AN ANALYSIS OF APICAL PROLIFERATION IN THE FORELIMB REGENERATION BLASTEMA OF THE AXOLOTL AMBYSTOMA MEXICANUM

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY

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1966

This is to certify that the

thesis entitled

An Analysis of Apical Profileration in the Forelimb Regeneration Blastema of the Axolotl, Ambystoma mexicanum.

presented by

Lester Leroy Hearson

has been accepted towards fulfillment of the requirements for

Ph. D. **degree in** Zoology

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Date March 29, 1966

ABSTRACT

AN ANALYSIS OF APICAL PROLIFERATION IN THE FORELIMB REGENERATION BLASTEMA OF THE AXOLOTL AMBYSTOMA MEXICANUM

by Lester L. Hearson

It has been demonstrated in this study using the precise experimental methods of mitotic index calculations and statistical analyses that an apical dominance in mitotic activity exists among the most distally located mesenchymatous cells of the forelimb blastema of the axolotl Ambystoma mexicanum. The high apical mitotic rate was evident throughout the rapid growth phase of the blastema (10 to 16 days of regeneration) and produced a dense apical mass of mesenchymatous cells. As proposed by Faber (1960, 1965) this apical dense mass of proliferating mesenchymatous cells may be regarded as the "apical proliferation center."

Regional patterns in mitotic activity and cell density were also looked for using regenerating asymmetrical blastemata of the axolotl and regenerating aneurogenic blastemata of Ambystoma opacum. An apical peak in the mitotic activity of the mesenchymatous cells was also found for these regenerating limbs.

In the axolotl forelimb blastema a striking temporal relationship which might represent a causal relation, was found to exist between the mitotic activity of the wound epithelium and the initial accumulation and regional distribution of mitoses of the underlying blastemal cells. A similar relationship in the mitotic activities between the wound epithelium and the mesenchymatous cells in the asymmetrical and aneurogenic blastemata was observed.

An additional part of this study was a check on the mitotic distributions of blastemal cells with relation to the major regenerating nerve trunks of the limb stump. No significant differences in mitotic activity between those regions surrounding the major nerve trunks and those which did not could be demonstrated.

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Zoology

1966

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Dr. Charles S. Thornton for suggesting the problem and also for his interest, direction, and encouragement during the course of its investigation.

Thanks are also extended to Dr. G. B. Wilson and Dr. J. R. Shaver for their helpful suggestions and for serving as committee members. Acknowledgment is made to Dr. R. N. Band for service as a committee member.

Special thanks are extended to Dr. W. E. Cooper for his interest and assistance in the statistical analysis and to Mrs. Mary Thornton for her technical assistance.

Special recognition and appreciation is made to my wife, Patricia, whose encouragement and sacrifices have made the completion of this study possible.

This research was supported by a Grant (GB-2618, National Science Foundation) administered by Dr. Charles S. Thornton.

TABLE OF CONTENTS

		Page
ACKNOWLEDGMENTS		ii
LIST OF TABLES		iv
LIST OF FIGURES		v
INTRODUCTION		1
MATERIALS AND METHODS		20
RESULTS		34
Series I:	General Morphology and Histology of the Regenerate	34
	The Wound Epithelium	38
Series II:	Analysis of Blastema Cell Proliferation along the Longitudinal Axis	41
Series III:	Analysis of Mitotic Activity within Regions of the Blastema Studied in Cross Section	53
Series IV:	Analysis of Mitotic Activity within Asymmetrical Blastemata	60
Series V:	Analysis of Mitotic Activity within Aneurogenic Limbs	65
DISCUSSION		69
SUMMARY		81
LITERATURE CITED		98

LIST OF TABLES

Table		Page
1	Changes in mitotic activity within the epidermis of the forelimb of the axolotl for three regeneration periods .	39
2	Two-way analysis of variance between regeneration times and zones along the vertical axis of the forelimb blastema of the axolotl	44
3	Two-way analysis of variance between regeneration times and regions of the forelimb blastema of the axolotl studied in cross section	57

LIST OF FIGURES

Figure		Page
1a	Diagrammatic drawing of the forelimb blastema illustrating the five sample levels for the cell counts	83
1b	Diagrammatic drawing of a center section of the forelimb blastema showing the four sample areas, A, B, C, and D for the cell counts	83
2	Twelve day aneurogenic blastema showing numerous apical mesenchymatous cell divisions	85
3	The apical wound epithelium of a 10 day axolotl regenerate possessing numerous epidermal cell divisions	85
4	A 13 day axolotl regenerate showing the intense mitotic activity of the apical mesenchyme	85
5	The prominent apical epidermal lobe of a 16 day axolotl regenerate	85
6	Longitudinal section of the "early bud stage" (10 day blastema) illustrating the arbitrary vertical zones	87
7	Longitudinal section of the "mound stage blastema" (13 days) showing the vertical zoning	87
8	Longitudinal section of the "cone blastema" (16 days of regeneration) showing the vertical zoning	87
9	Longitudinal section of the 19 day regenerate "paddle stage" showing the vertical zoning	87
10	Cross section near the distal tip of a 13 day blastema (early bud stage) . . .	89

Figure		Page
11	Sixteen day asymmetrical blastema illustrating the sample areas for the cell counts	89
12	Cross section near the center of a 13 day blastema showing the 5 regions for the cell counts	89
13	Cross section near the base of a 13 day blastema	89
14	Graphic representation of the mitotic index and cell density distributions of the 4 longitudinal zones for the 4 regeneration stages	91
15	Graphic representation of the mitotic index and cell density distributions of the 5 cross sectional regions for the 4 regeneration stages	93
16	Graphic representation of the mitotic index and cell density distributions of the 4 sample areas for the 3 regeneration stages of the asymmetrical limb blastema	95
17	Histogram showing the mitotic index and cell density distributions for the base and tip areas for the 4 aneurogenic blastemata	97

INTRODUCTION

The epimorphic regeneration of an amputated limb in urodele amphibians is dependent on the formation and differentiation of a regeneration bud or blastema. A significant problem in blastema formation has been the source of origin of its constituent cells. Several origins have been proposed and investigated during the last several decades. These have included the concept of an epithelial contribution to the underlying blastema (Godlewski, 1928; Rose, 1948a; Hay, 1952; and Rose, Quastler and Rose, 1953, 1955); a hematogenic contribution, (Hellmich, 1930, 1931; Kazancev, 1934; and Ide-Rozae, 1936); a connective tissue contribution, (Mettetal, 1939; Luther, 1948; Liebman, 1949; Manner, 1953; and Glade, 1963); a reserve cell contribution, (Brunst and Cherometieva, 1936; Weiss, 1939; and Needham, 1942); and a general contribution by a "dedifferentiation" of the stump tissues, (Thornton, 1938a, 1938b, 1942; Butler and O'Brien, 1942; Chalkley, 1954; Holtzer, et al., 1957; Hay, 1958, 1959; Bodemer and Everett, 1959; Hay and Fischman, 1960, 1961).

Godlewski observed for the salamander axoloti limb a reserve of indifferent cells located in the basal layer of the epidermis. In his opinion, these cells, following amputation, were stimulated to migrate internally and give rise to the blastema.

Rose, ~~(1948a)~~ described ~~tongues of~~ wound epithelial cells entering the developing blastema of the amputated amphibian limb and believed they ~~subsequently~~ eventually transformed into mesenchymatous cells of regeneration. He came to this conclusion as a result of observing that ~~supra~~vital staining of the wound epidermis subsequently gave rise to stained mesenchymatous cells. Furthermore, measurements of cell numbers in the epidermis and subepidermal regions at the time of young blastema formation revealed a sudden decrease in epidermal cells and the corresponding rapid increase in mesenchymatous cells. Finally, when the limb ~~of the newt~~ was exposed to x-rays, subsequent regeneration of the limb was reported after replacing the irradiated limb skin by unirradiated epidermis ~~(Rose, Quastler, and Rose, 1953). They~~ concluded that only epidermal cells could have contributed the blastema cells of regeneration.

The evidence of Hay, (1952) for an epidermal origin of mesenchymatous tissue was derived from the identification of cells in the early blastema which were apparent migrants from heteroploid skin transplants. In later blastemal stages, however, heteroploid cells could not be distinguished in the undifferentiated part of the blastema nor the differentiating internal tissues.

A number of investigators have re-evaluated the epidermal hypothesis and have concluded that such a contribution is highly unlikely. Heath, ~~(1953), for example~~, considered the epidermis a negligible source of blastemal

elements, basing his conclusion upon evidence from differential growth rates of ~~chimaeric~~ amphian limbs created by exchanges of epidermal and subepidermal limb-bud tissues between embryos with ~~markedly~~ different growth potentials. ~~Here~~, the experimental ~~design~~ provided evidence that the control of the growth rate of the organ resides ~~chiefly~~ in the limb mesoderm and ~~rules against~~ not the participation of the wound epidermis in blastemal formation.

The hypothesis of an epidermal origin of the developing blastema is also not supported by the work of ~~Manner~~, (~~1953~~). Through histological study and cell counts Manner was unable to reveal any evidence for an epidermal ingression but indicated instead a major contribution to the early blastema by fibroblasts. He emphasized a cytological similarity of the fibroblasts and the cells of the epidermal stratum germinativum and noted this similarity of character might easily engender confusion as to blastemal cell source.

Chalkley ~~has provided an excellent systematic study~~, (~~1954~~) and review, (1959) of the mechanism of ~~the production and accumulation of cells during blastema formation~~. He could detect no loss-gain relationship between epidermal and subepidermal components. The ~~abrupt~~ sudden rise in the number of subepidermal cells and the comparable drop in the number of cells in the overlying epidermis, as reported by Rose for the early blastema, could not be ~~corroborated~~ confirmed. ~~Chalkley has suggested that losses in epidermal cell numbers were probably a function of~~ desquamation rather than cellular ingressions.

The most convincing evidence against the epidermal hypothesis can be derived from Chalkley's discovery that dedifferentiating proximal mesodermal tissues become mitotically active during the very early phases of regeneration and at an equally early time cells produced by this mitotic proliferation migrate distally and accumulate to form the regeneration bud. This phenomenon can easily account for the increase in blastemal cell numbers.

Chalkley (1954) has interpreted epidermal intrusions into the underlying blastema as simply mechanical infoldings resulting from amputation. Other investigators, (Taban, 1955; Scheuing and Singer, 1957; Bodemer, 1958; Kamrin and Singer, 1959; Singer and Salpeter, 1961) have observed internal movements in the form of long chains or cords and in some cases dis-associations of epidermal cells. For the majority of these workers and especially Singer and Salpeter (1961) such responses are believed to be a measure of the sensitivity of the wound epidermis to the topographical and chemical composition of the wound tissues. Indeed, epidermal cells, singly or in clusters, have been observed to encapsulate and engulf cellular debris and foreign matter and to subsequently extrude these through the skin. It is the opinion of Singer and Salpeter (1961) that reactivity of this type, although common to all epithelia, is a more highly developed feature of the wound epidermis.

14

It is also of interest to note that Luther (1948), following distal irradiation of intact tails, observed that the dying irradiated epidermal cells were replaced by unirradiated proximal epidermal cells which were accompanied by subepidermal cells. A similar phenomenon could very well account for the results of Rose, Quastler, and Rose (1953).

Similarly, such a phenomenon would provide an alternative explanation to the results of Hay (1952). Indeed, Hay and Fischman (1961) have considered this possibility since, as they indicate, polyploid fibroblasts were components of the proximally located skin grafts used in Hay's 1952 work.

Perhaps the most damaging evidence against the epidermal origin of blastemal cells is that of Riddiford ~~(1960) and Hay and Fischman (1961).~~ He Riddiford exchanged labeled epidermis for unlabeled epidermis between labeled and unlabeled regenerates. The unlabeled epithelium covering the labeled blastema gradually became labeled ~~presumably~~ probably by movements of cells ~~and cellular debris~~ into the epidermis from below. In no instance did the ~~reverse~~ movement of label from the wound epithelium into the blastema take place. ~~In totum~~ all in all there appears to be little evidence in support of the epidermal hypothesis.

A second hypothesis concerning the origin of the blastema ~~which chronologically preceded the epithelial concept~~, was the idea of a hematogenic contribution ~~(Colucci, 1884, cited in Chalkley, 1959). Such a source was also suggested by Hellmich, (1930, 1931); Kazancev, (1934) and~~

~~Ido-Rozas, (1936)~~. Hellmich (~~1930~~) distinguished ~~on purely histological grounds~~ two general kinds of cellular components within the blastema--hematogenic and histogenic. For Hellmich, these types: erythrocytes, lymphocytes, ~~eosinophils~~, ~~special leucocytes~~, plasma cells, fibrocytes, wandering cells, mast cells, pigment cells, giant cells, and pericytes were all indispensable components for the restoration of the missing structure. Whatever the regenerating part, it was thought by Hellmich that regeneration was possible only through the physiological cooperation of the entire organism.

The idea that leucocytes and other elements of the ~~general~~ circulation were the source of blastemal cells was not supported by the irradiation experiments of Butler ~~(1933, 1935); Brunst and Cheremetieva (1936); Butler and O'Brien (1942) and Burnst (1950).~~ ~~Butler~~ ~~(1933, 1935)~~ he was able to show that larval Ambystoma limbs ~~situated~~ in shielded regions during x-ray exposure of the remaining body retained their ~~capacity~~ to regenerate. ~~Likewise,~~ unirradiated limbs transplanted homoplastically to irradiated hosts, entirely ~~incapable of~~ regenerat~~ion~~, retained the ability to regenerate following amputation.

~~In a similar manner Brunst and Cheremetieva (1936);~~ Butler and O'Brien ~~(1942); and Brunst (1950)~~ demonstrated that failure to regenerate occurs only in that region of the ~~appendage~~ actually exposed to x-rays. ~~Butler and O'Brien (1942)~~, for example, destroyed by local x-ray treatment the

regeneration capacity in the knee-joint region of the Amby-stoma larval limb while shielding the other portions of the limb. No regeneration occurred following amputation through the exposed part while normal regeneration was observed in the proximal and distal parts of the limb when subsequent amputations were performed in these regions. Conversely, Brunst and Cheremetieva (1936) destroyed by localized irradiation the regenerative capacity in both the proximal and distal parts of the limb, although the regenerative ability of a small intermediate protected area of the same limb was unaffected. Brunst (1950) found, as did Butler earlier (1933, 1935) for transplanted limbs, that normal regeneration buds when transplanted to treated body parts could regenerate normally.

Earlier, Hertwig (1927) obtained similar results by the simple technique of transplanting haploid limbs to limg stumps of diploid hosts. The resultant blastema was formed mainly from haploid elements.

The foregoing experiments provide proof beyond reasonable doubt that blastemal cells must be the products of tissue components in the immediate vicinity of the amputation surface. Thus, the evidence is clearly contrary to the idea that body parts in general contribute cellular elements to the regeneration site. How is it then that local stump tissue components give rise to blastema cells? Weiss (1939) has suggested that "reserve cells" apparently "left over" from embryonic development and situated somehow among

the formed tissues of the stump, are stimulated by amputational trauma to proliferate and form the blastema. To date, numerous detailed histological surveys have failed to reveal any such store of reserve elements. Their existence in Amphibia remains purely hypothetical.

The most widely held [redacted] theory of blastemal cell origin is that of redifferentiation of stump tissues. The injured cells of the mesodermal tissues of the amphibian limb, [redacted], undergo loss of histological structure during the first few days following amputation and "spill out" cells, [redacted], which can be traced into the blastema.

Thus, David (1934) followed distal loss of structure in the injured limb cartilage and described migration of cells, released by the histolysis of their capsules, into the developing blastema of the axolotl limb. These cells were subsequently thought to reconstitute the missing skeletal structures.

