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Forelimb Regeneration Study in the Adult Small-Mouthed Salamander (Ambystoma texanum)

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FORELIMB REGENERATION STUDY IN THE ADULT SMALL-MOUTHED SALAMANDER (*AMBYSTOMA TEXANUM*)

(TITLE)

BY

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THESIS

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INTRODUCTION

Regeneration of lost body parts in higher animals is in essence a compendium of embryonic events revisited upon the adult host. It involves the replacement of a body part possessing considerable structural complexity. This process is initiated by the accumulation of undifferentiated cells termed a blastema, and brought to completion by the control of differentiation in the blastema, whereby the new product bears close resemblance to the lost part. The regenerating organ or part passes through the majority of morphogenetic stages as did its embryonic counterpart.

Regeneration of structural units of the body is an integral function of many of the invertebrate phyla. This function is a rarity in the vertebrates. The class Amphibia, however, possesses the capacity for regeneration and great diversity exists in the regenerative power of the various groups within the class. The anuran amphibians (toads and frogs) forfeit their regenerative capacity after the metamorphosis, in contrast to most of the urodele amphibians, (newts and salamanders), which retain their regenerative powers when transformed into adults. Urodales can regenerate many parts of the body, namely; the forelimbs, hindlimbs, digits, tails, much of the eye (sic lens, iris, and retina), peripheral nerves, and the anterior part of the head.

This thesis will limit its range of investigation to forelimb regeneration in the adult urodelan salamander, Ambystoma texanum.

The purpose of this investigation is three-fold:

1. To perform an initial study of forelimb regeneration in the little-studied species, Ambystoma texanum, and establish whether regeneration does occur under the experimental conditions. This will provide evidence that another species of salamander possesses regenerative capacity and will perhaps advance the knowledge of the phylogenetic distribution of limb regeneration in the family Ambystomidae.
2. To follow the regeneration of the forelimb by describing the various stages of development and to establish the average time of inception for each stage.
3. To trace the external gross morphological changes and the internal minute histological changes of the regenerating limb.

LITERATURE REVIEW

The first recognition of amphibian regeneration is generally credited to Spallanzani (1768). Nicholas (1955) stated that the nineteenth century literature had many references to abnormalities created after regeneration, and that researchers of the late nineteenth and early twentieth centuries, such as T. H. Morgan, P. Weiss, R. Harrison, and W. Roux, initiated the early attempts to explain regeneration. Since 1900, many of the principles of regeneration have been determined, however, many paradoxes still exist.

Regeneration can be divided into phases which usually occur in a definite sequence or time. There exists no sharp demarcation between the phases and they may overlap. There can be different phases occurring at different levels of the regenerating limb. These phases include (1) wound-healing and apical cap formation, (2) dedifferentiation, (3) blastema formation, (4) differentiation and growth, (5) and control of morphogenesis. Most of the investigations used either various species of Ambystoma or the newt Triturus viredescens to discover the various principles of regeneration. Unless mentioned contrary, literature citings will deal with regeneration in adult urodelan salamander of either the previously mentioned genera.

The genus Ambystoma is also listed in the literature reviewed as Amblystoma. Ambystoma is the taxonomically correct name of the genus. This misnomer, Amblystoma, probably a spelling or printing error, was first detected in an article by Baird in (1859). The wide impact of Baird's work possibly caused the mistake to be perpetuated. Therefore,

in bibliographic information, Amblystoma will be maintained.

Wound Healing and Apical Epidermal Cap

When a limb is severed from the body of an amphibian, the epidermis is the first tissue to respond to the emergency. The epidermis serves two major roles in regeneration -- wound closure and apical cap formation. The presence of an epidermal layer is an absolute necessity for regeneration.

