the axial regions of the blastema is greater than that of lateral regions. Assuming that the label is equally available to all cells, some medial displacement of cells must occur, for cellular division is equally likely throughout the blastema. Assuming that the data on thymidine

incorporation are artifacts and that all cells are not as likely to incorporate the label, why does a dense core of cells exist in the blastema from the earliest stages of blastemal formation? Under both assumptions, there must be some reason why cells tend to aggregate in the axial regions of the blastema.

Hypothetically, if one assumes that the wound epithelium serves to confine the blastemal cells and that the outward pressure exerted on the wound epithelium by the growing blastema is equal at all points and that the wound epithelium resists this internal outward pressure equally, then one should expect that the blastema would be bulbous in shape throughout its growth, even if tissue dedifferentiation is more prevalent in the medial regions of the stump (Figure 13A). But this is not the case, for the blastema forms a mound which later develops into a cone shape. Why then does the blastema form a cone, better described as a paraboloid, instead of a bulb?

Thornton ('60) showed that the direction of blastemal growth is correlated with the position of the apical cap. If the cap is shifted to an eccentric position, the blastema develops eccentrically. Nerves play no role in this process (Thornton and Steen, '62). Why then does the growth of the blastema follow the apical cap?

Thornton ('65) suggested that the apical cap may be involved in the accumulation of mesenchymatous cells beneath

it and via an epithelial-mesenchymal interaction may assert some control on limb morphogenesis. However, the apical wound epithelium may influence the direction of blastemal growth in a much more passive manner.

Due to the apical cap's large number of cells, the apical wound epithelium may serve as a reservoir for additional epithelial cells while at the same time offer the "path of least resistance" to blastemal growth. As the blastema grows, cells may exert the same outward pressure on the wound epidermis. However, since the lateral wound epithelium is about the same thickness as normal epithelium, it may be at its minimal thickness and resist further stretching. But the apical wound epithelium may undergo additional stretching due to the fact that it has not yet attained the thickness of lateral wound epithelium. The blastema then may continue to grow distally at a rapid rate because the apical cap continues to offer the path of least resistance.

If the apical cap is in an eccentric position, the cap may still offer the path of least resistance, resulting in skewed blastemal growth. Indeed, the lower incidence of mitosis immediately beneath the apical cap may be due to the pressure exerted there by more proximal cells. Whether cells of the apical cap are derived from the distal migration of more proximal epithelial cells or to their own replenishment by local mitosis would make no difference,

for the point of least resistance may still be at the apical cap.

Study of the adepidermal membrane in the newt regenerate reveals an interesting correlation. Using electronmicroscopy, Salpeter and Singer ('60) showed that the adepidermal membrane is absent under the wound epithelium during the earliest stages of regeneration. However as the blastema forms, the adepidermal membrane begins to form beneath the proximal regions of the wound epithelium. During the rapid growth stages of regeneration, the adepidermal membrane is not found beneath the apical wound epithelium. As rapid blastemal growth subsides at the late bud stage, the adepidermal membrane begins to appear at the distal tip of the blastema.

The function of the adepidermal membrane is unknown. Some investigators believe that the membrane serves as an ionic barrier (Caesar and Edwards, '57; Ottoson et al, '53). Others feel that during regeneration direct contact between the apical wound epithelium and the mesenchymatous cells of the blastema may facilitate the movement of fluids and particulate substances (Singer and Salpeter, '61) and the communication between the apical cap and blastemal cells (Hearson, '66). However the adepidermal membrane may also serve as a cohesive cement, restricting additional stretch or displacement of the proximal wound epithelium, which has been generally accepted as its

function, at least for normal epithelium. If the adepidermal membrane does bind epithelial cells, then that region of the wound epithelium which lacks the membrane, namely the apical wound epithelium, may in fact be the "weakest" region of the wound epithelium.

The apical wound epithelium is thought to produce some chemical substance which attracts mesenchymatous cells (see Thornton, '68 for review). By treating the epidermis with actinomycin D, untreated mesodermal tissues either failed to regenerate or regenerated abnormally (Sprague and Thornton, '68). The actinomycin D is thought to block DNA-dependent RNA synthesis of a specific substance responsible for cell aggregation. Whether actinomycin D's only effect is on DNA-dependent RNA synthesis during regeneration is still unclear (Carlson, '67).

According to the above model, as cells are continually displaced distally, there may also be a tendency for them to be displaced axially. As studies of objects and substances moving through a confined space illustrate, the path of least resistance is along the center line (Rouse and Howe, '53). One of the reasons why mesenchymatous cells are denser in the axial regions of the blastema may be because they too, being in a confined space, may be displaced axially as they are displaced distally (Figure 13B). Certainly other factors may act to further aggregate cells axially, but the initial impetus towards the

medial regions may be due to the pure physical characteristics of the blastema.

Indeed, Umansky ('66) showed that in in vitro experiments on mesenchyme cells of the mouse limb "population-dependent aggregative patterns arising early in culture determined the nature of histodifferentiation and morphogenesis". He went on to say that chondrogenesis is associated with dense cellular populations whereas myogenesis occurs in less dense cultures. Curtis ('61) speculated that the more frequent contact between cells in dense populations may lead to changes in cell surface configuration affecting cell adhesive properties. However whether the aggregation of cells is in lieu of cell density or is due to initial differences in cells, is unknown. Also whether the in vitro condition accurately represents the events occuring in vivo is not known.

Blastemal morphogenesis is usually described in terms of chemical interactions. This is understandable, for surely these hypotheses are the easiest to explain and test given the present level of technology. But because technology limits analytical methods, there is no reason that it also restrict the formulation of new hypotheses.

Influence of Nerves

The nature of the nerve's influence during regeneration is currently expressed as a two-fold hypothesis:

(1) a minimal amount of innervation is required before regeneration commences; and (2) the nerves are capable of producing a "trophic factor" which initiates regeneration (Singer, '65). Both concepts stem from each other, one to explain the quantitative aspect of the nerves; the other to explain the qualitative influence of nerves.

Singer ('46b) postulated that the newt limb must have between one third to one half the total number of nerve fibers to regenerate. This variability in the nerve fiber number required for regeneration is called the "threshold" level of innervation and is thought to be the minimal number of nerve fibers required for regeneration. Since the number of nerve fibers may vary with the size of the limb, Singer expressed the minimal requirement in terms of the number of nerve fibers per tissue area (nerve:tissue ratio). Using this ratio, nonregenerating limbs have a lower nerve to tissue ratio than the threshold level of the newt. Other workers have found that the nerve:tissue ratio of the tadpole limb which can regenerate is above the threshold value of the newt, while the ratio of the non-regenerating post-metamorphic frog limb is below the minimal threshold value (Van Stone, '55). Furthermore, nerve augmentation of the post-metamorphic frog limb increases the nerve:tissue ratio above the threshold, resulting in partial limb regeneration (Singer, '51). Also nerve augmentation of the normally non-regenerating lizard limbs results in rudimentary limb formation (Singer, '61).

Using the relationship between the number of nerve fibers and the ability to regenerate, Singer ('59) hypothesized that the nerves produce a "trophic factor" which regulates the ability to regenerate. If the nerves do not produce enough trophic factor, regeneration is arrested. The amount of trophic factor at the wound surface is related to the number of nerve fibers, that is, the fewer nerve fibers there are, the less trophic factor at the wound surface. Singer's attempts ('60) to isolate a trophic factor have been unsuccessful, albeit at one time acetylcholine was thought to be the agent produced by the nerves which controlled regeneration.

Even though a correlation exists between the number of nerve fibers and the ability to regenerate, the threshold concept is not a steadfast rule. Harrison ('04) showed that embryonic limbs can develop without nerves. Polezajew ('39) reported that frog limbs, transplanted to the back, can regenerate without nerves. Rose ('44) showed that the post-metamorphic frog limb can be induced to partially regenerate by repeated exposure to hypertonic salt solutions. Bodemer ('64) demonstrated that electrical stimulation of the adult frog's spinal nerve can also induce partial regeneration. Singer and Mutterperl ('63) reported that newt limbs transplanted to the back regenerate with a sub-threshold number of nerve fibers. Rzehak and Singer ('66) found that the Xenopus laevis limb

partially regenerated with a sub-threshold number of nerve fibers. Based on these exceptions both the concept of nerve fiber requirements and the trophic factor have been revised.