Thornton (1938a, 1938b) in a careful and detailed histological investigation of Ambystoma limb regeneration described a multiple origin for the blastema involving the redifferentiation of cartilage, nerve sheath, perichondrium, muscle and general connective tissues. For example, he described a dissolution of the distal muscle fibers with separation of muscle cell nuclei surrounded by scanty cytoplasm which could be followed (by light microscope observation) into the developing blastema. In subsequent years there

followed a variety of studied focusing upon the mesodermal hypothesis (Thornton, 1942; Chalkley, 1954; Hay, 1958, 1959; Holtzer, et al., 1957; Bodemer and Everett, 1959; Hay and Fischman, 1960, 1961).

Thornton (1942) gained confirmatory evidence for his earlier conclusions on dedifferentiation by implanting unirradiated limb and tail musculature (muscle and also connective tissue) into irradiated limb stumps of adult Triturus. The transplanted musculature not only underwent partial dedifferentiation to form blastemal cells but was shown to have directed the development and differentiation of the formed blastema. It should be noted, however, that not only muscle per se, but also connective tissue elements took part in regeneration.

Chalkley (1954) by means of quantitative studies (cell counts) has proposed a tissue specific origin for the blastema. He indicated a substantial cellular contribution from the general connective tissue (fibroblast, etc.) and lesser contributions from other mesodermal components such as muscle, cartilage, perichondrium, etc. In his opinion, limited dedifferentiation, proliferation, and migration of proximal stump elements provided for the early growth of the blastema. Similar findings were reported by Manner (1954). Hay (1958, 1959) has confirmed and extended by electron microscopic analysis the earlier light microscope work of Thornton (1938a). Her studies show, for cartilage and muscel at least, that differentiated cell types can

lose their characteristic cytological properties and become mesenchymatous like regenerate components.

Criticisms (see Manner, 1953) that tissue "dedifferentiation" is really only tissue degeneration are met by the experiments of Bodemer and Everett (1959) and Hay and Fischman (1961). Using isotope labeling they demonstrated that as limb stump tissues dedifferentiate, the resulting cells enter into a phase of protein and nucleic acid synthesis, preparatory to active cell proliferation, which, of course, dying cells would not be expected to do.

An additional technique which has proved to be a valuable tool for investigating blastemal cell origins is that of fluorescent antibody labeling of specific components of stump tissues. Using fluorescent anti-myosin as label, Holtzer, Marshall and Finck, (1957) were able to trace dedifferentiated muscle cells into the blastema. Unfortunately, the label was not detectable during the period of blastemal cell proliferation (from approximately the 8th to the 12th day after amputation) after which the fluorescent label of differentiating muscle cells could again be detected. The 4 day gap in labeling thus prevents one from establishing the possibility of metaplasia in regeneration but does clearly answer the question of muscle participation in blastema origin.

It is now generally accepted by the majority of investigators that tissue dedifferentiation is a mandatory prerequisite to the appearance of a blastema. Thus, all

stump tissues ~~with the exception of the epidermis, blood elements~~ and ~~nerve tissue proper~~ provide cells of general ~~mesenchymatous~~ morphology for early blastemal growth. ~~The genetic potentialities of these cells is still in question and thus~~ the ~~problem of a possible metaplasia is yet to be established~~.

Whatever the ~~specific~~ origin of the blastema may be, it is clear that some kind of "blastema-forming" activity must ~~be in operation~~ take place if regeneration is to occur. Thus, stump tissue dedifferentiation and the appearance of mesenchymatous cells are ~~not in themselves sufficient~~ enough to achieve the regeneration of a salamander limb. These events all occur in the non-regenerating limb of the adult frog--histolysis of stump tissues and appearance of mesenchymatous cells--yet no blastema ever forms and ~~consequently~~ therefore regeneration fails. Even the salamander limb can be prevented from blastema formation and, thus, regeneration by ~~the simple experiment of~~ substituting head skin for the limb skin. Even though after amputation ~~through the skin graft tissue~~, dedifferentiation may occur, no blastema forms, and regeneration fails in a limb ~~which possesses the potentiality of regenerating (Thornton, 1962).~~

In both the frog limb and the salamander limb with head skin, wound healing ~~of the amputation surface~~ is abnormal in that the epidermal cells involved do not lose their fully developed histological characteristics. No wound epithelium consisting of epidermal cells of "dedifferentiated" morphology is produced. As a result ~~of a long~~

~~series of investigations~~, Thornton (~~1957-1965), has~~ developed the theory that the wound epithelium in some way ~~exerts an influence~~ on the aggregation and development of a blastema.

From experiments performed on tadpoles (Rana) ~~and Ambystoma~~ larvae, Thornton ~~(1954, 1956) has~~ ~~described~~ a distinct thickening of the wound epithelium, which he ~~has termed~~ the "apical cap," overlying the area where the dermis has been interrupted. ~~Subsequent to~~ this thickening and immediately ~~underlying~~ it develops the early accumulation of blastemal cells. The ~~temporal~~ relationship between these two ~~phenomena~~ appeared significant.

Insight into the ~~indispensable~~ nature of the "apical cap" was gained through two simple ~~but clear-cut~~ experiments (~~Thornton, 1957, 1958~~). In ~~these~~ experiments there was a total failure of blastemal cell accumulation following ~~conditions of~~ either "cap" removal or "cap" treatment with localized ultraviolet irradiation. Also, by experimentally inducing the formation of eccentric apical caps in ~~both~~ normal ~~and excessively regressed~~ larval limbs, Thornton (~~1960~~) produced ~~correspondingly~~ eccentric blastemata. Eccentric apical caps, however, may be innervated by eccentric sensory nerve fibers which could serve as migration pathways for mesenchymatous cells. It follows from this that aneurogenic limbs with eccentric apical caps should show no eccentric blastema formation. However, Thornton and Steen (1962) demonstrated that aneurogenic limbs did produce eccentric blastemata directly correlated with eccentric

apical caps. Therefore, nerve axons are not necessary for directing cells beneath the epidermal cap.

Also
~~Finally~~, Thornton ~~and Thornton~~ (~~1965~~) transplanted epithelial caps ~~autoplastically~~ to the base of developing blastemata. Successfully grafted caps were found to support secondary blastemal development and ~~supernumerary~~ limb parts were differentiated. Thus, the ~~wound skin both in the normal and aneurogenic regeneration milieu has~~ apical caps have specifically been indicated as having an essential role in blastemal formation.

In addition to the concept that the wound epidermis functions to "direct" the accumulation ~~to~~ of blastemal cells, a number of other theories concerning its ~~role~~ function in regeneration have been suggested. ~~These include~~ Among these are histolysis, mechanical regulation and the control of morphogenesis. Of particular interest ~~in this connection~~ is the ~~recent~~ suggestion by Faber (~~1960~~) that the apical wound epidermis might play a role in the ~~establishment~~ formation of an "apical proliferation center" among the ~~most distally located~~ more distant mesenchyme~~atous~~ cells of the blastema.

Once the regenerating blastema is formed, its further growth has been ~~generally~~ considered to be by means of the rapid proliferation of its constituent cells. Not much attention has been focused on the patterns of mitosis within the various regions of the blastema, although studies ~~of mitotic proliferation~~ of the blastema as a whole have been made, ~~(Litwiller, 1939; Polezajew and Ginsburg, 1943; Forsyth, 1946; Manner, 1953; Chalkley, 1954).~~ However, some

studies of regional growth patterns in embryonic limb buds have been completed. Thus, the anuran hindlimb bud (Tschumi, 1955, 1956, 1957) and the avian limb bud (Saunders, 1948; Zwilling, 1956), as well as regenerating fin of the fish (Haas, 1962) are believed to grow by apical proliferation. The phenomenon of apical proliferation points up another parallel in the mode of growth which exists between regeneration blastemata and embryonic and larval limb buds. A most striking feature in all of these systems is the presence at the apex of the growing rudiment of a thickening of the epithelium.

The characteristic properties of this thickening has been variously described by a number of investigators. Thornton (1954) has described an "apical cap" for regenerating limbs, while Tschumi (1955), Saunders (1948) and Zwilling (1956) have observed a thickening in the form of an "apical ectodermal ridge" for developing limb buds. An ectodermal ridge has as well been demonstrated for the limb buds of some reptilian and mammalian forms (Hinrichsen, 1956; Milaire, 1956, 1957; O'Rahilly, Gardner and Gray, 1956). Lastly, Faber (1960) has described a distinctive "epidermal lobe" at the apex of the regenerating forelimb in Ambystoma mexicanum. He has noted a possible causal relationship between the appearance of the "apical epidermal lobe" and the active proliferation of the underlying mesenchyme.

It is necessary to note here that the experiments of Tschumi (1951), Saunders (1948), Faber (1960) and others are suggestive rather than conclusive concerning the existence of a specific proliferation center. In all cases, both in regenerating blastemata and developing limb systems, the criterion of apical growth has been the localization of charcoal particles which were forced into the mesenchyme at the apex of the bud. Since individual cells, and therefore mitotic cells, are not marked by this technique, it cannot represent a precise measure of the actual patterns of cellular activity within the developing structure, a matter of considerable importance, for, as suggested by Faber (1960), such a proliferation center may constitute an autonomous organizational center which controls in some way the regional organization of distal limb parts. Through ~~a variety of~~ experiments performed with forelimb blastemata of the axolotl it has been shown ~~(Faber, 1960, 1962, 1965; Michael and Faber, 1961)~~ that the mesenchyme of the blastema isolated from the stump ~~acquires differentiation tendencies~~ becomes differentiated for structures which are ~~markedly~~ more distal than those which they normally would have produced. Thus, blastemata at various stages of development ~~as well as proximal and distal halves of palette stage~~ blastemata and proximal halves ~~of the palette stage~~ blastemata with a reversal of the ~~proximo-distal polarity~~, were transplanted to a ~~neutral~~ site ~~(pocket)~~ on the back of the animal. In all of these transplants, ~~and especially~~ the ~~noninverted~~ and inverted ~~proximal halves~~ of

the palette stage, there occurred dedifferentiation and disorganization of the developing tissues of the regenerate. Subsequently, as determined in most instances by carbon marking, apical proliferation was initiated and the grafts produced normally oriented limb outgrowths. The striking feature of these experiments was that the grafts predominantly formed distal structures (digits) for which, at least in the case of the early blastema stages, and proximal halves, no rudiments were present. Thus, as described by Faber, (1960), the distal predominance must have resulted from the action of an "apical organization center" or an organizing principle which was responsible for the self-organization of the mesenchyme. Faber, (1960) has, in addition, proposed that the "apical organization center" might be identical with the "apical proliferation center"; thus, cells formed in the apical proliferation center by division of dedifferentiated cells would intrinsically possess distal differentiation tendencies.

An important feature in all of these experiments was the fact that the self-organization of the transplanted mesenchymatous material was always associated with the occurrence of apical proliferation. Mesenchymatous cell proliferation, in turn, appeared to depend upon the covering of the wound surface by the wound epidermis, which was supplied either from the transplanted limb section itself or from the back skin at the transplantation site. Therefore, as has previously been demonstrated by Thornton

(1956-1958), a wound spithelial covering devoid of the underlying dermis, was necessary for regeneration to proceed. In view of this fact, Faber (1960) has also proposed that the wound epidermis, in some unknown manner, initiates the appearance from among the distal mesenchymatous cells of a center of proliferation and organization which functions to produce and organize, at least for the distal structures, the materials of the developing limb.

In view of this hypothesis it is apparent that a re-examination of apical proliferation is needed using more precise methods than charcoal marking, so that a clear and unequivocal picture of the blastemal cell proliferation patterns can be obtained. Although there have been a number of studies of the mitotic proliferation in the blastema, none have focused upon the patterns of the mitotic activity.

The most significant work to date on the regional distributions of mitoses was presented by Chalkley (1954) for the newt, but this study was primarily concerned with the problem of tissue dedifferentiation and the origin of blastemal cells. Thus, for both epidermal and mesodermal tissues, Chalkley has described a gradual distal shift from the limb stump to the blastema in both cell number and mitotic index. It was not until the 19th day, however, that the peak of miotic activity was totally resident within the blastema. At this time the mesenchymal mitotic index was highest in roughly the distal half of the blastema with the

peak of mitotic distribution still extending into the stump portion.

In an earlier study (Litwiller, 1939) of the blastema of the Japanese newt, a peak of mitotic activity was demonstrated for the base of the young blastema which shifted distally in older regenerates. The gradual shift in mitotic intensity within the blastema was associated with and preceded differentiation. Only in a vague manner were recurrent proximo-distal mitotic patterns between regeneration stages made evident in this work.

Polezajew and Ginsburg (1943), Forsyth (1946) and Manner (1953) have shown that mitosis of the mesenchyme cells is infrequent in the very early blastema, but that it increases rapidly and reaches a high degree of intensity during the period of rapid blastemal growth. Although no attempt was made to reveal mitotic patterns within the blastema, Polezajew and Ginsburg (1943) demonstrated for the axolotl a maximum in cell divisions at the cone stage, which then decreased and remained relatively constant until digital indentations were formed.

The present investigation, therefore, was undertaken to examine the regional distributions of mitoses within the blastema and to test experimentally by mitotic index calculations the hypothesis proposed by Faber (1960) that there exists within the distal mesenchyme in the limb blastema of the axolotl an "apical proliferation center."

The investigation was attempted in addition to determine if a correlation might, as suggested by Faber (1960), exist between the appearance of the apical epidermal lobe and the mitotic proliferation patterns within the blastema.

MATERIALS AND METHODS

Larvae of the Mexican axolotl Ambystoma mexicanum and larvae of Ambystoma opacum were used in this investigation. Axolotls were reared from eggs or newly hatched larvae kindly supplied by Dr. R. R. Humphrey of the Department of Zoology of the University of Indiana. The Ambystoma opacum larvae were reared from eggs obtained from Mr. Glenn Gentry of the Tennessee Conservation Commission.

The axolotls were fed brine shrimp and Enchytrae worms during their early growth phases and were later hand fed beef liver 3 times weekly. Animals were maintained in individual plastic containers in a Model 805 Precision Incubator at 20 $\pm$ .5 degrees C. during the experimental tests.

The Operations

Axolotl larvae of 4 to 6 centimeters snout-anal length were anesthetized in a 1:2,000 concentration of MS 222 (Tricaine methanesulphonate, Sandoz), and both forelimbs were amputated through the elbow under the binocular dissecting microscope. Following the initial amputation, which resulted in the retraction of the soft tissues, the protruding cartilage was reamputated so as to provide as nearly as possible an even amputation surface perpendicular to the limb axis. The remaining humerus stump comprised

approximately three fourths of its original length. Since it is possible that the mitotic activity of regenerating amphibian limbs might undergo daily periodic fluctuations all amputations were carried out on predetermined dates at 12:00 M. Indeed, Litwiller (1940) while studying regeneration in the forelimb of the newt (Triturus pyrrhogaster), discovered a daily rhythm in the mitotic rate which corresponded with specific lighting conditions. He found, for example, high mitotic peaks at 12:00 M. under the conditions of either constant darkness or normal day-night illumination. In contrast, the 12:00 M. mitotic peak was depressed considerably and a new peak occurred at mid-night under constant illumination. The light source in this experiment was a 60 watt light bulb suspended at a distance of 5 feet above the animal containers.

Cameron (1936), on the other hand, found no correlation between mitotic indices and the time of day in Ambystoma larvae and Rana tadpoles.