Amphibian (urodelan) wound healing differs from mammalian wound healing, in that the dermis does not contribute to the healing in the amphibian. Lash (1955), using a vital stain, observed epidermal cells during wound healing. He found that wound closure was accomplished in about one day. There was detachment of epidermal cells from the basement lamella bordering the wound. A sheet of epidermal cells moved toward the center of the wound in a coordinated mass movement, rather than individual amoeboid movement. There was simultaneous arrest of mass movement once closure was effected. Chiakulas (1952) has shown that there is tissue specificity in wound healing. Several epidermal transplants were shown to be incompatible with the host site epidermis. In these cases, the two tissues would not fuse or cooperate in closure of wounds. These cells may differ slightly in their chemical, or more specifically, stereochemical composition. Therefore, wound closure may be a complicated chemical-physical phenomena instead of a purely mechanical manifestation.

The wound epidermis may have several functions. It protects the stump tissues by minimizing loss of body fluids and ingress of foreign

materials. Needham (1941) and Thornton (1949, 1950, 1951) proposed that the injuried epidermal and stump cells may secrete a wound hormone which may trigger the entire regeneration response. Beryllium nitrate, a compound which inhibits regeneration, was used in their investigations. It was found that beryllium application was effective only when applied to the amputation surface immediately following amputation. Beryllium applied six hours before or after amputation had no detrimental effect on regeneration. These investigators hypothesized that beryllium destroyed the wound hormone produced at the amputation surface. The hormone either had an effect of short duration, triggered irreversibly the regeneration phenomena, or was quickly protected by the wound closure.

Singer (1959a) stated that wound closure and other wound reactions such as tissue breakdown, intense phagocytosis, and formation of scar tissue occurred within the first five days. The epidermal layer that closed the wound started to thicken and became the apical epidermal cap. Singer (1959) demonstrated that the apical cap had 12 or more layers of cells in contrast to three or four layers in the normal limb.

One of the few rules of regeneration states that the epidermal layer must have an unobstructed contact with the internal mesodermal structures for regeneration to occur. A dermal layer or basement membrance interposed between the epidermis and mesodermal tissue will stop regeneration.

Many investigators have correlated the dermis with non-regeneration. Rose (1964) reviewed the works of Tornier (1906), Schawal (1921), and Godlewski (1928) who discovered that regeneration was prevented by

placing whole skin over the surface of the wound immediately after amputation. This demonstrated that the dermis might be retarding regeneration. Once the wound was covered by epidermis, however, whole skin transplants had no effect on the regeneration. Schotté and Harland (1943) and Rose (1944) demonstrated that in adult frogs, which do not regenerate limbs, a dermis layer was always present in wound healing. This showed that the dermis plays a large role in adult anuran wound healing. Rose (1944, 1945) induced regeneration in adult frogs by obtaining a dermis free epidermis by treating the stump with sodium chloride solutions.

The epidermis has been shown to be necessary for dedifferentiation and blastema formation by Thornton (1957). He removed the epidermal layer or apical cap daily from several species of Ambystoma, and dedifferentiation and regeneration were prevented. Singer and Salpeter (1961) proposed that the role of the apical epidermal cap in dedifferentiation was one of phagocytotic and organization destroying nature. Several authors have reported indirect evidence for migration of apical cap cells into blastema. Scheuing and Singer (1957) injected beryllium nitrate into blastemas which resulted in necrosis and cellular destruction. Apical cells were observed migrating into the stump and engulfing products of cellular destruction. A dermal layer would be a barrier to this migration and inhibit regeneration.

Another requirement for regeneration includes intimate contact between epidermal cells and nerve fibers. The role of nerves are vitally important to regeneration. Singer (1949) found that normal epidermis is poorly innervated, but wound epidermis has a very rich neural supply.

Hay (1960) observed that the intimate contact between the nerve and cells were similar to the type found at synapses. Rose (1948a, 1948b) and Singer (1959a) suggest that the dermal layer would stop this intimate contact and correlate this with non-regeneration.