Singer now feels that the axon area, not fiber number, is critical in determining the flow of "trophic factor" to the tissues. Singer, Rzehak, and Maier ('67) found that although the number of nerve fibers per tissue area in the regenerating Xenopus limb was below the threshold number, the cross sectional area of the axons per tissue area is within the threshold axon area:tissue area ratio of the newt. Both the number of nerve fibers per tissue area and the axon area per tissue area of non-regenerating Rana limbs fall below the threshold ratio of the newt. Thus, it is reasoned that the larger caliber of Xenopus nerve fibers transports enough trophic factor to initiate regeneration. However, they were not clear as to whether the axon calibers are calculated from a number of different nerve bundles or from the same nerve bundle, for possibly the fiber bundle they sampled does have these relationships, but other fiber bundles may not. Furthermore whether the threshold level for newts is also applicable to Xenopus, Rana, Anolis, and mice is questionable. Indeed whether the abnormal partial regenerates of anurans and lacertilians are comparable to complete urodele regenerates is likewise extremely questionable.

As for the trophic factor concept, Singer ('65) proposed that non-neural tissues also have the ability to produce trophic factor. Upon innervation the tissues become "addicted" to the nerves for the trophic factor and relinquish the production of the trophic factor to neural tissues. However, addicted non-neural tissues may again resume the production of trophic factor after severe trauma, thus theoretically explaining why nerveless embryonic limbs, transplanted limbs, and normally non-regenerating limbs are capable of regeneration.

The nerve does have some trophic influences on non-neural tissues. Denervation of muscles results in muscle atrophy (see Guth '68 for review). Denervation of taste buds in mammals (Guth, '57) also results in their atrophy, but nerves may play no role in the maintenance of newt taste buds (Mintz and Stone, '34; Poritsky and Singer, '66; and Wright, '64). Reinnervation of these atrophied organs results in the restoration of lost structure and function. Likewise, denervation followed by reinnervation results in first the loss and then the restoration of regenerative abilities in urodele limbs (Schotte and Butler, '41). However wheher the morphological integrity of an organ or tissue is due to the "tonic" influence of nerves or to the transport of a specific "trophic factor" is still unresolved (see Eccles, '64). Indeed, the necessity to postulate a trophic factor is questionable, for the ability

of an organ to regenerate does not appear to reside in the trophic influence of the nerves, but in the tissues themselves.

Regeneration is the replacement of preexisting missing structures. Nerveless embryonic limbs beget nerveless regenerates. Aneurogenic limbs begen aneurogenic regenerates. Innervated limbs beget innervated regenerates. Indeed no differences exist between the regeneration of innervated and aneurogenic limbs, for they both replace preexisting structures. If a difference does exist, it is only that aneurogenic limbs need not replace nerves, for they never existed in the limb.

On the other hand, innervated limbs which have been denervated, do not beget aneurogenic regenerates. Possibly, denervated limbs fail to regenerate, not because the source of the "trophic factor" has been severed, but because one of the preexisting structures is physically prevented from participating in the regeneration process.

The whole relationship of tissues and cells to each other may have to be reorganized if a nerveless regenerate is to replace a previously innervated limb. Some kind of organization surely exists within the limb before amputation. Regeneration appears to be the reestablishment of the original relationships between tissues which existed prior to amputation.

The regeneration of previously innervated trans-

planted limbs with few nerve fibers and also the fact that a certain period of time must elapse before transplanted aneurogenic limbs which are allowed to be innervated, become "dependent" on nerves (Thornton, personal communication) suggest that new relationships may be established among the tissues over time. However these new relationships are formed before the limb is amputated, not during regeneration itself. Possibly once the new relationships exist, the limb may be able to regenerate, not because non-neural tissues begin to synthesize a "trophic factor" or because the non-neural synthesis of the trophic factor is supplanted by the nerves, but because the limb, now a reorganized structure, is able to replace those preexisting missing structures which were present before the limb was amputated.

It is proposed that the ability to regenerate may be determined by whether a component of each <u>preexisting</u> structure is represented during the regenerative process. The limiting factor of regeneration may then be whether all components of the blastema are brought together at a critical phase of regeneration. Thus, it is hypothesized that no one factor is responsible either for the ability to regenerate or for the rate of regeneration.

Indeed, the growth phase of regeneration may be the critical stage during which all components of the preexisting structure must be represented. Studies on the influence

of nerves, hormones, and the wound epithelium suggest that the growth phase is the stage most sensitive to experimental manipulations and also the stage at which removal of any one component of the regenerate results in a gross alteration of the regenerative process (see Singer, '52, '60; Rose, '64; Hay, '66; and Thornton, '68, for reviews). The inability of extra nerves to substitute for hormones in hypophysectomized newts (Shuraleff and Tassava, unpublished) and the inability of growth hormone to stimulate regeneration in denervated newt limbs (DeFazio and Thornton, '68) suggest that one component of the regenerate can not be substituted by another component.

Since regeneration is a balanced interrelationship of events, a change in the rate of one process may influence the rate of development of another. In order to reestablish the original balanced relationship and to maintain an equilibrium between the various components of the regenerate, an alteration of any one process during regeneration may result in the alteration of other processes. For example, an abundant ingrowth of nerve fibers into the blastema may result in the increase of the rate of cellular proliferation in order to maintain a balanced relationship between the amount of innervation and the blastemal mass. Likewise, partially denervated limbs may regenerate at a slower rate because more nerve fibers must be replaced to reach the proper proportion of blastemal components. This

relationship need not be restricted to just the nerves. Thus, the regeneration process is altered not because one component of the regenerative process adds more or less of a specific substance which in turn affects one specific component of the regenerate, but because the whole relationship of the blastemal components has been changed. The nature of the cohesive organizational force which maintains the limb and the regenerate as a unit, is currently being mused by the author.

SUMMARY

A series of experiments was conducted to determine the influence of superinnervation, nerve augmentation, on the appearance of mesenchymatous cells at the wound surface, on the rate of blastemal growth and limb morphogenesis, and on the rate of cellular proliferation within the regeneration blastema of the hindlimb of the axolotl, Ambystoma mexicanum. Also a detailed study was performed on the regionalization within the blastema of cellular mitosis, cellular density, and thymidine incorporation over time, in relation to the amount of innervation and to specific sample sites within the blastema. Finally the relationships between mitosis, cellular density, and thymidine incorporation were studied.

The results of this study revealed eight primary relationships: (1) augmentation of the nerve supply is correlated with an accelerated growth and limb morphogenesis, but not with the final size of the regenerate; (2) superinnervation is correlated with an increase in mesenchymatous cell proliferation; (3) superinnervation is not correlated with the rate of appearance of mesenchymatous cells at the wound surface; (4) mesenchymatous cell proliferation is uniform throughout the regeneration blastema, except immediately proximal to the apical wound epithelium,

where proliferation is markedly reduced; (5) superinnervation does not alter the pattern of mitosis within the blastema, just the frequency; (6) the blastema has a substructure during the proliferative stage, a cylindrically shaped dense core of cells; (7) cellular proliferation is not correlated with the density of mesenchymatous cells; and (8) an inverse relationship exists between cellular density and thymidine incorporation.

The hypothesis was proposed that the apical wound epithelium may additionally serve as the path of least resistance to the outward pressures of the growing blastema, accounting for both the gross morphological shape and the internal cellular substructure of the blastema. It was proposed that the physical characteristics of the growing blastema establish a template upon which limb morphogenesis proceeds.

The notion was rejected that the nerves produce a specific "trophic factor" which mediates the ability and the rate of regeneration. The hypothesis was proposed that the limb regenerates only when all the preexisting components of the missing structure are represented during the proliferative stage of regeneration, deemed to be the critical stage. Also the hypothesis was proposed that the rate of regeneration is not regulated by any one component of the regenerate, but by the speed these components are brought together during the critical stage of regeneration.

Figure 1.--Example of Ocular Grid Superimposition Used in Determining the Appearence of Mesenchymatous Cells at the Amputation Surface.

Figure 1.

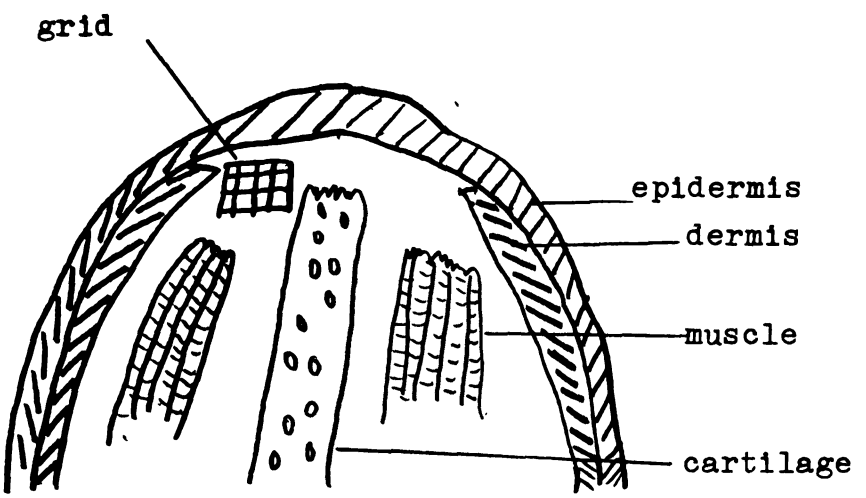


Figure 2.--Position of Sections Used in Sampling Blastemata Sectioned Longitudinally.