The larvae used in this study were kept under conditions of low intensity, constant illumination in order to obtain maximal feeding and growth. The light source used was a 10 watt bulb located 30 inches above the animals which were maintained in plastic containers housed on, and covered with, 18" x 24" metal trays. Under these conditions, those animals closest to the light source (at the periphery of the trays) were exposed to 1 footcandle of light as measured by a Weston Model 416 light meter. This situation

cannot be compared to the conditions reported by Litwiller (1940) since he did not describe his experimental method. Measurements in this laboratory indicate the light intensity of a 60 watt bulb at a distance of 5 feet to be 6.5 foot candles of light. As will be explained below, the average mitotic index at the apex of rapidly growing blastemata of the axolotl (16 days regeneration) is 3.0 percent under low constant light conditions. These results indicate either that light has no inhibiting effect on the mitotic activity of axolotl blastema cells or that the light source utilized was of an insufficient intensity to cause any alteration in the mitotic patterns. To test the possibility, however, that the light intensity employed might have affected the mitotic activity at 12:00 M., a small group of 6 animals was kept in constant darkness (except for feeding periods) for a 16 day regeneration interval. The mitotic indices of these animals were then compared with 16 day regenerates maintained under one foot candle of constant illumination. No differences in the pattern of mitotic activity could be detected between the two groups allowing for normal range of variability.

For the present study, 4 series of regenerating axolotl limbs were prepared for histological examination. Group I comprised axolotl forelimbs of post-amputation periods between one and 25 days. Daily camera lucida drawings were made of the regenerating limbs in order to provide records of the gross morphological changes for this series.

Limbs were fixed in Bouin's fluid and sectioned at 10 microns parallel to the long axis. Sections were stained for general histological features by Heidenhain's iron-hematoxylin method.

Series II included 10, 13, 16, and 19 day forelimb regenerates. Limbs were fixed in Zenker's (acetic acid) fluid and differentiated for chromosome detail by means of the Feulgen method. Longitudinal sections of the paraffin mounts were prepared at 10 microns. For sectioning, the limbs were not deliberately oriented with respect to the dorso-ventral, anterior-posterior axes.

In series III regenerated axolotl forelimbs were amputated and fixed beginning at 10 days and thereafter at 3 day intervals up to 19 days post-amputation. Limbs were fixed in either Pienaar's or Zenker's fixatives and sectioned at 10 microns. Sectioning was at right angles (cross sectioned) to the dorso-ventral axis of each limb. Preparations were stained with Schiff's reagent (Feulgen technique) for the localization of DNA.

For series IV, axolotl limbs were allowed to regenerate for 14, 16, and 18 day periods. During the regeneration period, the established epidermal cap of these limbs was shifted to an accentric position according to the technique of Thornton (1960). The cap was shifted from its normal position at the apex of the limb stump to the anterior edge of the initial amputation surface by removing a narrow strip of skin (dermis and epidermis) approximately 1.5 mm.

wide and .6 mm. in length running parallel and adjacent to the anterior border of the amputation surface of the limb. Operated limbs were approximately 9.5 mm. in diameter. The first skin removal was at 7 days post-amputation when the apical cap first becomes established and before a blastema has formed, and the procedure was repeated once, 2 days later to insure an adequate cap shift in these large limbs. Correspondingly eccentric blastemata were subsequently formed at the tips of these limb stumps. Zenker's fixative and Schiff's reagent were used for these limbs. Longitudinal sections were prepared at 10 microns, the plane of sectioning being as nearly parallel to the long axis of the eccentric blastema and limb stump as possible.

In series V, (Ambystoma opacum), aneurogenic forelimbs were produced (see Thornton and Steen, 1962) and at the 4 digit stage were amputated at the elbow and fixed from 10 to 16 days post-amputation in Zenker's fluid and stained for DNA localization with Schiff's reagent. These limbs were sectioned longitudinally at 10 microns.

The Cell Counts

Since the primary focus of this study is on blastemal cell proliferation, considerable care has been devoted to the method of counting cells and mitotic figures. For counting nuclei and as a basis for cell density determinations, an ocular grid ruled into 100 square units was used. Each grid unit, at 225X, was found to be .027 mm. on a side.

When the grid was superimposed over a field of the blastema (see below) all nuclei, with the exception of small nuclear fragments, red blood cells, distorted nuclei and identifiable leucocytes lying within each small grid unit surveyed, were scored as either interphasic or mitotic cells. Those nuclei with one half their volume within the outer boundaries of the total area surveyed were included in the counts. A total of 100 (interphasic + mitotic) nuclei were counted for each area sampled and the number of small grid units covered to obtain this total were also recorded. It should be noted that except for large aggregates, those units occupied by cell fragments, red cells, etc., were not subtracted from the total grid units recorded. For these reasons, density recordings represent approximate values only. They are, however, sufficiently accurate for the purposes of this study.

In order to determine if 100 cells was a sufficiently high figure for detecting differences in mitotic rate between regenerating limbs, pilot counts of 100 cells at randomly selected sites for six 16 day regenerates were made. These counts produced results indicating an average deviation of $\pm$ .34 in the number of mitotic figures counted. From a statistical point of view, the small variability in the number of mitoses between limbs obviated the necessity for counting a larger number of cells. The mitotic categories identified were mid and late prophase, metaphase, anaphase, and early telophase figures. Late telophase

figures were counted as one mitosis. Early prophase and late telophase figures were scored as interphasic cells. Where there was doubt concerning the identity of a scored mitotic figure, the cell in question was re-examined at a magnification of 430 X for positive identification. Visual periodic checks were made to determine if the same mitotic figure was being recorded in the different sections. In no instance was this found to be the case.

An inherent variable in a study of this type is the degree of subjectivity on the part of the scorer with reference to the criteria being used. Indeed, early counts undertaken to establish a consistent sampling technique showed a progressive decrease in density scoring and a slight increase in the number of mitotic figures recorded. These earlier counts served to standardize the more precise counts recorded in this thesis. Nevertheless, as a check on the accuracy of the scoring, periodic recounts of sample areas selected at random were made. Such counts revealed an error of ± 3 percent in the recorded figures.

The Blastema Areas Sampled

For those limbs sectioned longitudinally, mitotic index determinations were carried out on two sections each interspaced by one section at 5 different levels within the blastema. Sample level #1 was at the center of the blastema and usually corresponded to the median plane through the humerus stump while levels #2 and #3 were located

parasagitally through the right and left halves of the blastema. Sample levels #4 and #5 were located at the lateral edges of the blastema lateral to sample levels #2 and #3 respectively (Figure 1a).

Based upon histological topography, the regenerating portion of the forelimb was arbitrarily divided into 2 zones; a distal blastema and a proximal dedifferentiation area. The dedifferentiation area comprised the cellular elements located between the base of the blastema proper and the level of the differentiated tissues of the stump. The base (the approximate primary amputation plane) of the blastema was determined using the more proximal edge of the dermis as a landmark. This method provides a base level which corresponds fairly accurately with the base line used for blastemata cut in cross section. An occasional level difference between the lateral dermal elements was practically unaviodable. It should be noted in this regard that because of unequal stump tissue masses which apparently cause differential tissue retraction and probably differential tissue dedifferentiation, dermal structures never indicate exactly the primary level of amputation but serve as an approximate indication.

In the longitudinally sectioned blastemata a maximum of 4 sample areas (Figure 1b) was selected for mitotic density calculations. Sample area A was at the tip of the blastema immediately beneath the epithelial cap; area B was at the center at the base of the blastema;

area C was at the left periphery at two thirds of the distance from the base line through the blastema; area D was at the right periphery at one third of the distance from the base line. The positions of sample areas varied slightly for the smaller blastemata. In the 10 day regenerates, for example, the sample areas were located within 4 equal zones along the length of the blastema as illustrated in Figure 4.

To avoid the possibility that the areas counted in the smaller blastemata (10 and 13 day regenerates) might overlap one another, density and mitotic counts were spread, in some cases, over several sections. The levels for the counts were kept constant between each of the sections scored.

For cell counts at the tip (area A) the ocular grid was superimposed over the blastema and oriented under the tip curvature so that its distal edge or corners, depending upon the blastemal morphology, just touched the base line of the wound tip epithelium. For the base calculations (sample area B) the ocular grid was superimposed over the blastema so that the base of the grid coincided with the base line of the blastema. The mid-line of the grid was located so as to correspond with the medial point of the blastema. At sample areas C and D, the grid was superimposed over the blastema so that the lateral grid border at its center touched the bordering wound epithelium at the level of the one third or two thirds base-distal measurements (Figure 1b).

This manner of selection of the sample levels and areas thus provided an accurate 3 dimensional survey of the developing blastema. As a result of the random orientation of those limbs sectioned longitudinally several points on a vertical (proximo-distal) axis and on a 360 degree circumferential plane for each regeneration time were surveyed, thereby allowing comparison of mitotic intensity and cellular density distributions between external and internal locations as well as between the base and the tip.

Cell counts on limbs cut in cross section were made at 3 levels: At the top; at the center; and at the base of the blastema (Figures 10, 12, and 13). The base line for the blastema was determined as the most proximal cross section from the tip possessing a continuous dermis. If in any case differentiated stump tissues were contained within the section, selected in this manner, the first distal section not possessing differentiated stump tissue was used as the base line. Two sections, each interspaced by one section, were sampled at the center and base level. At the tip of the blastema cross sections each interspaced by one section were surveyed until a total of 200 cells had been counted. With the exception of the tip, each section to be scored was divided into 5 regions (an axial center and 4 quadrants--dorsal, ventral, anterior, and posterior (Figure 12). With the aid of the ocular grid, each region was examined for mitotic activity and cell density. At the center and base of the blastema, the ocular grid was

superimposed over the cross sections of the blastema within the 4 quadrants, (dorsal, anterior, ventral and posterior) near the center region of the limb. Care was taken to avoid any overlap in scoring between adjacent quadrants. Thus, the cross sectional material added still another dimension to the survey of the blastema providing information on the internal distribution of cell density and cell division at 2 proximo-distal levels.

For surveying the asymmetrical blastemata, sample levels at 3 different locations were selected. The center level was selected as the best possible section based upon size through the eccentric blastema which usually corresponded to the medial section through the humerus of the limb stump. The other levels studied were located 8 sections bilateral to the center level. The sample areas were located within the sections as illustrated in Figure 11. Sample area #1 was located at the tip; area #2 was at the base of the blastema at its center and adjacent to the remaining humerus stump; area #3 was located laterally adjacent to the wound epithelium and distal to the most distally located dermis; area #4 was located laterally opposite to and at the same level from the tip as area #3.

Because of their small size, aneurogenic limbs were sampled at two locations only--at the tip and at the base of the blastema. The base sample was located at the center of the blastema immediately distal to the cartilaginous humerus. Beginning at the center of the blastema and

moving bilaterally, several sections, each interspaced by one section, were surveyed for mitotic activity until a total of from 150 to 250 cells had been scored. More than half of each blastema was surveyed by this method.

Mitotic index determinations were completed for each limb series by dividing the total number of cells into the total number undergoing mitosis and multiplying by 1,000. Densities were determined for all limb groups as cells per unit surface area, the unit of area being the area under one small square of the ocular grid (.027 $mm.^2$). The number of cells per unit area was determined by dividing the total number of cells by the total number of ocular grid units.

The relationship between mitotic activity and "vertical and horizontal" position within the regenerating limb bud as a function of time from initiation of developmental activity was demonstrated by a standard two-way analysis of variance with equal replication. Measurements from which the statistical treatment effects for longitudinal sections were derived have been made between 4 time periods and 4 physical locations on a disto-proximal axis. Identical measurements were made for the cross sectional material, but between 4 time periods and 5 physical (central, dorsal, ventral, anterior, and posterior) locations. There was a relationship between variance and mean within the cells analyzed. The mean points of the cells, however, were distributed in a significant linear relationship so

that the estimated error term was not biased. An analysis of this type obviated, especially for limbs cut longitudinally, the consideration of geometrical variation of blastemal morphology which characteristically developed.

H^3 thymidine treatment has not been used in the present analysis for several reasons. Paramount among these was that isotope labeling does not provide specific information on the exact number of cells undergoing division. This fact was made clear from the autoradiography studies of Messier and Leblond (1960) who found that the radioactive index (percent labeled cells) is not a true measure of proliferation rate since it is also influenced by the rate of incorporation of the label into the intermediate compounds leading to DNA synthesis and by the duration of the period of DNA synthesis.

Although tritiated thymidine is known to be incorporated into the DNA molecule, there remains the possibility that it might be taken up by non-dividing cells such as phagocytes, etc., and metabolized via a variety of enzyme pathways. The availability of the tritium label to cold limb tissues has been demonstrated by the experiments of Riddiford (1960), in which she exchanged newt blastema epithelium between labeled and unlabeled blastemata. Subsequently, in the blastemata provided with H^3 thymidine, the isotope was identified in the formerly unlabeled skin.

Barr (1963) has in addition noted for Hela cell populations an alteration in the duration of the mitotic cycle following exposure to exogenous H^3 thymidine. Thus, under some conditions, the thymidine treatment caused a prolongation of metaphase. Therefore, it is possible that this phenomenon could give a false impression of a high degree of mitotic activity.

A specific problem in using tritiated thymidine for the study of proliferation rate among mesenchymal cells of the Amphibia is the time span between DNA synthesis and cellular division. As shown by Hay and Fischman (1961) this time lapse may constitute 3 to 4 days, a factor which probably allows for wide spread cellular displacement before mitosis. This characteristic of the label might well be useful in studies of the relation of DNA synthesis in developing blastema cells and their eventual locus of division. For the purpose of the present study, however, in which specific mitotic indices are compared between various levels of the blastema and between various stages of blastemal development, the classical, if more laborious, methods promise the more clear-cut interpretations.

RESULTS

Series I:

General Morphology and Histology of the Regenerate

Although aspects of the regeneration of the forelimb of the axolotl, Ambystoma mexicanum, have been previously described (Kazancev, 1934; Faber, 1960), no general review of the major morphological and histological features of the process is available. Since the detailed analysis of cellular proliferation patterns in the blastema is meaningful only in relation to the regeneration process as a whole, the following description seems necessary.

Following amputation through the distal one third of the humerus, there is early closure of the wound and at 24 hours post amputation, the amputation surface is covered by epidermal cell layers equal in thickness (6 to 7 cells) to the epidermis of the normal stump skin. During the first 3 days there occurs a period of phagocytosis of cellular debris resulting from the trauma of amputation. Numerous leucocytes (phagocytes) are present and cellular fragments and debris begin to appear in the wound epithelium. Tissue debris and pycnotic nuclei are evident in the wound skin in some limbs as long as 10 to 13 days after amputation,

apparently arising from the general histolysis of stump tissues during the period of dedifferentiation. Debris of various sorts within the intercellular spaces of the epidermis has previously been reported for Ambystoma larvae (Thornton, 1938a) and in the newt (see review by Singer and Salpeter, 1961).

By 4 to 5 days after amputation, sarcolysis of the transected muscle is apparent and general histolytic changes of the cartilage are beginning. This period constitutes, therefore, the beginning of the "dedifferentiation" phase, (Thornton, 1938a; Hay, 1959), a process which may continue in some limbs as late as 10 to 14 days after amputation. These observations are similar to those of Polezajew and Ginsburg (1943) who noted in the forelimb of the axolotl that tissue dedifferentiation was detectable even on the 14th to 16th day of regeneration.

Blastema cell aggregation is first seen at approximately 6 to 7 days after amputation and is accompanied by the accumulation of a mesh of connective tissue fibers between the wound epithelium and humerus stump. In subsequent stages the connective tissue mesh is obscured except at the periphery by the aggregation of the blastemal cells. It is possible, but has not been demonstrated, that the connective tissue network may serve as a substructure upon which the blastema cells migrate.