Thornton (1954), in his study of supernumerary limbs, created by deviated nerve supply, found that the basement membrane dissolves at junctions of nerves and epidermal cells, the apical cap forms, and mesenchymal tissue accumulates. Rose (1964) states that if not enough afferent nerves make contact with epidermis, regeneration does not occur. Regeneration will still proceed if there is an augmented efferent nerve supply which does not have to enter the epidermis. This demonstrated that some other tissue was working in concert with the nerves to take over the function of the epidermis.

Thornton (1956) summarizes by stating that an apical epidermal cap free of dermis is needed for dedifferentiation, blastema accumulation, and possibly one further step associated with differentiation. The apical cap must have intimate contact with mesodermal tissue to possibly transmit wound hormone message, to contribute migratory phagocytotic cells to help in dedifferentiation and/or to establish close with neural pathways.

Dedifferentiation.

Following wound healing, the stump tissues undergo dedifferentiation which means the return of cells and tissues to a morphologically simple state resembling embryonic cells. The stimulus which triggers dedifferentiation has not yet been elucidated. It might not be a

single factor, but a number of factors working in concert. Release of wound hormone, action of migratory organization destroying epidermal cells, stress of operation, and the change in stump physiology due to amputation have all been proposed as the initiative factor of dedifferentiation. Needham (1941, 1952) proposed the existence of a wound hormone or regeneration promoting factors released by stump cells immediately after amputation. Possibly, this factor sets dedifferentiation into motion. The wound hormone is only conjectural and is far from being detected directly or chemically identified.

The amputation or injury alters the environment of the stump which creates conditions conducive to dedifferentiation. The stump is a site of great changes and upheavals in metabolism, biochemistry, and physiology. Goss (1969) and Needham (1952) describe the physical and biochemical changes in the stump after amputation. Blood supply is curtailed lowering the amount of oxygen. Macrophages and lymphocytes are mobilized for defense and demolition. Metabolism via the Kreb's cycle is replaced by the glycolytic and/or pentose phosphate shunt pathway. This shift in metabolism was shown by Johnson and Singer (1964) when they demonstrated that lactic dehydrogenase was present in higher concentrations than succinic dehydrogenase and other enzymes of the Kreb's cycle. The shift is probably due to the lack of oxygen. Anaerobic respiration by the glycolytic pathways produces lactic acid. Lactic acid accumulation and other autolytic activity lowers the pH. Proteolytic enzymes such as cathepsins, peptidases, and collagenases are found in the stump. The shift in pH is beneficial for many catabolic enzymes have optimum efficiency in the acid range. Acid phosphatase

found in lysosomes is found in the stump and epidermis. Goss (1969) and Schmidt and Weidman (1964) demonstrated that enzymes associated with the pentose phosphate shunt are found in dedifferentiated cells. This indicates that pentose phosphates which could be used to produce energy in the glycolytic pathway are being directed into channels of nucleotide or nucleic acid synthesis. Constructive events are taking precedence over energy production.

Chalkley (1954) using mitotic indices showed for the first time that all tissues contribute cells for dedifferentiation and that there is enough cell division to account for the increase in cells that produce the new limb.

It is questioned why the tissue must dedifferentiate. Why can't the stump tissues simply grow out to complete the limb? Goss (1969) states that dedifferentiation facilitates mitosis and enhances pluripotency. Blastema formation requires a tremendous increase in cellular division to give rise to all cells needed. Most cells can not undergo the mitoses required, because the intricate cytoplasmic structures which are acquired due to differentiation are incompatible with mitosis. The cells must revert to a more immature or primitive form to undergo the many mitoses that are necessary. It is obvious that bone and cartilage tissue must be dedifferentiated first to release the osteocytes or chondrocytes from the matrices in order to replicate. Thus these dedifferentiated cells are well equipped and streamlined for the ensuing proliferation of the large cellular mass, the blastema.