Figure 3.--Ideal Longitudinal Section Divided into a 3 X 3 Quadrant with Examples of Ocular Grid Superimposition.

Figure 2.

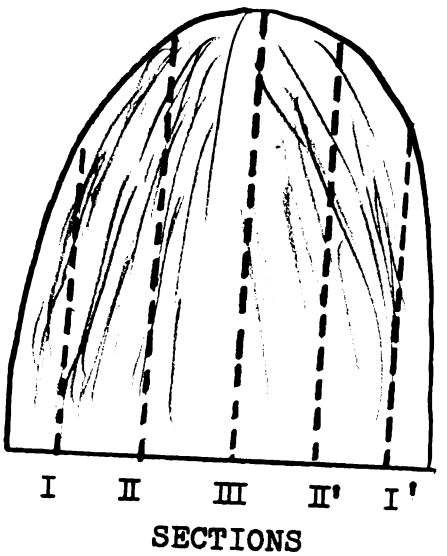


Figure 3.

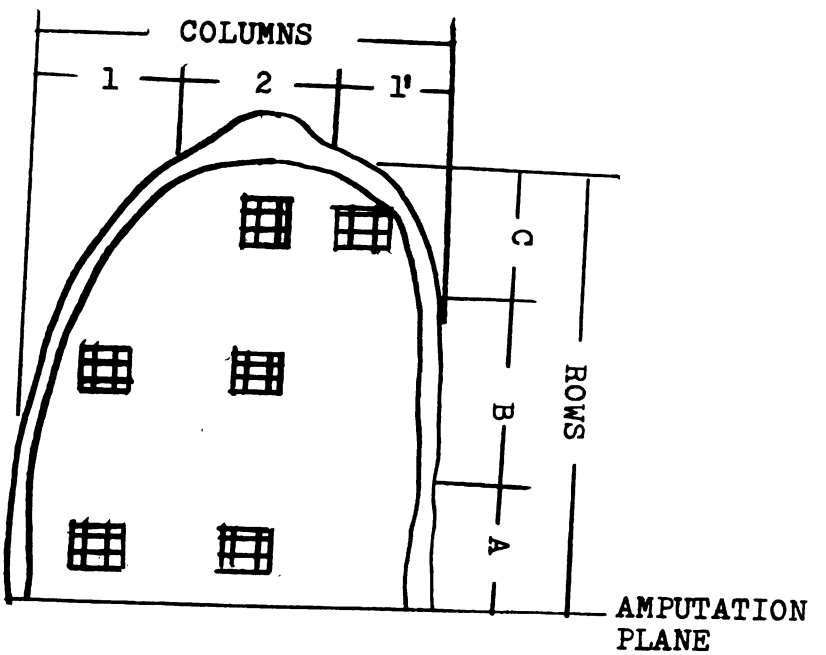


Figure 4.--Position of Sections Used in Sampling Blastemata Sectioned Transversely.

Figure 5.--Ideal Transverse Section Divided into Three Concentric Circles with Examples of Grid Superimpositions.

Figure 4.

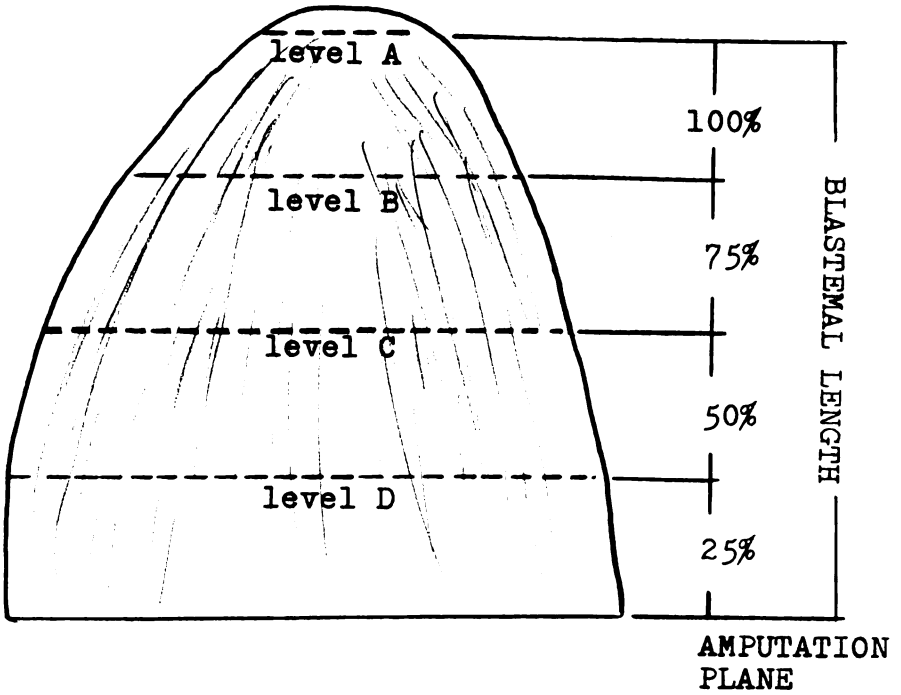


Figure 5.

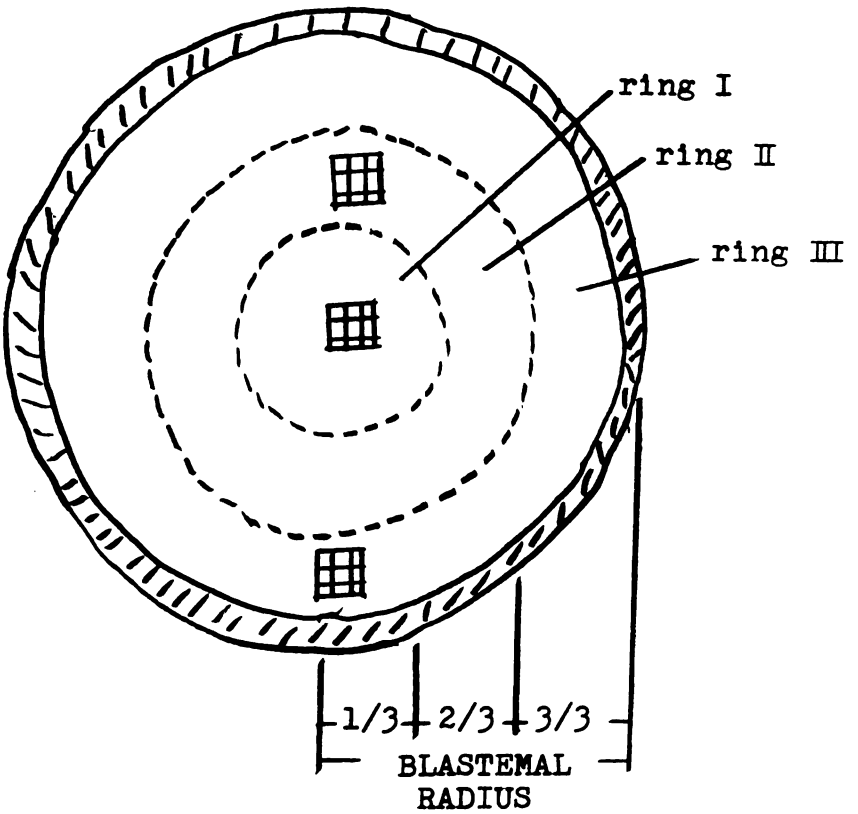


Figure 6.-- Semi-Log Graph of Blastemal Outline Traces over Time.