An interesting feature of limb regeneration in the axolotl is the appearance at an early stage (8 to 10 days) of free melanocytes among the accumulating cells of the blastema. For the most part these cells tend to remain in topographical relation to their former (dorsal and anterior) location, and thus are found primarily in the dorsal and anterior regions of the regenerate immediately beneath the wound epidermis (Figure 13). In many cases, the pigment granules from melanocytes are identifiable within the wound epidermis (Figure 5).

During the period of early blastema accumulation (6 to 7 days) mitotic figures although not numerous, are found among the distally aggregating mesenchymatous cells. Infrequent mitotic figures are also observed among the dedifferentiating tissues proximal to the amputation plane. Thus, although no quantitative data on the early regeneration stages was obtained in this study, blastemal formation must be, in part at least, the product of early cell proliferations. These observations on the existence of cell division during the early regenerative phases agree with the data of earlier experiments (Polezajaw and Ginsburg, 1943) for the axolotl and for the newt (Chalkley, 1954; Hay and Fischman, 1961).

Once mesenchymatous cell aggregation is well underway, it is possible to classify the developing blastema into several morphological stages which are depicted in figures 6 to 9. The stages--"early bud," "mound," "cone,"

and "paddle"--are equivalent to the Stages I, II, III, and IV previously described for the axolotl forelimb by Faber (1960). Under the conditions of this study (controlled temperature, 20° C., uniform age and size of animals used, and optimal feeding) the regeneration times for these stages were 10, 13, 16, and 19 days respectively. The shape of the early bud stage (10 days) is roughly conical and its constituent mesenchymatous cells are relatively homogeneous in distribution and not very densely aggregated (Figure 6). Based upon calculations from 6 limbs, the average length for a blastema at this stage is .31 mm. as measured from amputation plane to distal tip of the mesenchymatous mass.

By 13 days the blastema has enlarged considerably (average length = .60 mm.) and is mound shaped. As can be seen from Figure 7, a blastema at this stage possesses a definite pattern of blastemal cell density with the distal one half having a considerably more densely aggregated mass of cells than the proximal half. At 16 days, the regenerate is cone shaped and begins to show some anteroposterior flattening at the distal tip. The blastema at this stage has grown to an average proximo-distal length of 1.12 mm. The number of mesenchymatous cells is considerably higher distally and this high cellular density extends over approximately two-thirds of the blastema. In the more advanced 16 day blastemata there is some evidence of axial mesenchymatous cell condensation in conjunction with the stump humerus (Figure 8).

Continued antero-posterior flattening and distal growth produce by 19 days a paddle shaped regenerate whose average length is 1.41 mm. As can be seen from Figure 9, a considerable proximal differentiation of the humerus rudiment has occurred and mesenchymatous cell condensation has produced distinct patterns indicating the development of the radius, the ulna, and digits #1 and #2. During later stages of development (days 22 and 25) digits #3 and #4 will arise from the mesenchymatous mass (arrow) at the posterior periphery of the 19 day regenerate (Figure 9). Visual observations on the distal digital condensations, and also those destined to produce digits 3 and 4, revealed numerous mitotic figures for these regions. It would seem likely, although it remains to be demonstrated, that these regions grow by apical cellular proliferation.

The Wound Epithelium

With this brief general account of limb regeneration in the axolotl as background, we can now focus on one of its aspects which has received little attention in the past. This is the activity of the wound epidermis which covers the amputation surface. During the first few days after amputation, a gradual thickening of the wound epidermis occurs and by 6 to 7 days an "apical cap" 10 to 12 cell layers thick has been produced. Apical proliferation within the wound epidermis becomes especially prominent at the 7, 10, and 13 day stages studied (Table 1) and there exists a

Table 1.--Changes in mitotic activity at three levels within the forelimb epidermis of the axolotl for three regeneration periods.

Days Regeneration	Levels		
	*Site 1	Site 2	Site 3
	**MI	MI	MI
7 Days	1.0	.7	.6
10 Days	2.5	1.0	.8
13 Days	1.8	.8	.9

* Site 1 = Apical epithelium; Site 2 = Stump epithelium just proximal to the amputation plane; Site 3 = Stump epithelium 1 mm. proximal to the amputation plane.

** The mitotic index (MI) represents 3,000 (interphasic + mitotic) cells for each sample site.

striking difference in the mitotic rate in the apical cap as compared to the 2 proximal stump levels of the normal skin. As can be seen in Figure 3, there are numerous cell divisions within the apical wound skin of a 10 day regenerate. It should be noted that the cell counts reported in Table 1 were obtained by counting 1,000 cells for each sample location on 3 different limbs near their center for each regeneration time. Thus, a total of 3,000 cells were scored for each limb. It would appear from the data of Table 1 that it is mainly through the proliferation of the wound epithelial cells that the apical cap increases in thickness from 10 to 12 layers of cells at 7 days, to 17 to 20 layers at 10 days of regeneration (Figure 6) Continued epithelial

proliferation apparently is the cause of the formation of a unique and prominent "epidermal lobe" which is first seen at the 13 day stage, (Figure 7), and which usually reaches a maximum size (35 to 40 cells, (cap + lobe) at approximately 16 days of regeneration, (Figure 5). Epidermal tongues have been reported for the regenerating anuran limb stumps (Van Stone, 1955) but the axolotl seems to be the only urodele reported to possess this interesting structure. It should be noted that the lobe as a distinct entity does not develop on every regenerating limb, but limbs lacking the lobe possess an apical cap-like thickening, (Figure 8). It is important to note that no significant difference other than normal variability in mitotic activity or cell density could be discovered between those limbs lacking the lobe and those possessing it.

In later developmental stages, after 16 days of regeneration, the apical lobe normally decreases in size and gradually disappears. It may still be evident in some cases as late as 22 days of regeneration and in these instances is usually associated with the first digit projection. Thornton (1962) has reported for a variety of salamanders other than the axolotl, that Leydig cells are not found in wound epithelia which support limb regeneration. He has found, on the other hand, that wound epidermis which fails to support limb regeneration is heavily laden with Leydig cells. It is of great interest, therefore, to find that Leydig cells are very numerous in the wound epithelium and apical lobe

of the regenerating axolotl limb (Figures 3 and 5). Kazancev (1934) observed Leydig cells in axolotl wound epithelium and Faber (1960) did also. The latter author reported a degeneration of these Leydig cells, however, and did not find them ever to be very numerous in wound epithelium. Degeneration of Leydig cells in the wound epithelium has never been observed in the present study. Indeed, considerable mitotic activity has been found in these cells in the wound epithelium throughout regeneration.

It has recently been demonstrated (Kelley, unpublished) that Leydig cells are a characteristic component of larval skin and probably function in storing mucin materials. The Leydig cells are thought to reach a peak in numbers at the mid-larval stage and then gradually disappear, and are totally absent in the adult skin. The significance of the Leydig cells, if any, in the regeneration process is not apparent. It is evident, at least for the axolotl, that they do not function to inhibit the regeneration process since they were very numerous in the wound skin during rapid growth phase of the blastema.

Series II:

Analysis of Blastema Cell Proliferation Along the Longitudinal Axis

The object of this experimental series was to determine the mitotic index and cell density distribution along the disto-proximal axis within the blastema.

Twenty-four axolotl forelimbs, 6 for each of the regeneration stages of 10, 13, 16, and 19 days, were used in this experiment.

For the purpose of demonstrating differences in mitotic activity and cell density along the longitudinal axis of the blastema and as an aid in the statistical analysis, the blastema was arbitrarily divided, using a center section (sample level #1) of the blastema as the base for the divisions, into 4 disto-proximal zones which corresponded to the 4 disto-proximal sample areas at that level, (see Materials and Methods, page 26, Figure 1b and Figures 6 to 9). The division times were drawn half way between the points of the 4 sample areas. The cell counts from the 4 sample areas of each longitudinal section surveyed, thus from the 5 sample levels (10 longitudinal sections), were then tabulated into the appropriate zones (I, II, III, and IV) previously established for the entire blastema. For example, because of the geometrical design (roughly cone shaped) of the blastema, which causes the longitudinal section at sample levels #2 and #3 (Figure 1a) to be shorter than those at the center of the blastema, the tip counts for these levels were grouped with Zone II of the blastema. Also the cell counts from the other sample areas at these levels, for example B, C, and D and also the sample areas of sample levels #4 and #5 were, in like manner, tabulated into the blastemal zones which corresponded to their individual lengths.

The statistical treatment of the data collected by the method described above is represented in Table 2. As can be seen, each block cell of the table contains the observed mean of mitotic activity, the expected mean of the mitotic activity, assuming that zones are independent of time, and the corresponding increments of the sums of squares for the interaction term. For example, in the block cell at the upper left corner of the table (zone I, day 10) the figures represented are:

Observed mean = $\bar{X}$ = 3.723

Expected mean = $\bar{X}$ = 2.849

Sums of squares = SS = .764

Thus, the magnitude and the sign of the deviation between the 2 $\bar{X}$'s or the SS value indicate the degree of deviation from the assumed lack of interaction. The interaction term may be defined as the expression of the relationship between the different zones which changes as a function of time. Thus, Zones I, II, and III at 10 days of regeneration and Zones I, II, and III at 19 days of regeneration show a significant interaction. Expressed in a slightly different manner, it can be stated that at 10 days of regeneration, Zone I possessed a greater number of mitotically dividing cells than could be explained by the row and column $\bar{X}$'s or the expected mean value. The exact reverse of this situation can be noted at 19 days regeneration whereas in Zone I fewer than expected mitotically dividing mesenchymatous cells were observed. Therefore, for these zones and times there

Table 2.--Two-way analysis of variance between regeneration times and zones along the vertical axis of the forelimb blastema of the axolotl.

	Days of Regeneration				
Zones	10	13	16	19	
I	$\overline{X}$ = 3.723 $\overline{X}$ = 2.849 SS= .764	$\overline{X}$ = 3.362 $\overline{X}$ = 3.260 SS= .010	$\overline{X}$ = 3.070 $\overline{X}$ = 3.074 SS= .000	$\overline{X}$ = 1.514 $\overline{X}$ = 2.486 SS= .945	X = 2.917
II	$\overline{X}$ = 1.945 $\overline{X}$ = 2.362 SS= .174	$\overline{X}$ = 2.702 $\overline{X}$ = 2.773 SS= .005	$\overline{X}$ = 2.472 $\overline{X}$ = 2.587 SS= .013	$\overline{X}$ = 2.602 $\overline{X}$ = 1.999 SS= .364	$\overline{X}$ = 2.430
III	$\overline{X}$ = 1.280 $\overline{X}$ = 1.674 SS= .155	$\overline{X}$ = 2.158 $\overline{X}$ = 2.085 SS= .005	$\overline{X}$ = 2.019 $\overline{X}$ = 1.899 SS= .014	$\overline{X}$ = 1.514 $\overline{X}$ = 1.311 SS= .041	$\overline{X}$ = 1.742
IV	$\overline{X}$ = .945 $\overline{X}$ = 1.005 SS= .004	$\overline{X}$ = 1.312 $\overline{X}$ = 1.416 SS= .011	$\overline{X}$ = 1.229 $\overline{X}$ = 1.230 SS= .000	$\overline{X}$ = .805 $\overline{X}$ = .642 SS= .027	$\overline{X}$ = 1.073
	$\overline{X}$ = 1.972	$\overline{X}$ = 2.383	$\overline{X}$ = 2.197	$\overline{X}$ = 1.609	$\overline{X}$ = 2.040

Source	df	MSS	F
Sites	3	15.556	25.090*
Days	3	2.665	4.298*
Interaction	9	1.688	2.723
Error	80	.620	
Total	95		

* Significance at the 1 per cent level

The Expected Mean (Day 10, Zone I) = The Grand Total Mean adjusted for days [$\overline{X} + (\overline{X}d_{10} - \overline{X})$] or [2.040 = (1.972 - 2.0400] and then adjusted for zones [$\overline{X} + (\overline{X}d_{10} - \overline{X}) + (\overline{X}_{z1} - \overline{X})$] or [2.040 - .068 + (2.917 - 2.040)]. The expected mean therefore equals 2.040 - .068 + .877 = 2.849.

has been an effect upon the mitotic rate which has caused a deviation from the expected mitotic incidence, indicating that interaction has occurred between time and zone of the regenerating blastema.

With this general information as background, it is now possible to consider the mitotic patterns which are apparent for the individual regeneration times, 10, 13, 16, and 19 days. It should be reemphasized here that the analysis presented is an experimental test of the hypothesis for apical proliferation proposed by Faber (1960) and therefore regeneration stages equivalent to those used in his experiments (Stages I, II, III, and IV) were selected for this study.

At the "early bud stage" (10 days regeneration), which represents the first measurable accumulation of mesenchymatous cells, a striking pattern in the mitotic activity has already been established. It can be seen (Figure 14 and Table 2) that at this early time there exists a definite linear difference in blastemal cell mitotic activity, and the mitotic index is obviously highest at the tip (Zone 1). The proliferation rate at the tip represents a 300 percent higher rate than the base index. As stated above, the interaction term (SS = + .764) of Zone I at 10 days of regeneration (Table 2) indicates that a much higher than expected rate of mitotic activity was found. Significant, however, is the fact that just the reverse effect can be noted for Zones II and III where lower than the expected

rates in proliferation were observed. Thus, there exists an effect between these 3 zones (sites) and the time in regeneration. No clear explanation for this deviation from the expected pattern is evident, but it is interesting to note that the tip region is particularly close to the epidermal cap. All mesenchymatous cells in Zone I (tip region) are more closely associated with the apical wound epithelium than are the majority of cells which occupy the other zones of the 10 day blastema. This observation has been borne out by specific measurements of these zones with the aid of the ocular micrometer. To illustrate this point, all of the mesenchymatous cells in the tip zone of the blastema shown in Figure 6 lie within .067 mm. of the wound epithelium while, on the other hand, more than half of the cells counted in Zone II were more than .067 mm. from the nearest part of the wound epithelium.

What is again striking for the 10 day blastema is that the apical wound epithelium which is associated with Zone I shows an intense rate of cell proliferations which is not found, as determined by cell counts, for the more proximal levels of the wound epithelium adjacent to Zones II, III, and IV of the blastema. Again using the blastema represented in Figure 6, a mitotic index of 2.7 per cent was observed for the tip epithelium while the proximal wound epithelium possessed a rate of 1.2 per cent which represents, therefore, a 150 per cent difference in mitotic activity. It is interesting to speculate on the possibility that the

observed temporal relationships between the intense mitotic activity of the apical wound epithelium and the underlying apical mesenchymatous cells may also represent a causal relationship (see also Faber, 1965).

In contrast to the proliferation patterns at the 10 day stage is the distribution of the mesenchymatous blastema cells. No apparent pattern is evident as the cells are distributed relatively equally at all disto-proximal levels and the density is not very great (Figure 14). This feature of homogeneity in cell density at this stage seems to indicate that the high rate of proliferation, as found for the tip zone, may just have begun.

Three days later at the "mound stage" blastema the mitotic rate still shows a disto-proximal gradient and there are substantial increases in the proliferation rates within Zones II and III while Zone IV shows a small increase (+ .367 per cent) in the mitotic rate. For Zone I a slight decrease (- .361 per cent) in the mitotic rate from the high rate of the previous stage was noted, but the rate was still considerably higher (over 200 per cent) than that for the base zone. The most striking feature for the "mound stage" blastema is the observed increases in proliferation rates for Zone II and III. It would appear that the stimulus for cell proliferation, whatever it may be, has by this stage, "diffused" into the more proximal areas (Zones II and III) of the blastema. It is most interesting to note, in this regard, that the majority of the mitotic figures recorded

for Zone III were found within the dense mass of mesenchymatous cells which extended proximally from Zone II into this zone (Figure 7).