It is believed that a majority of cells or certain cell types are not pluripotent. They retain the heritage that they possessed before

dedifferentiation. Steen (1968) demonstrated by labeling cells with tritiated thymidine and/or triploid nuclei that chondrocytes when dedifferentiating were almost always chondrocytes. There is evidence that some cells possess pluripotency, but this will be covered in a later section.

Morphologically, the dedifferentiating cells lose all resemblance of their previous identity. The dedifferentiation of muscle will be selected as a representative example of dedifferentiation and described. Thorton (1938) first described muscle fiber dedifferentiation. However, it was convincingly confirmed in the electron micrographs studies of Hay (1959). She demonstrated that the syncytial muscle fibers at the stump started to fragment transversely two to six days after amputation. The fragmentation created mononucleate and anucleate units. The anucleate units did not survive and were probably phagocytized or lysed. The myofibrils in the mononucleate units fragmented along the A and I bands and then disintegrated. The Z- substance behaved as an extra filamentous component.

The mononucleated unit then appeared as a primitive mesenchymal cell showing no clue to its origin. Schotté (1940), Hay (1958, 1959, 1962) and Butler (1935) all verified that there was no differences in these cells to indicate tissue of origin. Hay (1959, 1962) described the totally dedifferentiated muscle cell which would appear identical to all other blastemal cells. The nuclei became round and larger, nucleoli was prominent, chromatin was more diffusely granular, and nucleus to cytoplasm ratio was increased due to cytoplasmic loss during fragmentation. The complex endoplasmic reticulum characteristic of

differentiated cells broke up into smaller units; it also derived the new plasma membrane. Numerous free ribonucleoprotein granules were present. This discovery plus the nuclear changes demonstrated that dedifferentiating cells were undergoing active cytoplasmic protein synthesis. Bodemer and Everett (1959), using autoradiography, demonstrated that active protein synthesis was a significant feature of dedifferentiation as well as differentiation. Goss (1969) indicated that these proteins may be proteolytic enzymes manufactured to mediate the cell's own degeneration.

After dedifferentiation had occurred there must be again a modification in physiology. Needham (1952) stated that oxygen is necessary for mitosis and some proteolytic enzymes have optimum efficiency at higher pH.

Therefore, another law applying to amphibians has been derived. No dedifferentiation--No regeneration.

These dedifferentiated cells accumulate under the wound epithelium to form the next state--the blastema.

Blastema

Cellular dedifferentiation produces a structure termed a blastema. The blastema is an increasing accumulation of dedifferentiated cells between the apical epidermal cap and the stump tissue. The origin of these cells and mechanism of formation of the blastema has long been a source of controversy. Nicholas (1955) mentioned the theories advanced to explain blastema formation. They include (1) regeneration by extension as in the tail, (2) hematogenic origin of dedifferentiated cells, (3)

origin of blastema cells from reserve mesenchymal cells, (4) dedifferentiation of epidermal cells create blastema, and (5) origin of blastemal cells from all stump tissue at amputation site.

Regeneration by extension is not observed in the limb since this is restricted to structures composed of metameric segments as in the tail. Theories of origin from hematogenic or reserve cells were seriously discredited by the work of Butler (1931, 1933, 1935) and Butler and O'Brien (1952). These investigators utilized x-radiation which has a growth and mitotic destructive effect which inhibits regeneration. Salamanders were irradiated in two ways. One group was entirely irradiated except for an area a few millimeters above and below the amputation level, and the other group was irradiated only in an area a few millimeters above and below the amputation level. The first group had normal regeneration, but the latter had no regeneration. Therefore, it was demonstrated that the blastemal cells were local in origin, not immigrant cells.

Finally, there is the controversy over which tissue contributes most cells to the blastema -- epidermis and mesodermal stump tissue. Most investigators do not recognize a major epidermal contribution to the blastema.