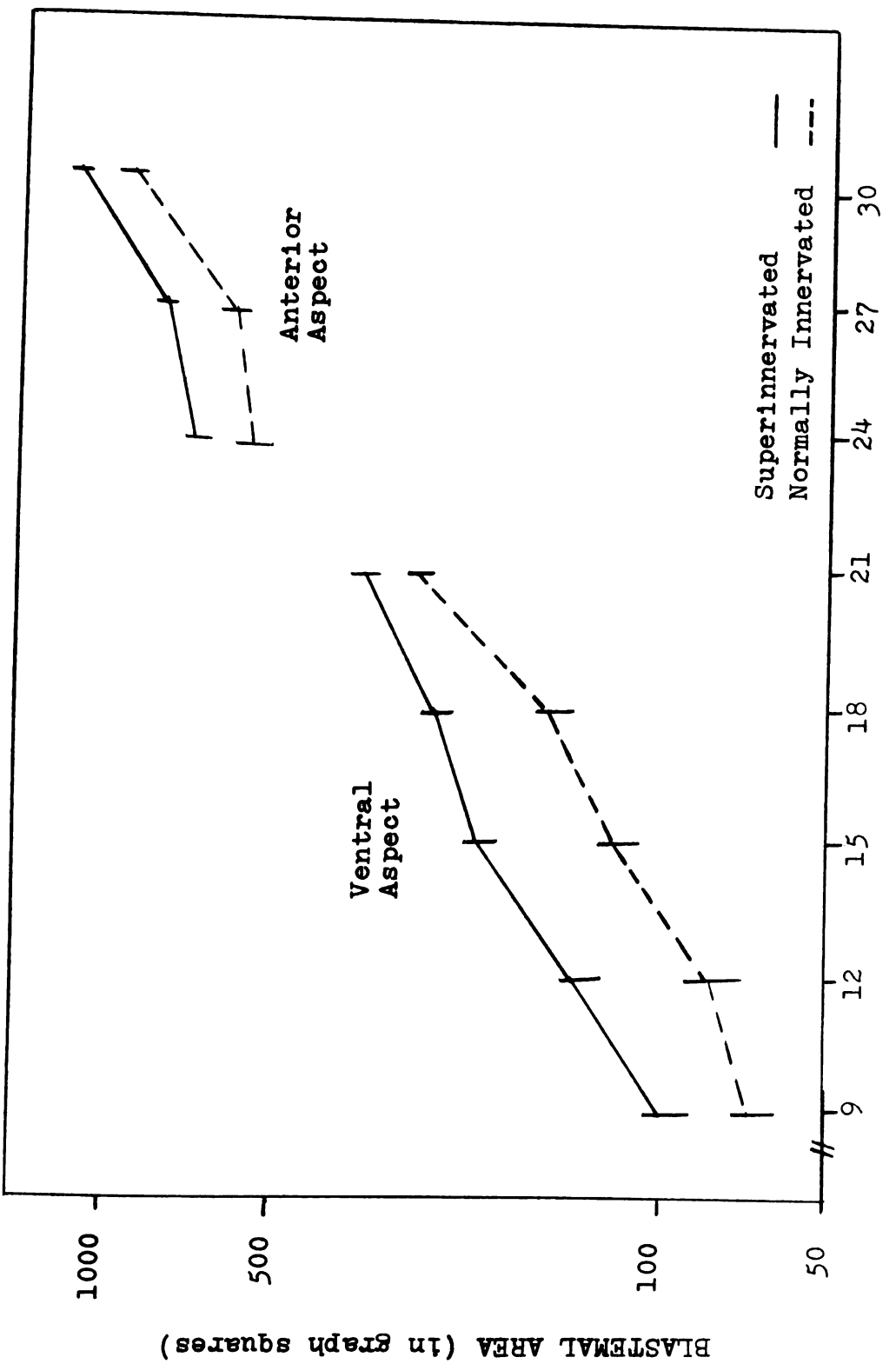

BLASTEMAL AREA (in graph squares)
1000
500
100
50
9
12
15
18
21
24
27
30
DAYS POST-AMPUTATION
Anterior Aspect
Ventral Aspect
Superinnervated
Normally Innervated

Figure 6.

Figure 7.-- Histogram of the Percent Proportional Change in Traced Blastemal Area of Superinnervated and Normally Innervated Blastemata.

Figure 7.

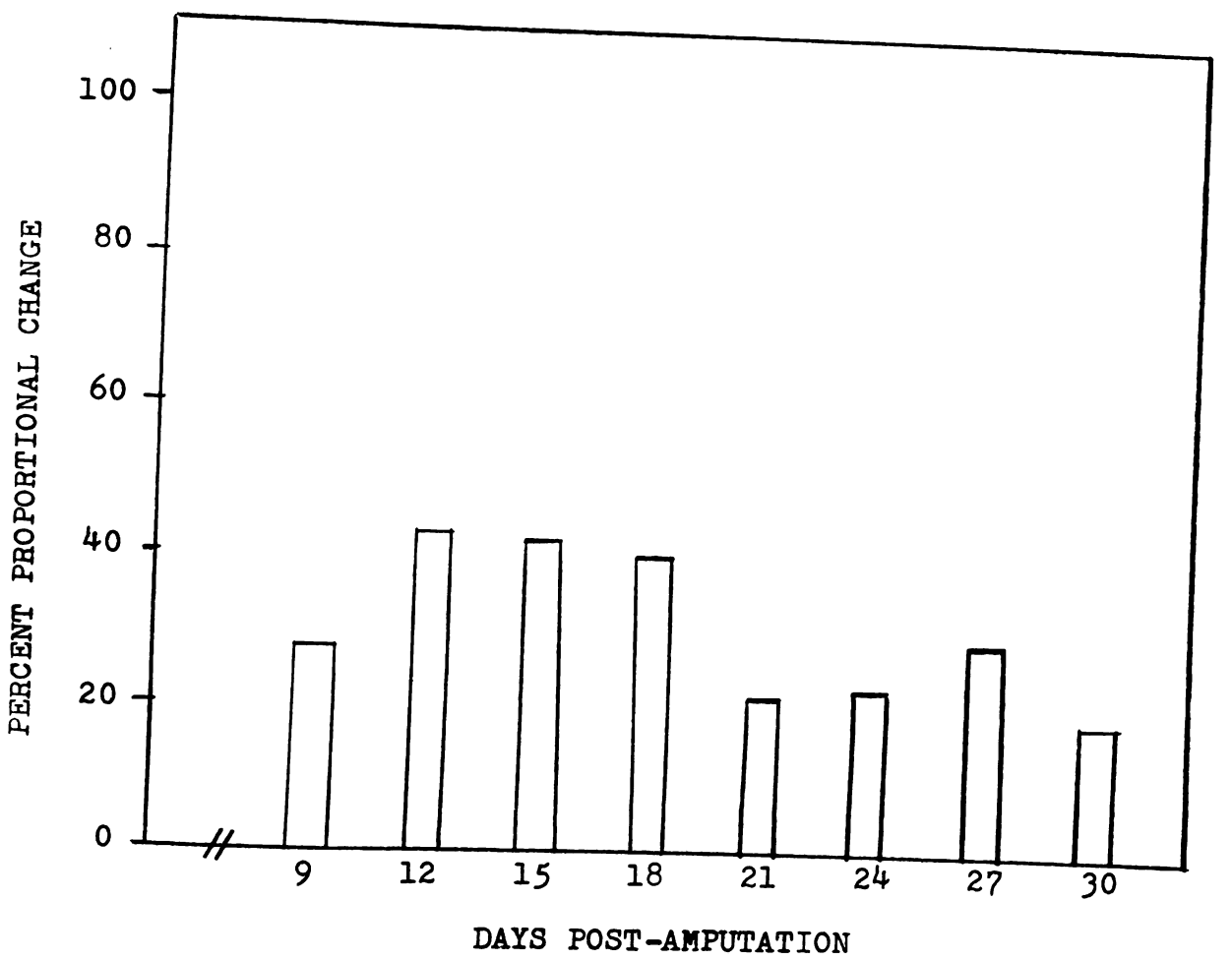

100
80
60
40
20
0
PERCENT PROPORTIONAL CHANGE
9
12
15
18
21
24
27
30
DAYS POST-AMPUTATION

Figure 8.--Histogram of Percent Regenerates Reaching Palette Stage.