As can be seen from Figure 14 and illustrated by the photomicrograph of Figure 7, the density of the mesenchymatous cells at this stage is greatest in roughly the distal half of the blastema; thus Zones I and II are more densely populated than are Zones III and IV. However, there is no sharp transition in density but rather a gradual disto-proximal decline in the number of mesenchymatous cells across Zone III. These findings seem to indicate a correlation between the density and the active proliferation of the mesenchymatous cells. Such a correlation seems to be borne out by the fact that the greatest difference and thus the greatest decline in mitotic divisions along the disto-proximal axis of the blastema has occurred between Zone III and Zone IV (Table 2). It should be noted, while considering the "mound stage" regenerate, that Faber (1960) has identified this stage (Stage II in his study), due to the dense accumulation of mesenchymatous cells at the distal tip, as the approximate time for the beginning of apical proliferation.

It is significant to note here that the wound epithelium at 13 days regeneration does not possess the striking difference in mitotic activity between the tip and the base that was observed for the 10 day stage. Indeed, cell counts involving a total of 1,000 cells within the apical wound

epithelium adjacent to Zones II, III, and IV for the blastema shown in Figure 7, revealed no difference in the mitotic index between these 2 levels. Each location possessed a 2.0 per cent rate in cell divisions. In view of this fact it is interesting that there has been an overall increase in mitotic proliferation within the blastema with very noticeable increases for Zones II and III. It is also interesting that with the decrease in mitotic division in the apical wound epithelium (- .7 per cent) there has also been a decrease in proliferation in the apical mesenchyme of Zone I (- .292 per cent).

The gradient in the mitotic rate along the disto-proximal length of the blastema is still very much evident at the "cone stage" blastema (16 days regeneration). All zones at this time have undergone slight decreases in the number of mitotic divisions, but there still remains a two-fold difference in the proliferation rates between the tip and the base regions of the blastema. A very significant aspect of the observed decreases in mitotic activity at this stage is that Zones III and IV decreased less in mitotic activity as compared with the previous stage than did Zones I and II. A most interesting parallel to this situation was the observed decreases in the mitotic rates within the wound epithelium. Although decreases were found in both the apical and proximal regions of the wound epidermis, a larger decrease was noted for the apical part. Indeed, cell counts of 1,000 cells for both these regions showed a

1.4 per cent mitotic index for the tip epithelium and an index of 1.6 per cent for the proximal part. Both regions possessed rates of 2.0 percent at 13 days regeneration. Thus, a striking correlation between the mitotic activities of the wound epithelium and the underlying mesenchymatous cells has, as it was for the 10 and 13 day regenerates, again been revealed.

The mesenchymatous cells at the "cone stage" continue to form dense accumulations, particularly throughout the distal two-thirds of the blastema. The most pronounced increases in cell density were evident for Zones I and II which apparently reflect the high degree of mitotic activity observed for those zones at the previous stage (13 day regeneration). Increases in cell density were also found for Zones III and IV and the base increase (Zone IV is apparently a measure of the axial "condensation" of mesenchymatous cells, which is beginning at this stage (Figure 8). It should be pointed out that the term "condensation" as used in this thesis does not imply cell differentiation but rather is used to describe the dense aggregations of the mesenchymatous cells which form the preliminary patterns of the future cartilage elements of the regenerating limb. The inference that these cells are not differentiating has been strengthened by the repeated observations that the cells in these early condensations are undergoing divisions.

At 19 days regeneration, the "paddle stage," profound alterations have occurred in the mitotic patterns of the developing regenerate. This is especailly evident for the tip zone, where the proliferation rate is only one half as great as the mitotic value found for this zone at 16 days of regeneration. Zone II, on the other hand, shows a slight increase in mitotic rate over the preceding stage, while Zones III and IV both have undergone decreases in the number of mitotic divisions. Similar to the changes in mitotic patterns are the alterations in the cell density distributions at the paddle stage. A drop in cell density was recorded, for example, in Zone I and an increase was determined for Zone II. Zones II and IV, however, both were found to be more densely populated than the equivalent zones of the 16 day stage. As can be noted from Figure 9, considerable cartilage differentiation is underway for Zone IV.

Referring to the "interaction" term, or the sums of squares (Table 2), it is apparent that Zones I, II, and III of the 19 day blastema have deviated considerably from the expected mitotic rates. The mitotic activity for these zones represents the exact reversal of the mitotic activity found for the "early bud stage," (10 days regeneration). Thus, at Zone I the mitotic rate is considerably lower than the expected value and Zones II and III are slightly lower than the expected mitotic rates. These differences between the actual and expected values are perhaps explainable on

the basis of the histological alterations which have occurred within the developing regenerate by 19 days of regeneration. The tip region, for example, no longer possesses a homogeneously distributed dense mass of mesenchymatous cells which was evident at the cone stage, but rather shows "condensation" patterns for the future digits (#1 and #2). The position of the sample area for Zone I at 19 days of regeneration, for experimental consistency, was the same as that used for the other stages; that is, at the tip and near the center of the blastema which was therefore between the two digital "condensations." It was thought possible that a high rate in mitotic activity might be associated with the digital "condensations." To test this possibility, 1,000 cells from the rudimentary digital masses of the 19 day regenerate represented in Figure 9 were scored. The result of this count revealed a significant 3.3 per cent mitotic index for these regions which is a striking 180 per cent higher than the rate found for Zone I at 19 days regeneration (Table 2).

The higher mitotic rate (+ .6) than the expected rate for Zone II is due perhaps to the fact that this zone at 19 days of regeneration includes not only the distal portion of the mesenchymatous condensation for the future radial and ulnar cartilages but also the dense mass of mesenchyme (arrow, Figure 9) which is destined to give rise to the cartilages of digits 3 and 4. Thus, the calculated mitotic rate for Zone II is a measure of the intense mitotic

activity of the mesenchymatous cells of the future limb skeleton. The mitotic rate for Zone III, which is slightly higher than the expected rate, can be attributed to the proliferation of blastemal cells accumulating to form the proximal parts of the developing autopodium (radius and ulna).

In conclusion, it is clear (Table 2) that a longitudinal gradient of mitotic activity is present in the regenerating axolotl forelimb. The mitotic rate is highest at the blastemal tip at 10 days following amputation and, although it progressively narrows, this mitotic differential is maintained until the 19 day regenerate. In the latter stage, also, if one includes the digital mesenchymal aggregations, the tip still maintains a higher rate than Zone II in actively dividing cells.

Series III:

Analysis of Mitotic Activity within Regions of the Blastema Studied in Cross-Section

In a recent investigation of blastemal growth in the newt, Triturus viridescens, it has been suggested, (Singer, Ray and Peadon, 1964) that the extent of cellular movement and multiplication of mesenchymatous cells, during the period of most rapid growth of the blastema is not the same everywhere in the blastema but is greatest near the regenerating nerve trunks of the limb. The evidence for this was derived from experiments in which cross-sectional regions (central, dorsal, anterior, ventral, and posterior)

of an early regeneration stage, which Singer and his co-workers have termed "moderately early" were infused with small quantities of Nile blue sulphate. Subsequently, during the rapid growth phase of the blastema, it was observed, by following the displacement of the dye, that some regions, (ventral, posterior and the adjacent central region), gave rise to large areas of the new limb, whereas the other regions (dorsal and anterior) contributed very little material to the developing structure. According to these investigators, cells derived from the ventral, posterior, and central areas of the early regenerate produced the hand and fingers and most of the lower arm. In other words, the cells of these regions formed the anterior and dorsal parts of the regenerate as well as the ventral and posterior structures.

Histological studies (Singer et al., loc. cit., 1964) revealed that the 3 major nerve trunks, in the distal part of the forelimb, are located mainly ventrally and posteriorly within the soft tissues near the bone. They also discovered that although regenerated nerve sprouts were present throughout the blastema, the fiber concentrations were more abundant in those areas overlying the regions (mainly ventral and posterior) possessing the major nerve trunks. In view of this fact it seemed apparent that the contribution of blastemal cells from the individual cross-sectional regions of the early blastema to the formation of the new limb was correlated with the position of the

nerve fibers and trunks. This was further emphasized when nerves were deviated preceding amputation from their normal positions (ventral and posterior) into the dorsal and anterior regions of the limb, with subsequent increased cellular contribution to the blastema from these areas.

Although it was not the initial intent of the present study to test for a correlation between cellular proliferations and nerve fiber concentration, in view of the experiments reported above and especially since the axolotl blastema was found to grow by apical proliferation, it seemed important to re-examine the blastema for mitotic activity using sampling patterns which would approximate the dye infusion regions studied by Singer, Ray and Peadon, (1964).

For this purpose axolotl regenerates, 2 for each time period, were sampled at 10, 13, 16 and 19 days after amputation. Cell counts were made on cross-sections at the center and at the base of the blastema within 5 cross-sectional regions, (center, dorsal, anterior, ventral, and posterior), (see Materials and Methods and also Figure 12). It is important to note that the precise dorso-ventral and antero=posterior orientation of the cross-sections was checked using the pigmentation patterns of the blastema and also by the identification within proximal cross-sections of the positions of the major nerve trunks and other stump tissues.

The statistical treatment of the data (mitotic activity between the 5 regions for each regenerate stage) is presented in Table 3 and the mitotic indices and cell density distributions are illustrated in Figure 15. For the individual regeneration times it can be observed from Figure 15 that there exists no significant difference between the regions, especially those regions which overlie the major nerve trunk and those which do not. Thus, for day 10 a relatively high rate of mitotic activity is apparent for the dorsal (1.1 per cent) and anterior (1.4 per cent) regions and the central 1.0 per cent) and posterior (1.0 per cent) regions are slightly lower. What is most significant is the fact that the proliferation rate of the ventral region which overlies the greatest concentration of nerve fibers is considerably lower (.6 per cent) than either of the other regions and especially the dorsal and anterior regions.

At the 13 day stage, which represents the most rapidly growing period of the blastema, no clear pattern in mitotic activity nor cell density between the cross-sectional regions has developed. The anterior region was found to be the most active (2.5 per cent) and was followed in activity by the ventral region (2.0 per cent), then the posterior region (1.9 per cent), the dorsal region (1.8 per cent) and lastly the central area (1.0 per cent). Thus, the average rate of mitotic activity (2.1 per cent) for the anterior and dorsal regions is considerably higher than the other 3 regions, (overlying the nerve trunks) which collectively possessed an average rate of 1.3 per cent.

Table 3.--Two-way analysis of variance between regeneration times and regions of the forelimb blastema of the axolotl studied in cross section.

Source	df	MSS	F
Time	3	2.193	9.37*
Quadrant	4	.808	3.543
T x Q	12	.135	.577
Error	20	.243	
Total	39		

* Significance at the 1 per cent level.

Regions	Center	Ventral	Dorsal	Posterior	Anterior
$\bar{X}$'s	1.083	1.375	1.406	1.625	1.938**

** The continuous line indicates a lack of significance between the mean mitotic values of the regions which overlie it.

Test for Significance according to Duncan's New Multiple Range Test.

At 16 days regeneration a drop in the proliferation rate was noted for all regions but the magnitude in mitotic activity remained essentially the same between the 5 regions. Thus, the anterior region was highest in mitotic divisions and was followed by the ventral and posterior (which were equal), then the dorsal and central areas. The recorded drop in the mitotic activity for all regions at this stage (Figure 15)

might be due to changes in cell distribution, especially at the base of the blastema. For example, the peripheral regions of the blastema (dorsal, anterior, ventral, and posterior) have become sparsely populated in comparison with the previous stage while the center region has increased in density. This effect is apparently the result of the axial blastemal cell condensation which is beginning by 16 days of regeneration.

For the 19 day stage, which is after the rapid growth phase of the blastema, the patterns in mitotic activity appear to reflect the orientation of the mesenchymatous cells which are aggregating to form the rudimentary patterns of the future skeletal parts. The anterior and posterior regions at this stage are equal and highest in mitotic activity, and it should be noticed (Figure 15) that they are quite densely populated with mesenchymatous cells. These results are apparently due to the antero-posterior flattening of the regenerate by this time. The dorsal and ventral regions are also equal in cell proliferation but are considerably lower as compared to the anterior and posterior regions. The center region is the lowest in mitotic proliferation but considerably more dense than the other regions.

In summary, as determined from the statistical analysis of the mean mitotic values (lower row of Table 3) there exists no significant differences between the individual regions of the growing blastema. However, significant

differences were noted between two combinations of the 5 regions. For example, the dorsal, ventral, central, and posterior regions, which are not significantly different from one another, are collectively different from the anterior region. The posterior region, however, is not significantly different from the anterior region. The most significant feature to be noted here is the fact that the mitotic indices of the regions overlying the major nerve trunks are not significantly different from those which do not. What is particularly important to note is the fact the mean mitotic value for the ventral region (overlying the major nerve trunks) of the blastema is considerably lower than the other regions, except the central region, and also that the anterior region which apparently does not overlie any major nerves possesses the highest mean mitotic rate. Thus, no correlation between the trunk nerve fibers and the multiplication and density of cells within the various regions of the blastema could be found. Indeed, just the reverse was observed, with more mitotic divisions recorded for those regions which did not surround the nerve trunks. This was not an unexpected result since it was previously demonstrated that following the early accumulation of the blastema cells the apical proliferation of these cells produces the major part of the growing blastema.

It is likely that the observed differences between the findings of the present study and those of Singer, Ray and Peadon (1964) are due mainly to species differences

between the animals used for the experiments (Singer and his co-workers used the adult newt) and also to differences in the regeneration responses between adult and larval forms. Other experiments, for example, especially those involving the phenomenon of regression (the larval limb regresses following amputation and denervation while adult limbs do not) have made it common knowledge that there are important differences between adult salamander limbs and larval salamander limbs in their responses to injury.

Series IV:

Analysis of Mitotic Activity within Asymmetrical Blastemata

It has been demonstrated in a preceding section (Series II) that the axolotl blastema grows by apical proliferation and since a striking relation between the mitotic activity of the wound epithelium, especially the apical portion, and that of the underlying blastemal cells was indicated, a further investigation of this relationship using asymmetrical regenerating blastemata seemed important.

Asymmetrical blastemata were produced by the removal of a strip of skin (dermis and epidermis) running parallel and adjacent to the border of the original amputation plane of the limb, (see Materials and Methods). It should be noted that to produce asymmetrically oriented blastemata on the large limbs used in this study, 2 skin removal operations were necessary. The first operation was

undertaken at the time of apical cap production (6 to 7 days of regeneration) and was followed by one additional operation 2 or 3 days later at 9 or 10 days after amputation. It should be noted that in some limbs there was a tendency, with time, for the angle of asymmetry of the blastema with the limb stump gradually to be reduced. The asymmetry of the regenerates was expressed as a deviation in the direction of outgrowth of the blastema so that the regenerate formed an angle with the limb stump which varied from approximately 45 to 75 degrees.

Three asymmetrical blastemata for 3 different time periods (14, 16, and 18 days of regeneration) were sampled (3 sample levels) for mitotic activity and cell density at 4 different sample areas (1, 2, 3, and 4) as illustrated in Figure 11. It should be emphasized that the sample areas were specifically selected to determine the changes in mitotic cell distributions with time and with relation to the orientation of the apical wound epithelium. Thus, counts were made at the tip and the base to determine the axial distribution of mitotic cells and at the anterior and posterior borders of the blastema (sample areas #3 and #4) to determine the lateral distributions of mitotic cells. Sample area #3 was selected as representing the approximate site of the blastema (apical to the limb stump) before shifting the apical cap, and sample area #4 was selected for the purpose of detecting any increases in mitotic activity and cell density which might develop in relation with the

extensive wound epithelial surface which existed on the anterior border (operated side) of the asymmetrical regenerate (Figure 11).