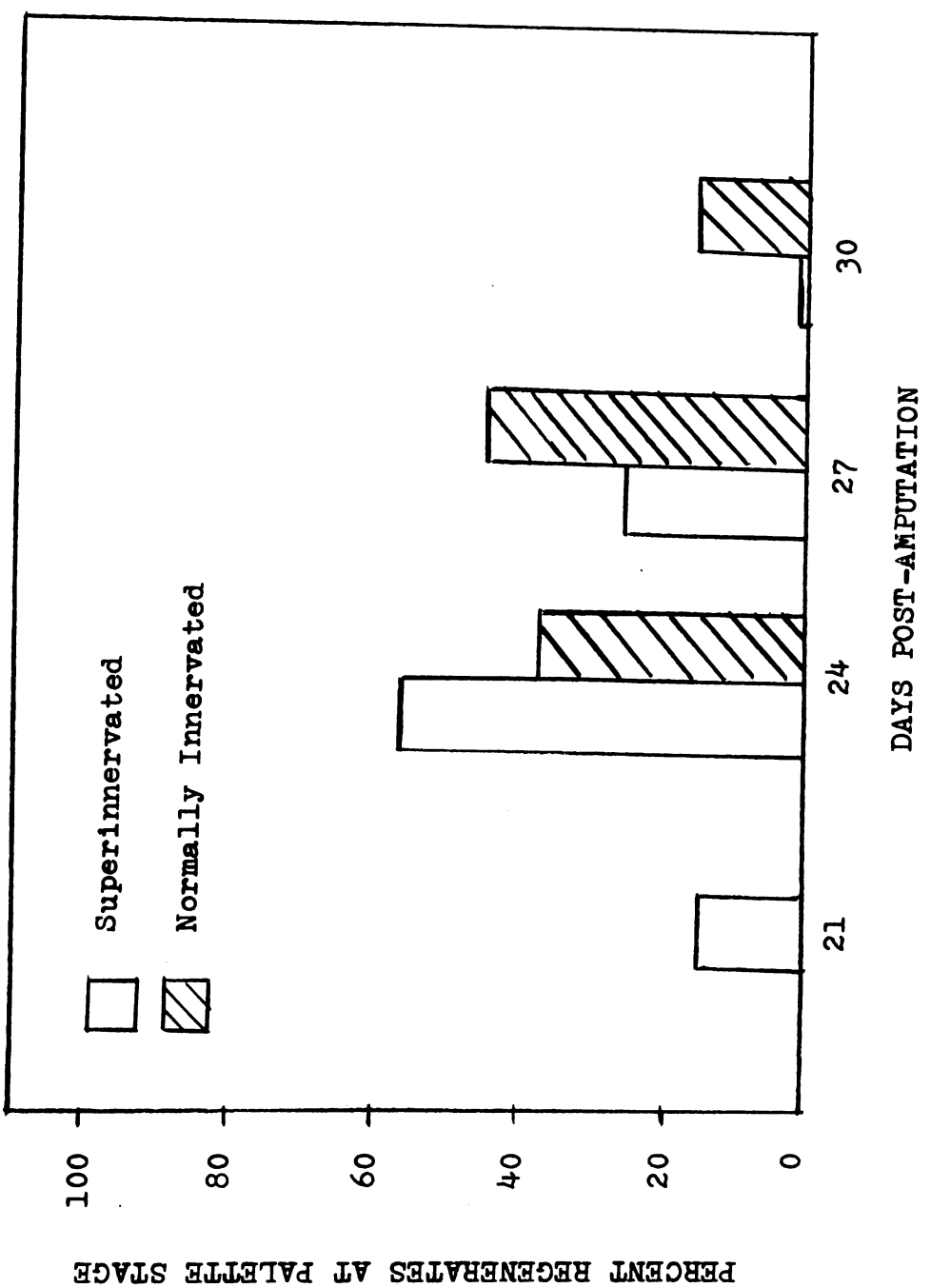

PERCENT REGENERATES AT PALETTE STAGE
100
80
60
40
20
0
Superinnervated
Normally Innervated
21
24
27
30
DAYS POST-AMPUTATION

Figure 8.

Figure 9.-- Histogram of Morphogenetic Stages of Partially Innervated and Totally Denervated Limbs Amputated Midway along the Tibia-Fibula.

Legend:

NR- No Regeneration
M- Mound Stage Regenerate
C- Cone Stage Regenerate
P- Palette Stage Regenerate

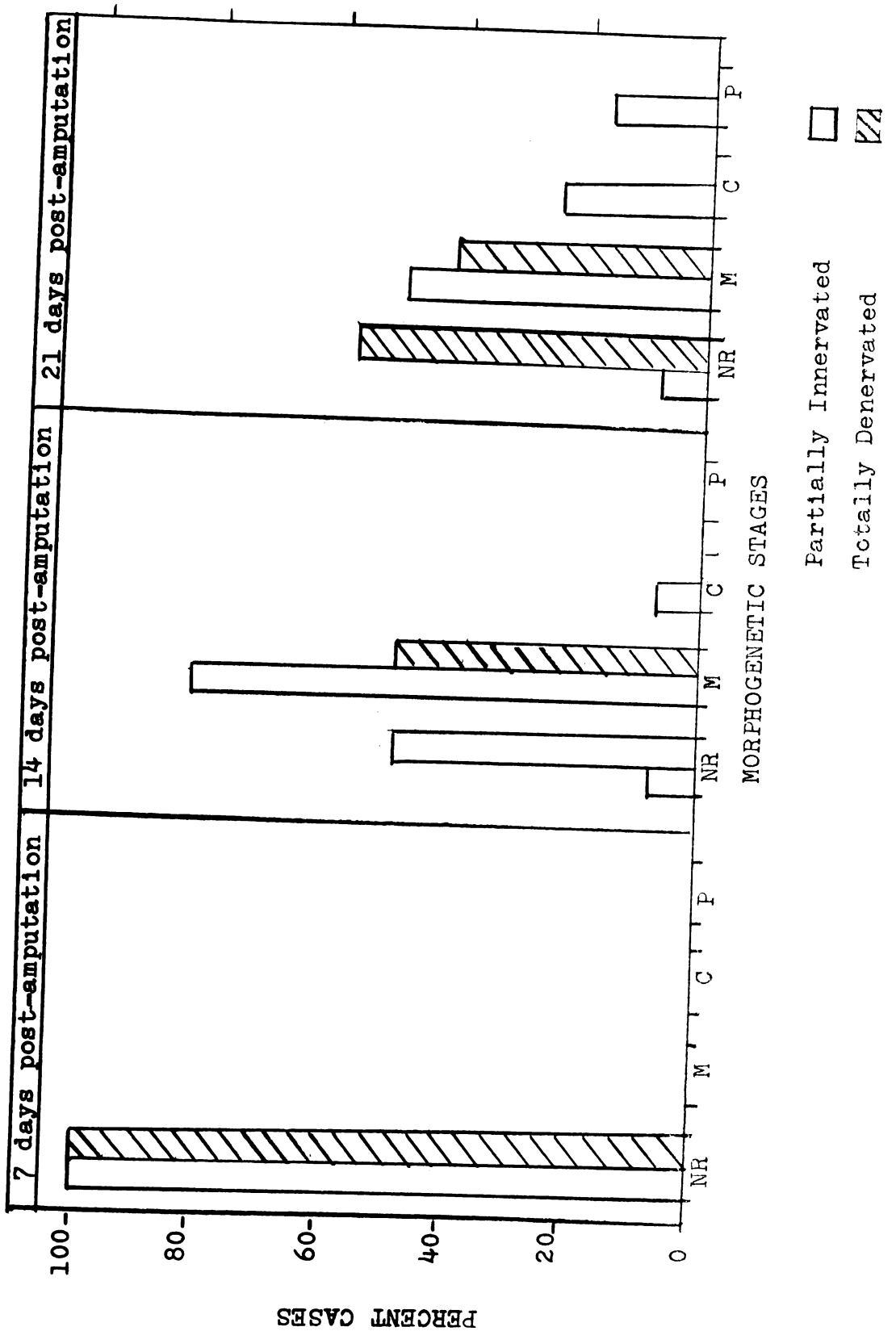

7 days post-amputation
14 days post-amputation
21 days post-amputation
PERCENT CASES
100
80
60
40
20
0
NR
M
C
P
MORPHOGENETIC STAGES
Partially Innervated
Totally Denervated

Figure 9.

Figure 10.--Changes in Limb Length of Partially Innervated and Totally Denervated Limbs Amputated along the Tibia-Fibula.

Figure 10.

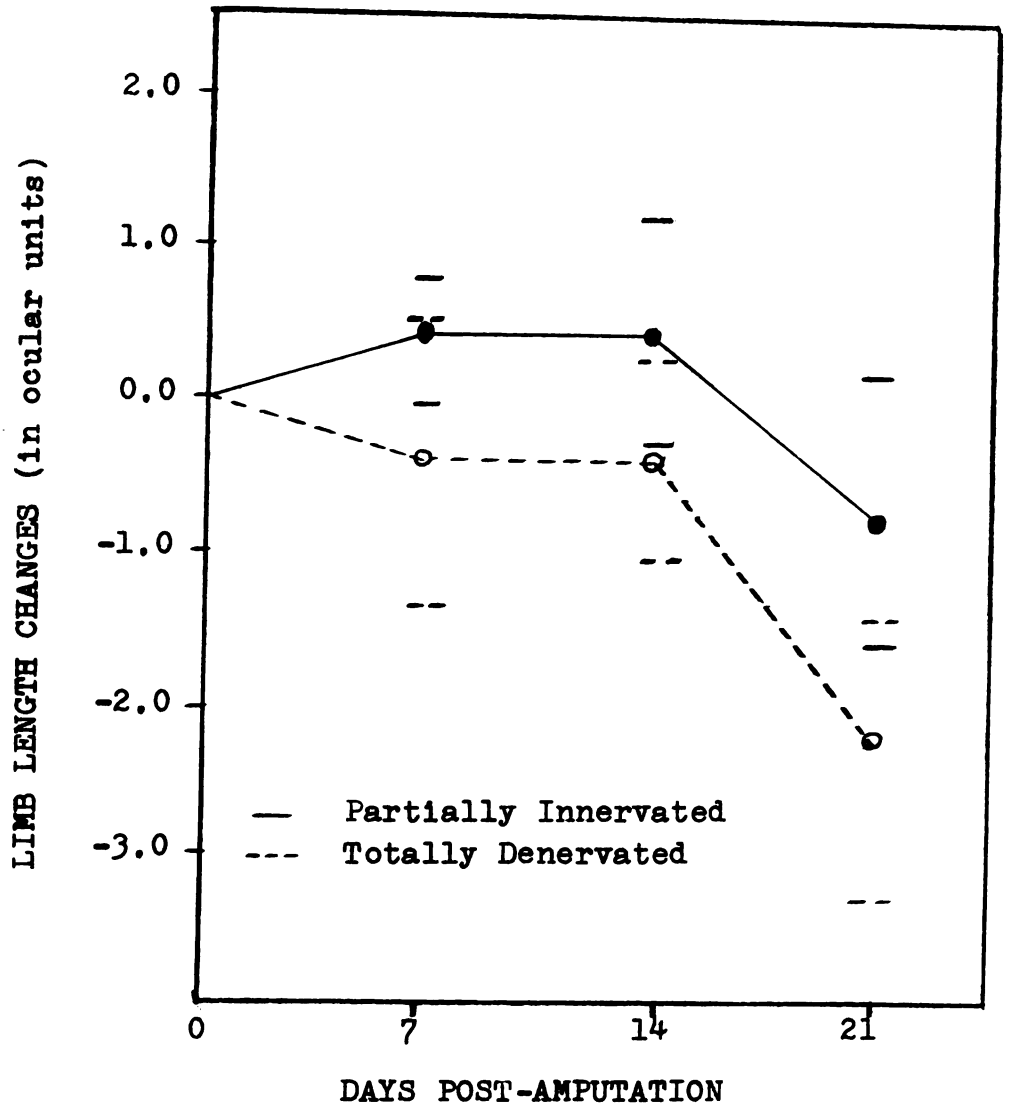

2.0
1.0
0.0
-1.0
-2.0
-3.0
LIMB LENGTH CHANGES (in ocular units)
Partially Innervated
Totally Denervated
0
7
14
21
DAYS POST-AMPUTATION

Figure 11.-- Correlation between Mitotic Index and Cellular Density of Mesenchymatous cells of Superinnervated and Normally Innervated Blastemata from 9 to 15 Days Post-Amputation.

Figure 11.

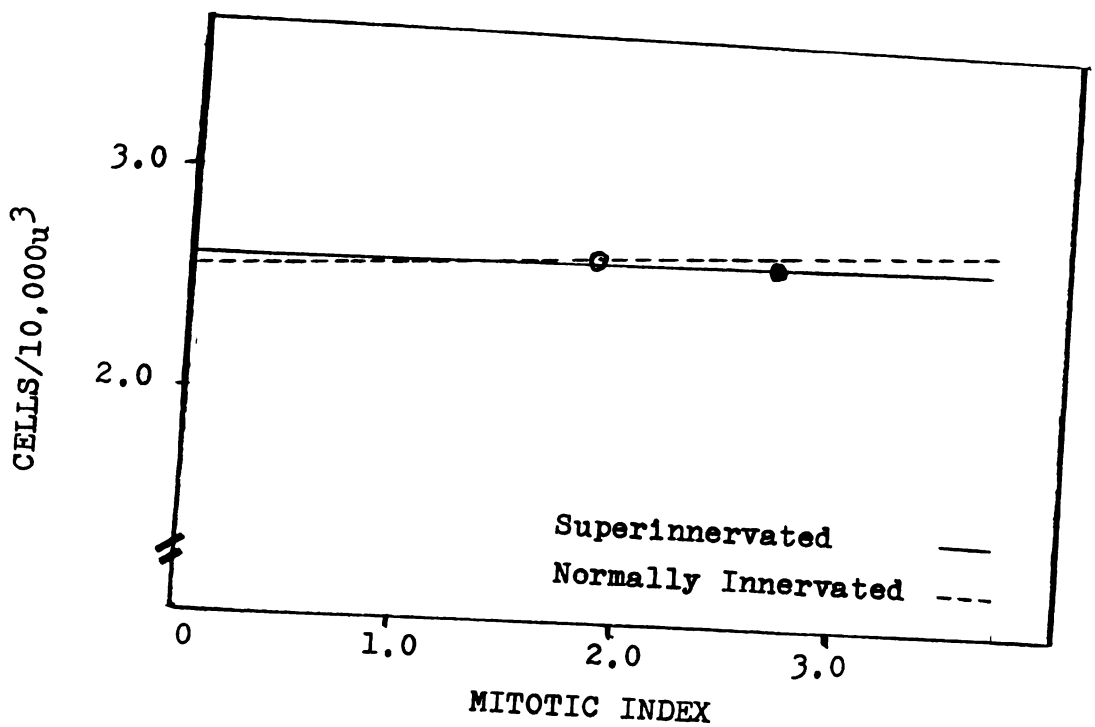

3.0
2.0
CELLS/10,000u3
Superinnervated
Normally Innervated
0
1.0
2.0
3.0
MITOTIC INDEX

Figure 12.-- Diagrammatic Model of the Appearence, Proliferation, and Distal Displacement of Mesenchymatous Cells Within the Regeneration Blastema.

Figure 12.

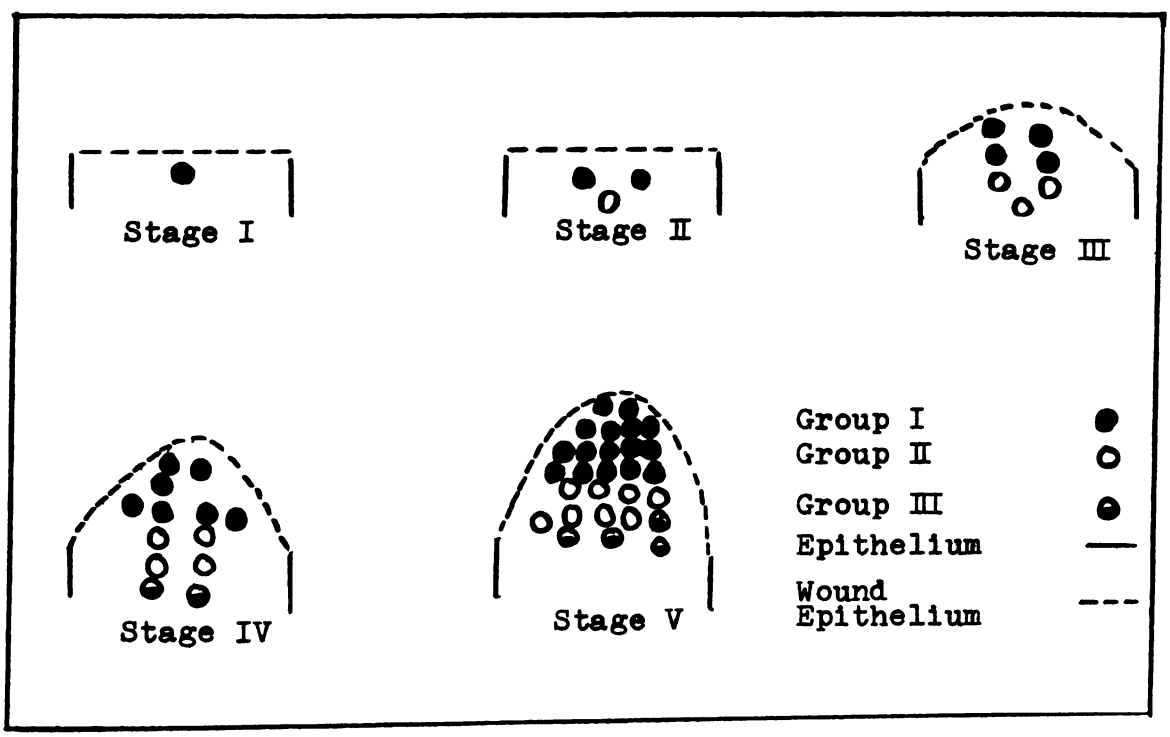

Stage I
Stage II
Stage III
Stage IV
Stage V
Group I
Group II
Group III
Epithelium
Wound Epithelium