It should be pointed out before considering the mitotic patterns and cell density distributions for the three regeneration times that because of the 2 operations for skin removal and also the several exposures to the anesthesia (MS 222), both of which were found to slow the regeneration process, the regeneration stages examined (14, 16, and 18 days) are not comparable to those used in the preceding section (Series II) but they do represent the approximate period for the rapid growth of the blastema.

As can be noted from Figure 16 for the first asymmetrical regeneration stage studies (14 days) there exists a high incidence of mitotic activity (2.9 per cent) at the tip and significantly lower rates at the base area and also area #4. If a comparison were made between the 14 day asymmetrical blastema and the regeneration stages of Series II, based upon size, the 14 day anymmetrical state is approximately equivalent to the 13 day undeviated regenerate. Histologically, however, the two stages are not strictly comparable, since the densely aggregated mass of mesenchymatous cells of the asymmetric blastema was found to be more closely associated with the stump tissues than is the case in the undeviated blastema. This is supported by the observation that each of the proximal sample areas, (2, 3, and 4) possesses a much higher mitotic rate

and cell density than could be found at equivalent distances from the stump tissues in normally oriented 13 day blastema. These unexpected results in mitotic activity and cell division were apparently a function of displacing the apical epithelial cap. What is most interesting, however, for the 14 day asymmetrical blastema, is that sample area 3 (apical to the humerus stump) was found to have a strikingly high mitotic rate of 2.3 per cent which is not greatly different from the apical blastemal rate (area #1). It was also noted by histological examination that the mesenchymatous cells were more closely aggregated along the distal wound epithelial border of the blastema than they were next to the wound epithelium at the anterior border (operated side) of the limb. It is possible that the unusually high rate of divisions at area #3 and the peculiar cell density distribution in the 14 day asymmetrical regenerate may represent the old patterns of blastemal cell orientation which developed before the apical epithelial cap was displaced.

At 16 days of regeneration considerable alteration in the mitotic and cell density patterns has become evident (Figure 16). There remains, however, a high rate of proliferation at the tip (sample area 1), but decreases in the mitotic rate were observed for areas 2, 3, and 4. The interesting feature here is the fact that sample area 3 (apical to the humerus stump) underwent the greatest decrease (- 1.4 per cent) while area 4 (adjacent to the wound epithelium at the anterior border of the regenerate) showed

the smallest drop (-.7 per cent). It appears, therefore, that there has been a reorientation of the dividing mesenchymatous cells toward the tip and anterior (operated side) border of the regenerate. Further support for this can be drawn from the observed changes in cell density (Figure 16). It can be seen, for example, that decreases in cell density have occurred for both sample areas #2 and #3 while increases were found for areas #1 (tip) and #4 (anterior border). The changes in density distributions are clearly apparent in Figure 11, which also demonstrates the unusually thick nature of the wound epithelium which has developed at the anterior border (operated side) of the regenerate.

At 18 days of regeneration the mitotic divisions at the tip have decreased (- .9 per cent) but are still more numerous than at any of the proximal regions. The most significant feature at this stage, as can be seen in Figure 16, is that the mitotic rate (+ .5 percent) and cell density have increased at area 4 (anterior border next to wound epithelium) while no change in mitotic activity and only a slight increase in density were apparent for area 3.

It would appear, therefore, that the asymmetrical disposition of the apical epithelium may somehow influence a corresponding asymmetry of blastemal cell proliferation, particularly since the change in proliferation patterns of the blastemal cells follows in time the change in location of the thickened apical epithelium.

Series V:

Analysis of Mitotic Activity within Aneurogenic Limbs

Since it has been demonstrated in a preceding section of this study (Series II) that innervated regenerating axolotl forelimbs grow by apical proliferations, the question was posed as to whether aneurogenic limbs also might regenerate by a similar mechanism. Because axolotl embryos were not available for this study, aneurogenic or sparsely innervated larvae were produced using Ambystoma opacum embryos instead. However, due to a high mortality rate, only 10 aneurogenic limbs were available for this experiment.

Aneurogenic limbs were sampled at 10, 12, 14, and 16 days after amputation and surveyed for mitotic activity and cell density in the distal third and basal third of the blastema. Two limbs each were scored for days 10 and 16 while 3 limbs each were sampled for days 12 and 14. The distributions of mitoses and cell densities found in the aneurogenic blastemata are shown in Figure 17. The number of cells counted for each of the 2 areas sampled (tip and base) depended upon the size of the blastema and ranged from 150 to 250 cells. Thus, for the small blastema (10 days of regeneration) 300 cells were scored for each limb and for the larger blastemata (12, 14, and 16 days of regeneration) 500 cells.

As can be determined from Figure 17, the overall pattern of growth for the regenerating aneurogenic limbs as determined from the mitotic activity shows a close parallel with the growth patterns established for the axolotl forelimb blastema. Even so, however, some differences are apparent and should be considered. Thus, at 10 days of regeneration, which is an "early mound" stage, the mitotic rate of the blastema is relatively low and there exists little difference in mitotic division between the tip and the base.

Due to its size, measuring only .11 mm. in length from the original amputation level to the tip of the apical mesenchyme, the 10 day aneurogenic blastema (at 16 degrees C.) apparently represents the initial accumulation of blastemal cells. This also seems to be borne out by the fact that the mesenchymatous cells at this early time are equally distributed throughout the blastema (Figure 17) and are not very densely aggregated (2.9 cells per unit of the ocular grid for both the tip and the base).

For the 12th day of regeneration, which approximates the development in both mitotic activity and shape that of the 13 day axolotl blastema, the mean mitotic rate has increased markedly showing a high degree of mitotic activity at the tip (mitotic index = 4.7 per cent) while the base rate shows only a slight increase (.5 per cent) over the 10 day stage. An interesting feature of the 12 day blastema is that the apical wound epithelium is much

more pronounced in its development than was observed for the 10 day stage. It is also to be noted that the apical wound epithelium at the 12 day stage was found to be highly active mitotically. This correlation between the mitotic divisions of the apical wound epithelium and the underlying blastemal cells is of interest since a similar relationship, although beginning at an earlier stage, was noted for the axolotl blastema. The mesenchymatous cell density distribution for the 12 day regenerates, as can be noted in Figure 17, begins to reflect the intense proliferation of the mesenchymatous cells in the distal area of the blastema. The cell density at the base of the blastema, on the other hand, has remained relatively constant as compared to the 19 day blastema.

At the "cone stage," 14 days regeneration, the mean mitotic index of the blastema has decreased by .3 per cent as compared to the 12 day regeneration stage; and a very noticeable drop in proliferation is evident for the distal third of the blastema (- 1.2 per cent) but the proximal third shows an increase (+ .4 per cent) over the preceding state (Figure 17). It is important to note that even with these changes in mitotic activity the distal rate is still considerably higher (+ 1.3 per cent) than the rate at the base. The cell density has increased in both the distal and proximal regions of the 14 day blastema but the cells are slightly more densely aggregated at the base than at the tip. This difference in cell density pattern is apparently a reflection of the axial "condensation" which is underway at this stage.

For the 16 day aneurogenic blastema (early paddle stage) the trend for a decrease in apical proliferation and the decrease in the basal rate is continuing. It should be noted that the base counts for this stage were made just distal to the axial differentiation of cartilage which is beginning at this time and represent, therefore, the high proliferation rate (Figure 17) of the mesenchymatous cells which are aggregating to form the future skeletal parts. As can be noted (Figure 17) the mesenchymatous cell density has increased for both the apical and basal areas.

In summary, the overall growth rate of the aneurogenic blastema shows a similar pattern to that found for the axolotl blastema. Based upon the total mitotic activity (tip and base) it can be observed from Figure 17 that the aneurogenic blastema shows a rapid growth increase from 10 days with a peak at 12 days of regeneration and then a gradual decline as development proceeds.

DISCUSSION

In the axolotl during the initial days of regeneration, there occurs at the apex of the limb stump a gradual thickening of the wound epidermis which constitutes, at 6 to 8 days post-amputation, an "apical cap" 10 to 12 cell layers thick. This apical thickening is apparently the product of both apical epidermal cell divisions, (see page 39) and the distal movements and divisions of cells from proximal epidermal levels. Concomitant with the formation of this thickening, and immediately underlying it, forms the initial collection of mesenchymatous cells for the blastema. For the axolotl then, as for other salamanders (see Thornton, 1960) the development of an apical epidermal thickening is correlated with blastemal cell aggregation.

Faber (1960) reported that preceding apical lobe development in the axolotl the thickening of the wound epithelium was not very pronounced so that he was unable to observe a distinct cap. The difference between Faber's findings and the present observations on early wound skin development is minor and might be resolved by a clearer definition of what constitutes an "apical cap." According to Thornton (1954) the epidermal cap thickening is at least twice that of the remainder of the wound epithelium at the apex of the regenerating limb. If this criterion is used,

a cap is easily discernible at the tip of the 6 to 8 day axolotl regenerate. Perhaps, however, we might simply consider the "apical cap" merely as the thickest part of the wound epithelium and not importantly different in function or structure (Thornton, personal communication).

At 10 days regeneration (early bud blastema) a high degree of mitotic activity of the apical epidermis was observed. Subsequent to this activity, and apparently resulting from it, there developed a secondary wound epidermal thickening--the "apical lobe." The possibility that rapid apical epidermal proliferation was the major source for apical lobe development was strengthened by counts of proximal and distal epidermal cells. The apical mitotic index at the early bud stage was found to be an impressive 200 per cent higher than that for the stump epidermis. Mitotic figures are found in the wound epidermis throughout regeneration. Indeed, even Leydig cells, a common component of both the wound epithelium and the apical lobe as determined in this investigation were repeatedly found in various stages of division.

These findings are apparently at variance with those reported for the adult newt Triturus viridescens (Chalkley, 1954; Hay and Fischman, 1961). These investigators observed a high degree of mitotic activity associated with the proximal epidermis but bound a limited number of divisions in the wound epithelium, especially the apical region. Chalkley (1954) demonstrated by cellular counts a

distinct peak in mitotic activity in the proximal epidermis at 7 and 13 days post-amputation. Only later, at 19 days regeneration, did the peak of activity move into the blastema. Even then the mitotic index remained closely related to that of the stump, being highest at the base of the blastema. A comparable stage of regeneration in the axolotl, as far as can be determined, would be the early bud (10 day) blastema, a stage in which considerable mitotic activity in the apical epidermis is found. Consistent with the mitotic index calculations of Chalkley are the autoradiographic studies of Hay and Fischman (1961) for DNA synthesis in limb regeneration in the adult newt. These workers reported epidermal incorporation of thymidine at levels proximal to the amputation surface, followed by distal migrations, and only in a few instances were cellular divisions found within the wound epithelium. Due to the dilution of the isotope label, it was determined that those cells which divide apically probably do so only once.

Although a systematic study of mitotic activity of the limb epidermis was not a feature of the present investigation, 3 periods of regeneration (7, 10, and 13 day regenerates) were surveyed for epidermal cell divisions. These stages represent critical periods in wound epithelial and blastemal development; at 7 days cap formation occurs; at 10 days the apical lobe is just about to begin its development; and at 13 days the intense mesenchymal proliferation within the blastema is underway. For each of these stages

a high degree of proliferation was revealed for the apical wound epidermis. The apical rate as illustrated in Table 1 was considerably higher than the rate for either of the proximal levels sampled. It is suggested that the incompatability of the findings of this study on epidermal divisions with those reported for the newt are due mainly to regeneration differences characteristic of the species used. Another possibility is that they represent regenerative differences which might exist between adult and larval forms. Whatever the underlying causes, it is apparent that the differences in epidermal mitotic activity as reported above are significant.

The influence of the wound epidermis on blastema formation and development has been studied extensively over the past several years and numerous theories have been offered to explain its activities (cf. Introduction). Paramount among these considerations was the hypothesis that the apical epidermis supports the accumulation of mesenchymatous cells and operates in the establishment of proliferation patterns within the blastema.

Thornton (1954, 1958) has described the apical epidermal cap as a transitory sturcture in the regenerating larval limbs of several species of Ambystoma. As an identifiable entity the cap was present only during the phase of limb regeneration which involves the accumulation of mesenchymatous cells for blastemal development. As indicated from cap deviation experiments (Thornton and Steen, 1962),

blastemal growth and differentiation may continue within the confines of cell patterns established during the epidermal cap phase of regeneration.

The concept that the wound epidermis may function in establishing a proliferation phase for blastemal growth has been proposed by Faber (1960). An increase in the apical epidermal mass, described in this study as a secondary epidermal thickening, or epidermal lobe, has been described by Faber (1960), and he has correlated its appearance with the onset of active proliferation within the developing blastema. He has likened its function to that ascribed to the embryonic ectodermal ridge (Tschumi, 1957) in the control of differentiation of the apical mesenchyme. Faber noted, as a result of the displacement of implanted carbon particles, that growth of the limb blastema of the axolotl is noticeably higher in distal than in proximal regions. In the early "bud" [or conical blastema, as Faber (1960) has designated it] the mesenchymal accumulation possesses a homogeneity in distribution and is not very dense. In later stages of development the density of the distal mesenchyme is considerably increased, a fact which might indicate that growth is by means of apical proliferation. Since the early bud blastema exhibits a homogeneous cell density pattern, Faber (1960, 1962, 1965) concluded that the "apical proliferation center" had not yet been established at this stage but subsequently appeared in relationship with the formation of the apical epidermal lobe. The evidence presented in the

present paper clearly demonstrates, however, that distal proliferation has already commenced by the early bud stage. The proliferation phase, therefore, precedes apical epidermal lobe development but is correlated with an intense mitotic phase of the apical cap. This temporal relationship suggests the possibility that distal cell division within the mesenchyme might be influenced by factors initiated by the wound epithelium. It also may indicate that the apical epidermal lobe as a discrete entity has no special role but is merely an outgrowth formed by continued high mitotic activity of the apical epidermis in general. In those cases in which the lobe did not develop, (see page 39), no obvious effect on regeneration could be observed.

It has been pointed out previously (Thornton, 1956-1958, 1960, 1962; Thornton and Steen, 1962) for both normally innervated and aneurogenic limbs of several Ambystoma species that the apical wound epithelium (the "epidermal cap") functions to "direct" the accumulation of mesenchymatous cells which produce the regeneration blastema. The evidence presented in the present paper clearly demonstrates for two Ambystoma species that within the mass of mesenchymatous cells which accumulates in association with the apical wound epithelium there develops at a very early time (10 days for the axolotl) in the regeneration process an apical dominance in the proliferation rate of the blastemal cells. Indeed, the high apical rate in mitotic divisions was evident throughout the rapid growth phase of the blastema

(between 10 - 16 days regeneration) and also for the 19 day regenerate (paddle stage) provided the mitotic counts were specifically focused upon the digital "condensation" area (see page 52). Reference should be made to Figure 4 for a view of the intense mitotic activity of the apical mesenchymatous cells of the mound blastema (13 days regeneration). It was also made evident from cell density calculations in this study that throughout the rapid growth phase of the blastema there was a continual increase in cell density in the distal part of the blastema. This increase in cell density might be regarded as additional evidence for the observed high incidence of apical mitotic activity. It was also noted that the increases in cellular proliferations at more proximal levels (mainly Zones II and III of the blastema) as regeneration progressed, were characteristically associated with the more proximal extension of this dense mass of mesenchymatous cells.

Thus, the results of the present investigation confirm the hypothesis of Faber (1960, 1965) that an "apical proliferation center," here identified as the apical dense mass of proliferating blastemal cells, is established within the limb blastema of the axolotl. The present study further provides evidence for the suggestion by Faber (1960) that the "apical proliferation center" functions in producing the cellular materials for the new tissue components of the regenerating limb. Although the present study clearly proves by precise mitotic index calculations and statistical

analyses that an "apical proliferation center" does exist, whereas Faber's carbon marking experiments only suggested the existence of the center; it was not designed to test the theory that an "apical organizational center" (Faber, 1960) is also operative in limb regeneration (cf. Introduction, p. 16).