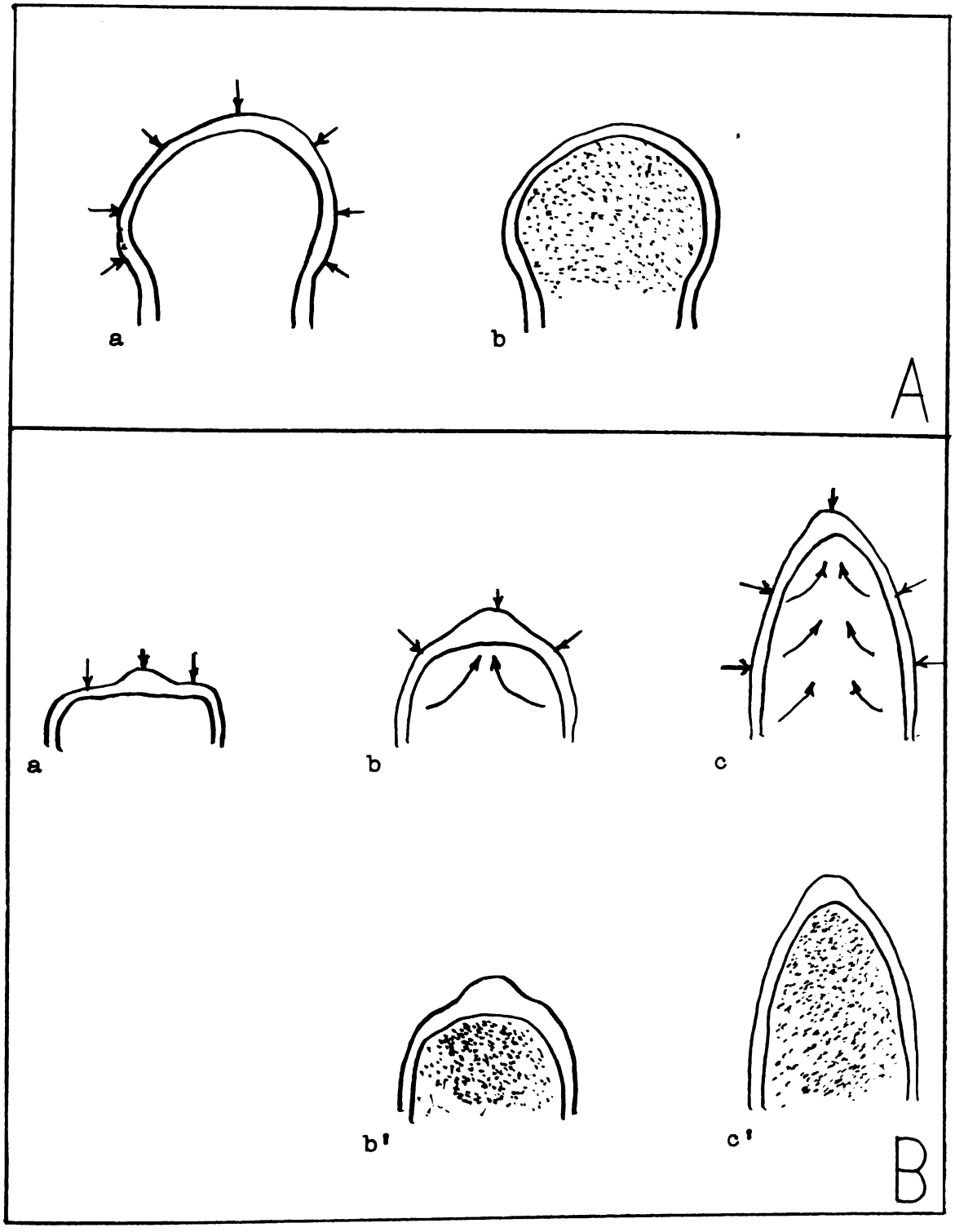
Figure 13A.--(a) Proposed Shape of Blastema if all Regions of the Wound Epithelium Had the Same Stretch Properties.

(b) Proposed Distribution of Mesenchymatous Cells within the Blastema if All Regions of the Wound Epithelium Had the Same Stretch Properties.

Figure 13B.--(a,b,c) Proposed Shape of Blastema if the Apical Wound Epithelium Was Less Resistant than More Proximal Regions of the Wound Epithelium to Stretch with Resultant Lines of Force Within the Blastema.

(b',c') Proposed Distribution of Mesenchymatous Cells Within the Blastema if Part of Apical Wound Epithelium Was Less Resistant to Stretch than Other Regions.

Figure 13.



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Appendix 1.--Basic Data for Outline Traces of Growing Blastemata.

Days Post-Amputation	Traced Blastemal Size (in graph squares)		Absolute Size Difference between Super and Normal Blastemata	% Proportional Size Difference
	Supers (S)	Normals (N)		
9	98.79** (9.56)*	70.78 (9.36)	28.01	28.35%
12	146.08 (12.81)	82.72 (10.35)	63.35	43.36%
15	221.42 (15.38)	127.85 (13.73)	93.57	42.26%
18	268.04 (16.97)	160.61 (12.53)	107.42	40.07%
21	351.43 (19.03)	276.24 (21.23)	75.19	21.39%
24	725.63 (40.03)	559.27 (47.99)	166.36	22.92%
27	835.32 (36.00)	591.36 (35.66)	243.95	29.20%
30	1145.32 (40.33)	933.33 (58.86)	211.99	18.50%

** Average ($\bar{X}$)
* Standard Error ($S_{\bar{X}}$)

Appendix 2.--Changes in Limb Length of Amputated Superinnervated and Normally Innervated Limbs Whose Endogenous Nerve Supply was Severed.

Days Post-Amputation	Changes in Limb Length	
	Super	Normal
7	0.41** (0.42)*	-0.41 (0.93)
14	0.42 (0.45)	-0.37 (0.64)
21	-0.79 (0.91)	-2.37 (0.94)

** Average change in ocular micrometer units from the day of amputation (1 millimeter = 2.632 units)

*Standard Error

Appendix 3.--Means and Standard Errors for Mitotic Indices of Longitudinally Sectioned Blastemata Sampled along the Transverse Axis.

	Days Post-Amputation		Sample Sites: Medial	P-Medial	Lateral	$\bar{\bar{X}}$
Superinnervated Blastemata	9		0.82** (0.35)*	3.37 (1.66)	2.05 (1.01)	2.08
	12		1.97 (0.34)	1.99 (0.26)	4.36 (1.20)	2.78
	15		2.59 (0.78)	1.98 (0.40)	3.05 (1.20)	2.59
		$\bar{\bar{X}}$	1.69	2.49	3.24	2.49
Normally Innervated Blastemata	9		0.82 (0.39)	0.74 (0.45)	1.11 (1.11)	0.89
	12		1.92 (0.39)	1.80 (0.46)	1.21 (0.21)	1.64
	15		2.50 (0.76)	1.84 (0.39)	1.72 (0.65)	2.02
		$\bar{\bar{X}}$	1.69	1.43	1.32	1.48

** Mean ($\bar{X}$)
* Standard Error ($S_{\bar{X}}$)

Appendix 4.--Means and Standard Errors for Cellular Densities of Longitudinally Sectioned Blastemata Sampled along the Transverse Axis.

	Days Post-Amputation		Sample Sites			$\bar{X}$
			Medial	P-Medial	Lateral	
Superinnervated Blastemata	9		0.98 (0.12)	1.17 (0.31)	0.75 (0.14)	0.97
	12		2.70 (0.27)	2.37 (0.26)	1.82 (0.18)	2.30
	15		3.59 (0.22)	3.68 (0.23)	2.15 (0.54)	3.04
		$\bar{X}$	2.28	2.22	1.55	2.00
Normally Innervated Blastemata	9		1.44 (0.27)	0.85 (0.17)	0.36 (0.11)	0.85
	12		2.47 (0.40)	2.71 (0.57)	2.67 (0.61)	2.62
	15		3.60 (0.34)	3.54 (0.29)	2.42 (0.31)	3.19
		$\bar{X}$	2.39	2.28	1.77	2.15

Appendix 5.--Means and Standard Errors for Mitotic Indices of Transversely Sectioned Blastemata Sampled along the Transverse Axis.