The possibility that the "apical epidermal lobe" might function to initiate the high degree of divisions among the apical mesenchymatous cells has been ruled out, since as was earlier indicated, a high rate in proliferations was found to precede the development of the epidermal lobe. The question arises, however, as to the possibility that the "epidermal cap" might in some way influence blastemal cell proliferation. A clue to this question might be derived from the experiments of Michael and Faber (1962) who found that for a renewal of growth and differentiation of limb transplants to occur, a wound epithelial covering (devoid of dermis) derived either from the transplanted limb stump itself or from the skin of the back (graft site) was required. A similar requirement for growth was noted for the normal blastema by Thornton (1957, 1958). In the experiments reported above the possibility that the wound epithelium and especially the cap region might serve to influence the underlying mesenchyme to become mitotically active has been investigated and some interesting correlations have been revealed. It was found, for example, that a striking relationship exists between the mitotic activities

of the wound epithelium and the underlying mesenchymatous cells. Indeed, for all blastemal stages, with the exception of the 19 day regenerate, which was not examined for epidermal mitosis, a striking parallel in the fluctuations in the mitotic activity between these two tissues was observed. Thus, intense mitotic activity in the apical wound epithlium (day 10) was accompanied by a high rate of proliferation within the adjacent region (Zone I) of the blastema. Subsequently, as the mitotic activity shifted into more proximal wound epithelium regions and decreased in the apical part (days 13 and 16) a similar change in cellular divisions was noted for the underlying mesenchymatous cells of the blastema. Thus, Zones II, III, and IV showed increases while Zone I underwent a slight decrease. Since high mitotic indices were found throughout the proliferation stages of regeneration in both the wound epidermis and the mesenchymatous cells of the blastema, the possibility must be considered that factors common to both tissues, and acting simultaneously on both, influence their mitotic proliferation. In other words, both wound epidermis and blastemal cells may be independently reacting to the same mitotic stimulant. This possibility, however, seems less likely in view of the following facts. There is a high mitotic activity, although not as intense as the 10 day stage, for the apical wound epithelium at 7 days regeneration (apical cap formation) which, interestingly enough, corresponds to the time for the beginning of blastemal cell accumulation. Cell counts

performed on the apical wound epithelium of the 5 day regenerate, on the other hand, revealed a much lower rate in mitotic activity as compared to the 7 day limb. Thus, beginning with the initial accumulation of blastemal cells there is a very noticeable increase in the mitotic activity within the apical wound epidermis (epidermal cap) which shows a peak in activity at the 10 day stage (early bud) commensurate with the burst of mitotic activity among the most distally located mesenchymatous cells of the developing blastema. Therefore, it can be shown that the heightened mitotic activity of the wound epidermal cells precedes that of the accumulating blastema cells and the relationship is especially obvious in the first 10 days of regeneration.

Evidence which would support the inference for a relationship between the activities of the wound epithelium and the underlying blastemal cells has been derived from two additional experiments. A most interesting correlation was found, for example, when the apical epidermis was shifted from its normal position (apical to the limb stump) to an eccentric position. It was highly interesting to observe that following the removal of the skin (epidermis and dermis), to produce asymmetry of the epidermal cap, the wound epithelium which migrated to cover the extensive wound surface became unusually thickened (Figure 11). As regeneration proceeded in these asymmetrical blastemata, the mesenchymatous cells of the blastema were found not only to aggregate beneath the thickened region of the wound

epithelium but a very noticeable increase (Figure 16) in the number of mitotic divisions among these cells was also found.

A similar relationship between the activities of the wound epithelium and the underlying blastemal cells was found for the aneurogenic limb. Indeed, as the numbers of cell layers of the apical wound epithelium in these regenerating limbs increased and became more active mitotically, a corresponding rapid increase in the mitotic activity among the most distally located mesenchymatous cells was observed (Figure 17, Figure 2). These results for the aneurogenic limbs are also particularly interesting since they demonstrate that blastemal cells aggregate distally (see Thornton and Steen, 1962) and then undergo an intense proliferation phase in the complete absence of nerve fibers. Thus, the findings for both the asymmetrically oriented blastema and the aneurogenic limb blastema, as well as the normal limb blastema, support and extend the hypotheses of Thornton and Faber that the wound epithelium functions, in some unknown manner, in both "directing" the accumulation of blastemal cells and in the "stimulation" of these cells to proliferate.

In this regard, it is important to note that a close association between the cells of the blastema and the wound epithelium, especially the apical part, was maintained throughout the rapid growth phase of the blastema (10 - 16 days of regeneration). As can be seen in Figure 4 a small space (30 to 40 microns across) separates the two

types of cells. By careful examination it was found that this space is criss-crossed by cytoplasmic strands which apparently originate from the mesenchymatous cells of the blastema. Thus, the space itself is sufficiently small enough to allow for the exchange of chemical materials (see Grobstein, 1963) between the two types of cells, but such an exchange could also be facilitated by the interconnecting cytoplasmic strands. I should like to suggest that the wound epithelium in the presence of wound tissues undergoes a "type" of dedifferentiation (see Thornton, 1962), and becomes highly active mitotically, as has been observed, and possibly highly active metabolically. As a result of this alteration, the wound epithelium may in turn "direct" and "stimulate," possibly through a chemically diffusible substance, the accumulation and then the active proliferation of the aggregated dedifferentiated blastemal cells. I should also like to propose that once the "initial information" has been supplied from the wound epithelium to the underlying blastema (10 to 16 days of regeneration) the blastemal cells continue their divisions until they are later organized into the limb parts by other influences which may originate from the "apical organization center" (see Faber, 1965) and also the proximal stump regions.

SUMMARY

It has been demonstrated in this study using the precise experimental methods of mitotic index calculations and statistical analyses that an apical dominance in mitotic activity exists among the most distally located mesenchymatous cells of the forelimb blastema of the axolotl. The high apical mitotic rate was evident throughout the rapid growth phase of the blastema (10 to 16 days of regeneration) and provided a dense apical mass of proliferating mesenchymatous cells. As proposed by Faber (1960, 1965) this apical dense mass of proliferating mesenchymatous cells may be regarded as the "apical proliferation center." An apical peak in the number of mesenchymatous cell mitoses was also demonstrated for the asymmetrically oriented blastema of the axolotl and the aneurogenic limb blastema of Ambystoma opacum. No correlation as suggested by Faber (1960), between the development of the prominent apical epidermal lobe and the rate of the proliferation of the mesenchymatous cells of the axolotl blastema could be found. A striking temporal relation, which may represent a causal relation, was found, however, to exist between the mitotic activity of the wound epithelium and the initial accumulation and regional distribution of bitoses of the underlying blastemal cells.

Figure 1a.--Diagrammatic drawing of the forelimb blastema illustrating the five sample levels for the cell counts.

Figure 1b.--Diagrammatic drawing of a center section of the forelimb blastema showing the four sample areas, A, B, C, and D for the cell counts.

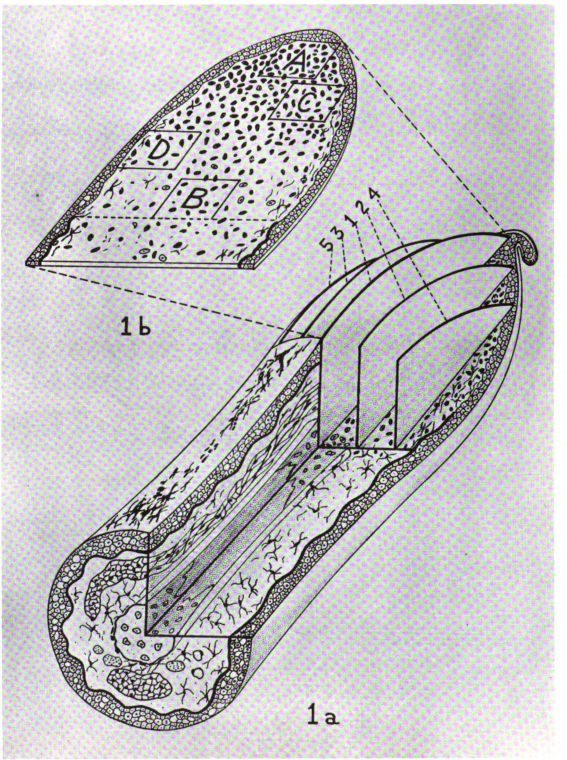
A
C
D
B
5
3
1
2
4
1b
1a

Figure 2.--Twelve day aneurogenic blastema showing numerous apical mesenchymatous cell divisions. 560X.

Figure 3.--The apical wound epithelium of a 10 day axolotl regenerate possessing numerous epidermal cell divisions. 560X.

Figure 4.--A 13 day axolotl regenerate showing the intensive mitotic activity of the apical mesenchyme. 590X.

Figure 5.--The prominent apical epidermal lobe of a 16 day axolotl regenerate. Note the excessive number of Leydig cells and also the pigment granules within the wound skin. 220X.

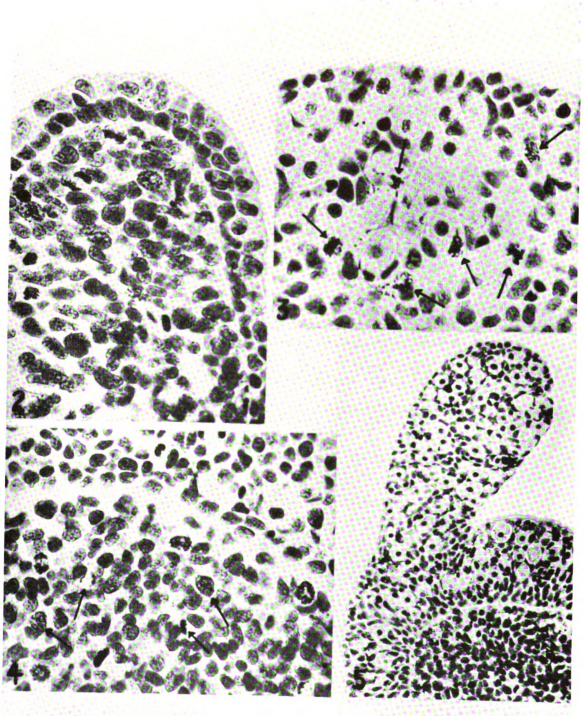

Figure 6.--Longitudinal section of the "early bud stage" (10 day blastema) illustrating the arbitrary vertical zones. Note the homogenous distribution of the mesenchymatous cells. 150X.

Figure 7.--Longitudinal section of the "mound stage blastema" (13 days) showing the vertical zoning. Note the dense apical mass of mesenchymatous cells and also the apical lobe. 135X.

Figure 8.--Longitudinal section of the "cone blastema" (16 days regeneration) showing the vertical zoning. Note the axial aggregation of blastemal cells which is beginning at the center and base of the blastema. 135X.

Figure 9.--Longitudinal section of the 19 day regenerate "paddle stage." Note the pattern of zoning and also the mesenchymatous cell condensations for digits 1 and 2 at the apex of the limb. The arrow indicates the mass of mesenchyme destined to produce digits 3 and 4. 120X.

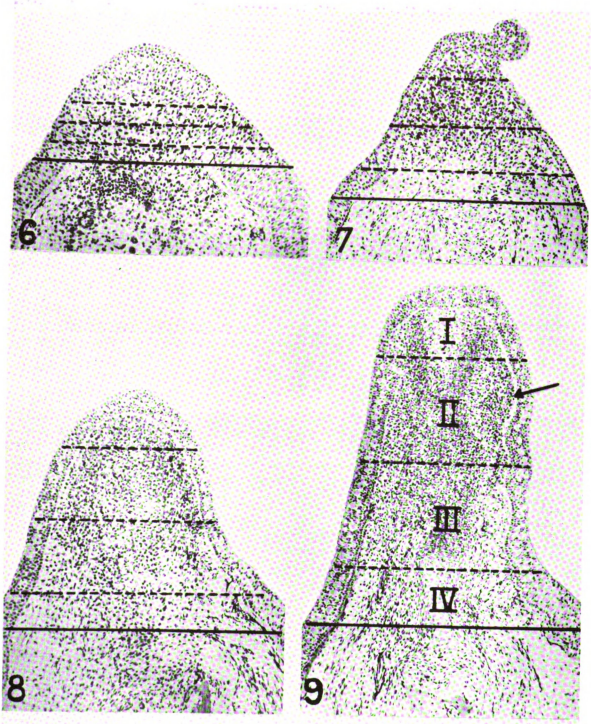
6
7
8
9
I
II
III
IV

Figure 10.--Cross section near the distal tip of a 13 day blastema (early bud stage). The upper arrow indicates the dorsal side and the bottom arrow the ventral side of the blastema. 380X.

Figure 11.--Sixteen day asymmetrical blastema illustrating the sample areas for the cell counts. Arrow indicates the normal axis of the limb. 110X.

Figure 12.--Cross section near the center of a 13 day blastema showing the 5 regions for the cell counts. 220X.

Figure 13.--Cross section near the base of a 13 day blastema. 150X.

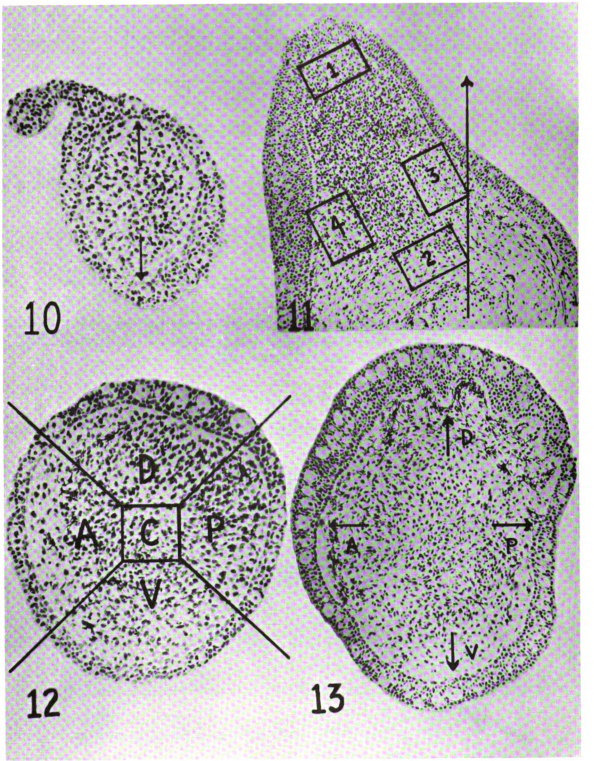
10
1
3
4
2
11
D
A
C
P
V
12
D
A
P
V
13

Figure 14.--Graphic representation of the mitotic index and cell density distribution of the 4 longitudinal zones for the 4 regeneration stages.