	Days Post-Amputation	Sample Sites: Medial	P-Medial	Lateral	$\bar{\bar{X}}$
Superinnervated Blastemata	9	1.65 (0.62)	1.76 (1.02)	2.70 (1.64)	2.04
	12	3.64 (0.57)	3.58 (0.83)	3.76 (0.64)	3.66
	15	1.66 (0.28)	2.67 (0.98)	1.58 (0.69)	1.97
	$\bar{\bar{X}}$	2.36	2.74	2.68	2.59
Normally Innervated Blastemata	9	0.85 (0.09)	1.77 (1.16)	0.00 (0.00)	0.87
	12	2.28 (0.28)	2.00 (0.56)	2.14 (1.20)	2.14
	15	1.47 (0.23)	2.35 (0.72)	0.44 (0.44)	1.42
	$\bar{\bar{X}}$	1.58	2.06	0.92	1.52

Appendix 6.-- Means and Standard Errors for Cellular Densities of Transversely Sectioned Blastemata Sampled along the Transverse Axis.

	Days Post-Amputation		Sample Sites: Medial	P-Medial	Lateral	$\bar{\bar{X}}$
Superinnervated Blastemata	9		1.06 (0.42)	0.92 (0.34)	1.00 (0.33)	0.99
	12		3.87 (0.38)	4.61 (0.43)	3.77 (0.32)	4.08
	15		3.88 (0.48)	3.88 (0.41)	2.90 (0.43)	3.55
		$\bar{\bar{X}}$	3.07	3.30	2.67	3.01
Normally Innervated Blastemata	9		1.80 (0.50)	1.34 (0.32)	1.29 (0.53)	1.48
	12		3.62 (0.24)	3.92 (0.21)	2.46 (0.06)	3.33
	15		3.35 (0.24)	3.02 (0.29)	2.37 (0.30)	2.91
		$\bar{\bar{X}}$	3.00	2.86	2.10	2.65

Appendix 7.--Means and Standard Errors for Mitotic Indices of Transversely Sectioned Blastemata Sampled along the Longitudinal Axis.

	Days Post-Amputation	Percent Blastemal Length 100%	75%	50%	25%	$\bar{\bar{X}}$
Superinnervated Blastemata	9	0.00	1.65 (0.83)	3.93 (2.25)	1.29 (0.76)	1.99
	12	0.74 (0.47)	3.96 (1.01)	3.46 (0.64)	4.61 (0.42)	3.19
	15	0.65 (0.65)	1.88 (0.60)	2.07 (0.73)	2.30 (0.66)	1.73
	$\bar{\bar{X}}$	0.63	2.62	3.03	2.84	2.36
Normally Innervated Blastemata	9	0.63 (0.63)	1.06 (0.69)	1.52 (0.69)	0.74 (0.32)	0.99
	12	1.98 (0.16)	2.72 (0.76)	2.14 (0.50)	2.10 (0.59)	2.23
	15	1.10 (0.49)	1.49 (0.79)	2.24 (0.46)	1.30 (0.46)	1.53
	$\bar{\bar{X}}$	1.28	1.81	2.00	1.43	1.63

Appendix 8.-- Means and Standard Errors for Cellular Densities of Transversely Sectioned Blastemata Sampled along the Longitudinal Axis.

	Days Post-Amputation	Percent Blastemal Length 75%	50%	25%	$\bar{\bar{X}}$
Superinnervated Blastemata	9	2.29 (0.29)	0.97 (0.25)	1.12 (0.38)	1.23
	12	3.54 (0.06)	4.52 (0.39)	3.38 (0.49)	4.04
	15	3.96 (0.60)	4.09 (0.23)	3.38 (0.51)	3.81
	$\bar{\bar{X}}$	3.27	3.53	2.96	3.25
Normally Innervated Blastemata	9	1.84 (0.56)	1.75 (0.56)	1.34 (0.38)	1.71
	12	3.82 (0.11)	3.92 (0.18)	3.18 (0.20)	3.64
	15	3.54 (0.26)	3.12 (0.12)	3.12 (0.20)	3.26
	$\bar{\bar{X}}$	3.21	3.02	2.63	2.96

Appendix 9.--Means and Standard Errors for Mitotic Indices of Longitudinally Sectioned Blastemata Sampled along the Longitudinal Axis.

	Days Post-Amputation	Sample Sites: Distal	Middle	Proximal	$\bar{\bar{X}}$
Superinnervated Blastemata	9		0.00 (0.00)	2.16 (0.92)	1.80
	12	2.38 (1.06)	2.23 (0.52)	2.92 (0.38)	2.51
	15	1.71 (0.54)	3.05 (0.52)	2.27 (0.38)	2.40
	$\bar{\bar{X}}$	2.16	2.33	2.49	2.35
Normally Innervated Blastemata	9		1.07 (1.07)	0.86 (0.55)	0.94
	12	2.00 (0.43)	1.38 (0.52)	1.45 (0.46)	1.76
	15	2.35 (0.33)	1.86 (0.26)	2.12 (0.66)	2.11
	$\bar{\bar{X}}$	2.18	1.67	1.43	1.69

Appendix 10.--Means and Standard Errors for Cellular Densities of Longitudinally Sectioned Blastemata Sampled along the Longitudinal Axis.

	Days Post-Amputation	Sample Sites: Distal	Middle	Proximal	$\bar{\bar{X}}$
Superinnervated Blastemata	9		1.53 (0.00)	0.99 (0.14)	1.08
	12	1.75 (0.32)	2.54 (0.29)	2.49 (0.14)	2.26
	15	3.08 (0.12)	3.15 (0.30)	3.43 (0.13)	3.23
	$\bar{X}$	2.12	2.67	2.24	2.36
Normally Innervated Blastemata	9		0.90 (0.40)	0.94 (0.14)	0.93
	12	3.46 (0.62)	2.54 (0.38)	2.45 (0.29)	2.77
	15	3.66 (0.35)	3.11 (0.15)	3.27 (0.31)	3.35
	$\bar{X}$	3.56	2.32	2.15	2.54

Appendix 11.-- Means and Standard Errors for Percent Labelled Cells of Transversely Sectioned Blastemata Sampled along the Transverse Axis.

	Days Post-Amputation		Sample Sites: Medial	P-Medial	Lateral	$\bar{\bar{X}}$
Super-Innervated Blastemata	12		10.27 (3.93)	8.97 (2.99)	18.05 (2.26)	11.73
	15		10.71	9.79	20.58	13.69
		$\bar{\bar{X}}$	10.38	9.18	18.89	12.26
Normally Innervated Blastemata	12		16.14 (1.37)	24.26 (7.63)	33.37 (1.80)	24.59
	15		8.39 (1.50)	10.28 (2.47)	10.60 (5.88)	9.76
		$\bar{\bar{X}}$	12.26	17.27	21.98	17.17

Appendix 12.--Means and Standard Errors for Percent Labelled Cells of Transversely Sectioned Blastemata Sampled along the Longitudinal Axis.

	Days Post-Amputation	Percent Blastemal Length 100%	75%	50%	25%	$\bar{\bar{X}}$
Super-Innervated Blastemata	12	2.54 (0.90)	8.59 (2.52)	7.77 (2.19)	16.49 (4.65)	8.71
	15	0.00	20.58	9.73	14.32	11.16
	$\bar{\bar{X}}$	1.69	11.59	8.26	15.77	9.41
Normally Innervated Blastemata	12	3.70 (3.70)	10.72 (2.03)	18.79 (6.72)	30.78 (2.72)	17.12
	15	3.94 (3.94)	8.58 (3.50)	12.38 (4.22)	8.65 (3.45)	8.79
	$\bar{\bar{X}}$	3.82	9.65	15.59	19.72	12.95

Appendix 13.--S/M Ratios for Superinnervated and Normally Innervated Blastemata Sampled along the Transverse Axis.

Innervation	Days Post-Amputation 12	15	$\bar{\bar{X}}$
Super	0.91	2.88	1.40
Normal	3.60	3.04	3.32
$\bar{\bar{X}}$	2.25	3.00	2.55

Appendix 14.--S/M Ratios for Superinnervated and Normally Innervated Blastemata Sampled along the Longitudinal Axis.

Innervation	Days Post-Amputation 12	15	$\bar{\bar{X}}$
Super	0.72	2.16	1.08
Normal	2.13	2.24	2.17
$\bar{\bar{X}}$	1.43	2.22	1.74