Figure 14

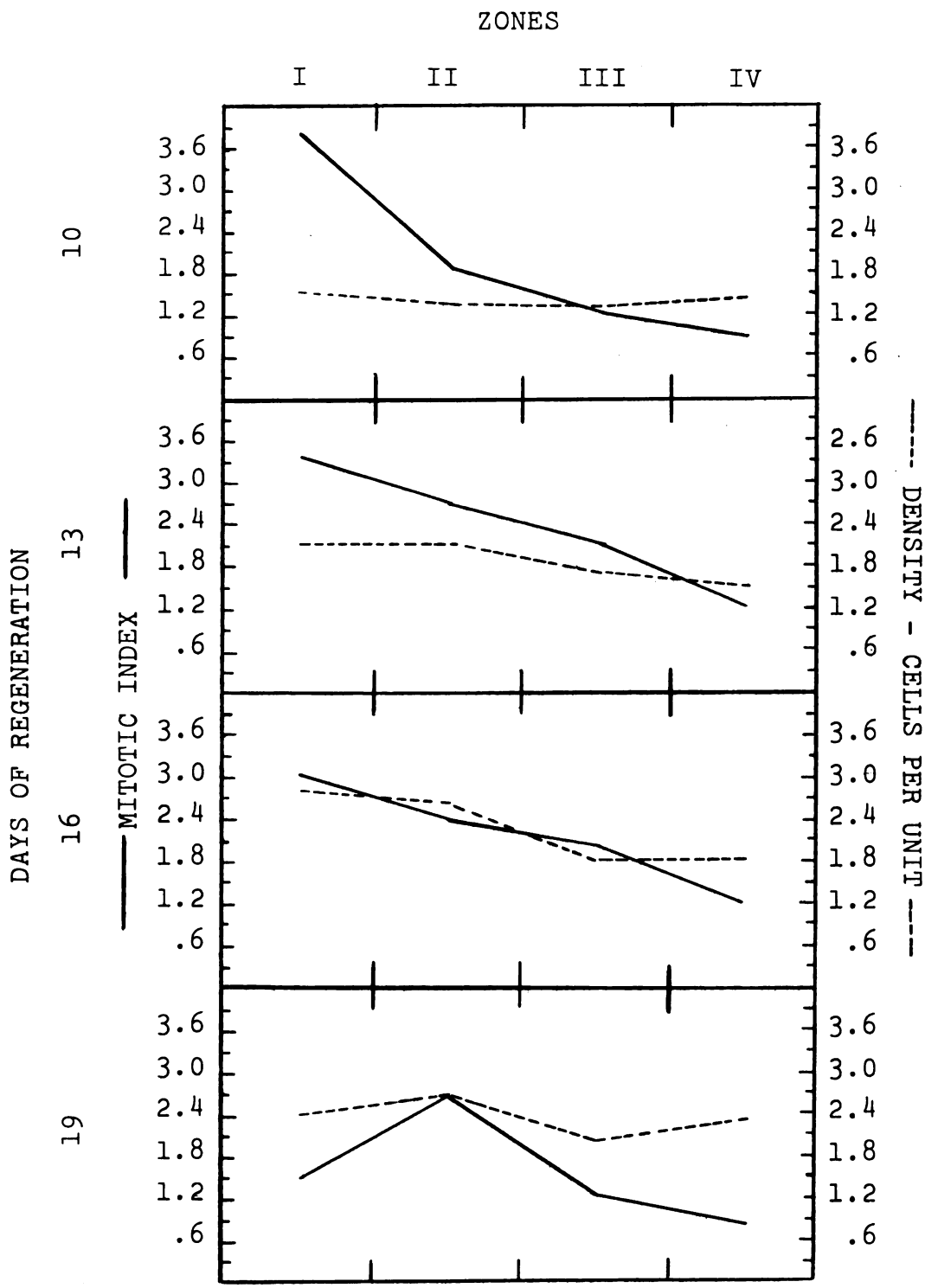
ZONES
I
II
III
IV
DAYS OF REGENERATION
10
13
16
19
MITOTIC INDEX
DENSITY - CELLS PER UNIT
3.6
3.0
2.4
1.8
1.2
.6
2.6

Figure 15.--Graphic representation of the mitotic index and cell density distribution of the 5 cross sectional regions for the 4 regeneration stages.

Figure 15

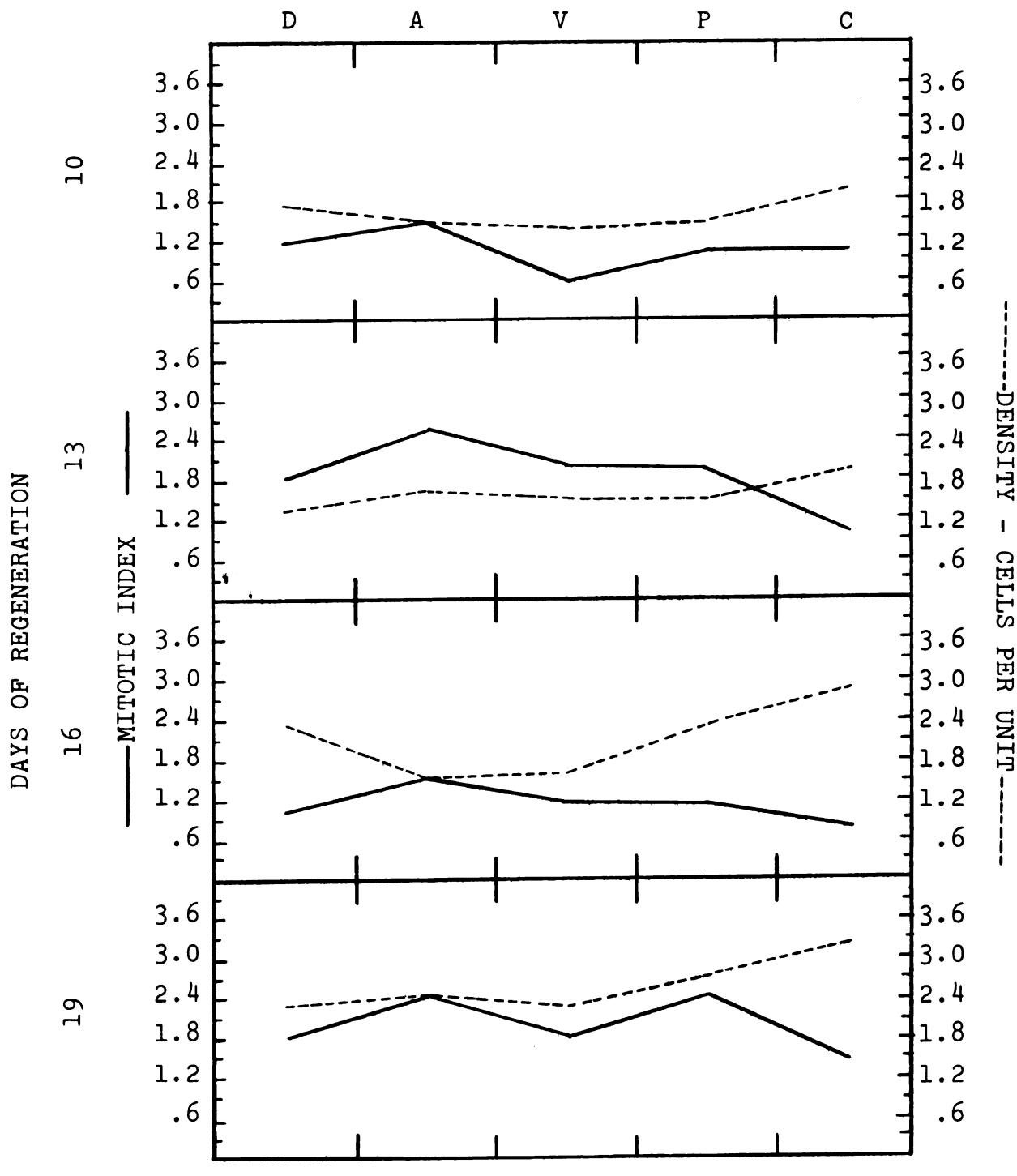
REGIONS
D
A
V
P
C
DAYS OF REGENERATION
10
13
16
19
MITOTIC INDEX
DENSITY - CELLS PER UNIT
3.6
3.0
2.4
1.8
1.2
.6

Figure 16.--Graphic representation of the mitotic index and cell density distribution of the 4 sample areas for the 3 regeneration stages of the asymmetrical blastema.

Figure 16

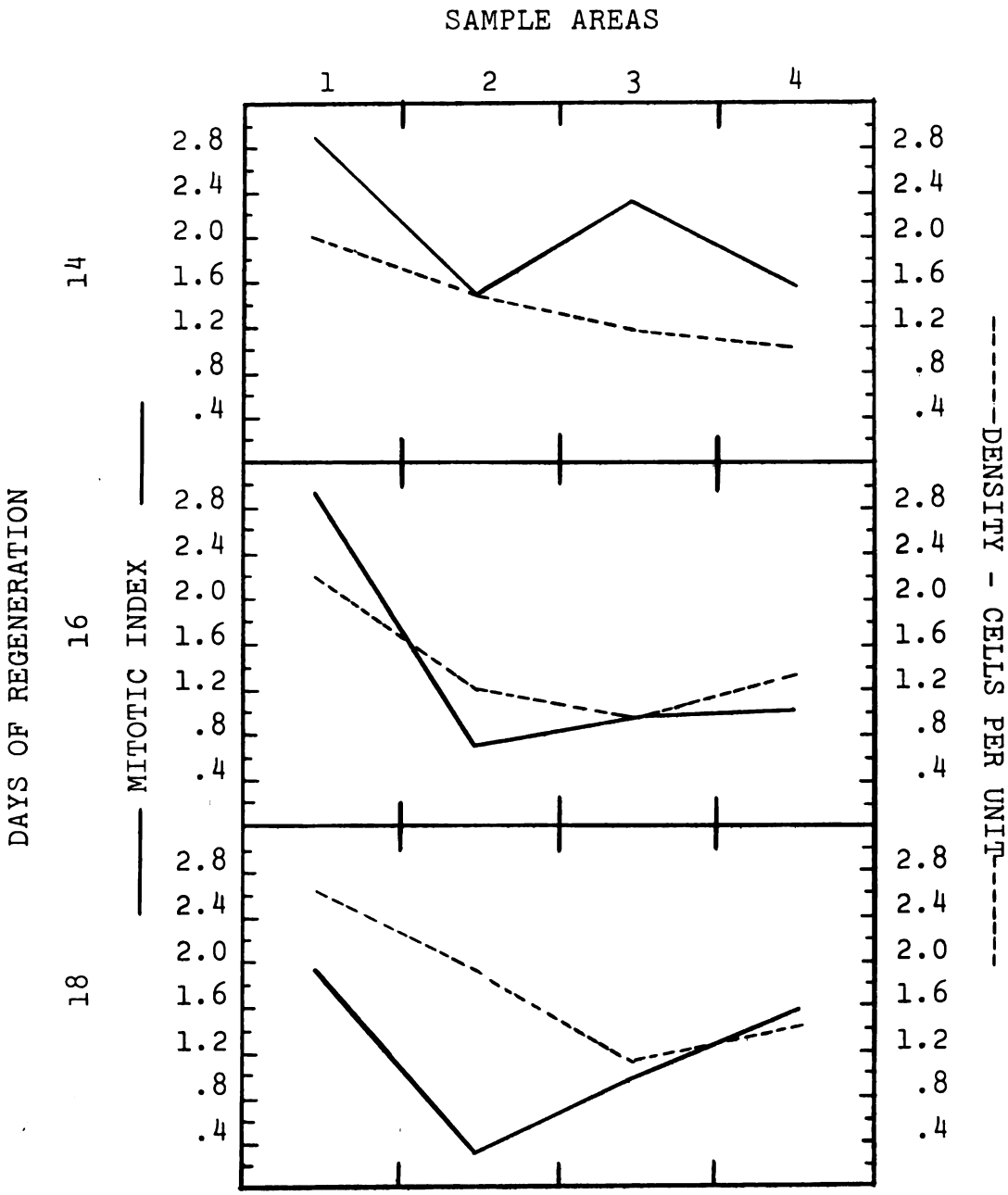
SAMPLE AREAS
1
2
3
4
DAYS OF REGENERATION
14
16
18
MITOTIC INDEX
DENSITY - CELLS PER UNIT
2.8
2.4
2.0
1.6
1.2
.8
.4

Figure 17.--Histogram showing the mitotic index and cell density distributions for the base and tip areas for the 4 aneurogenic blastemata.

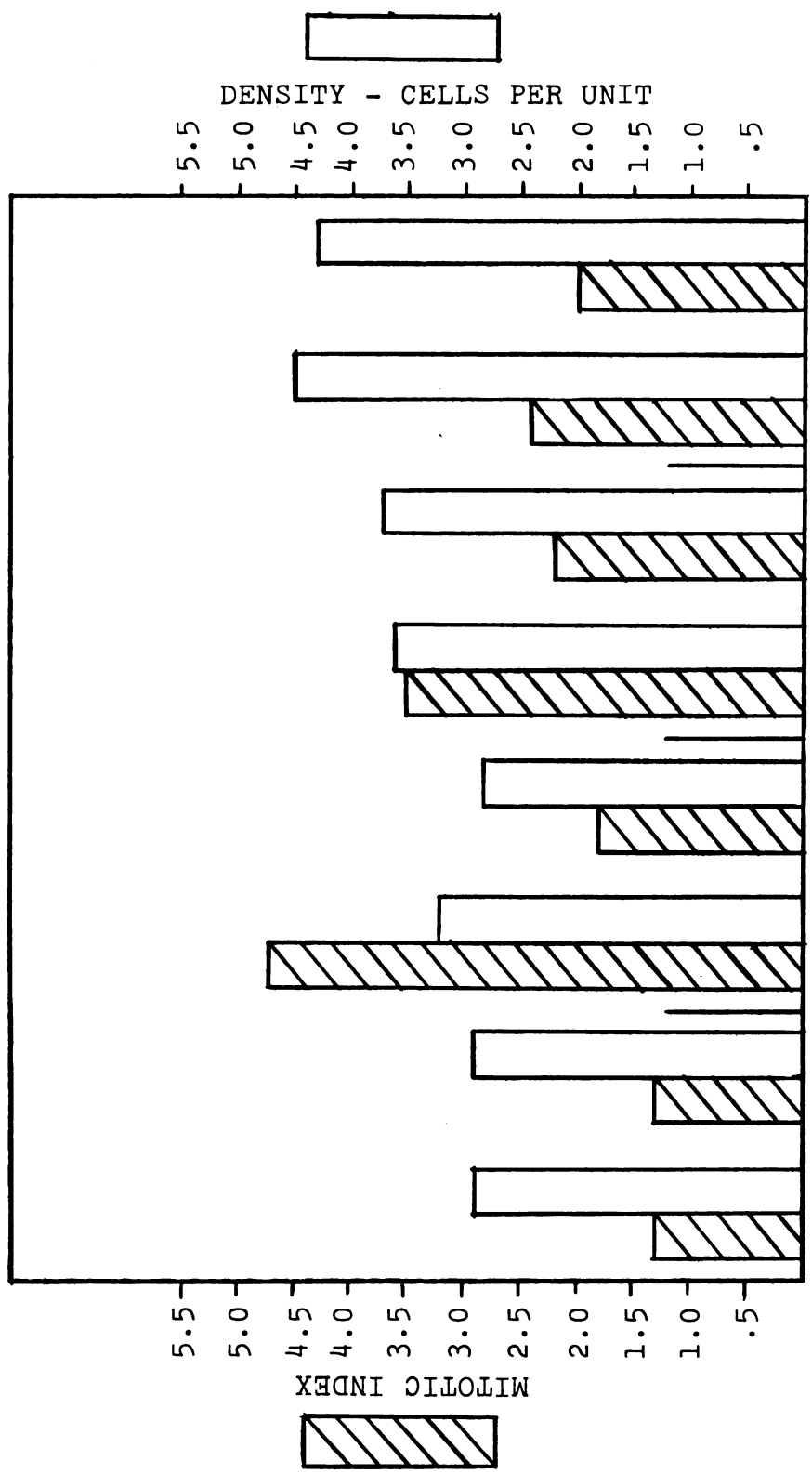
DENSITY - CELLS PER UNIT
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
MITOTIC INDEX
TIP
BASE
TIP
BASE
TIP
BASE
RIP
BASE
10
12
14
16
DAYS OF REGENERATION

Figure 17

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