Those who claimed considerable epidermal contributions were Rose (1948b), Hay (1952), and Scheuing and Singer (1957). Rose (1948b) reported an increase in blastemal cell with an equal decrease in epidermal cells. Hay (1952) and Scheuing and Singer (1957) counted mitotic figures and obtained an epidermal contribution. However, autoradiography studies of Riddiford (1961), Hay and Fischmann (1961), and Rose (1964)

demonstrated little epidermal contribution. Chalkley (1954, 1959) and Litwiller (1939) failed to find epidermal losses as did Rose (1948b). From the above evidence, it is now considered that the epidermis gives little to the blastema.

Chalkley (1959) stated that at present the theory on the origin of the blastema is that it is mainly formed from dedifferentiation of all the tissues at the amputation level. The epidermis contributes only a fraction of the cells, and the contributions from blood cells or reserve cells are minor.

The blastema has been shown to exert a control over dedifferentiation. This was discovered in X-ray and denervation inhibition investigations. Butler (1931, 1933, 1935) and Butler and Puckett (1940) noted that X-rays had a destructive effect as well as an inhibitory effect on regeneration. Stumps of larval salamanders X-rayed at amputation did not regenerate, however, dedifferentiation began and remained unchecked. It resulted in dedifferentiation, resorption, and regression of the entire stump proximally to the shoulder. X-radiation of a stump with a well formed blastema resulted in inactivation of regeneration, but none of the destructive dedifferentiation and resorption as in the previous case occurred. The blastema was especially sensitive to X-rays. It was hypothesized that X-rays stop differentiation by damaging the mitotic mechanism, causing the mitotic mechanism to disfunction, or causing failure to transmit morphogenetic information. Dedifferentiation is normally checked by action of the blastema. When the blastema is inactivated by X-ray at the time it is forming, dedifferentiation remains unchecked, goes wild, and destroys the stump. Irradiation of a well

formed blastema causes no excess dedifferentiation, because the blastema has already halted dedifferentiation. The above mechanism of removal of the regressing stump is unknown.

This same dedifferentiational regression has been observed in amputated denervated larval salamander limb by Butler and Schotté (1941, 1949) and Schotté, Butler, and Hood (1941). Denervation prevents the formation of blastema, thereby, dedifferentiation is unchecked and destroys the stump. Nervelessness is not the sole cause of regression, for amputation or injury is also required. The regression is similar to the above mentioned one. When reinnervation occurs, a blastema is induced, and dedifferentiation is brought to a halt. Schotté, Butler, and Hood (1941), by transplanting young blastemata to regressing limbs, stopped the wild dedifferentiation. However, once a blastema has started to show differentiation, it has lost the capacity to control dedifferentiation, if transplanted.

From the above examples, it is shown that the presence of a blastema is needed for cessation of dedifferentiation and to establish and maintain a balance between the processes of dedifferentiation and differentiation.

As the blastema cells accumulate, the regenerate appear like a conical projection. This conical projection will soon become dorsoventrally flattened and acquire a shovel or paddle-like appearance. This is indicative of the next, phase--differentiation.

Differentiation

The redifferentiation of tissue closely resembles the normal

histogenesis found in the embryo. The events of dedifferentiation are reversed. The nucleus reverts to a more mature form and the complex endoplasmic reticulum characteristic of each tissue starts to form. To illustrate differentiation, muscular tissue will be followed as an example. DeHann (1956) described the differentiation of muscle. There is elongation of undifferentiated cells into myoblasts. Following migration of the myoblasts into a string or aggregate, there is the appearance of granules in the myoblast cytoplasm which form the myofibrils. Finally, there is segmentation of myofibrils creating light and dark zones and alignment of segmented myofibrils side by side to form cross striations. DeHann demonstrated that early establishment of function while the tissue is still immature is of great survival value to the organism.

Goss (1969) described the biochemical changes in the regenerate. As soon as differentiation commences, lactic dehydrogenase, an enzyme of the glycolytic pathway, activity subsides and Kreb's cycle takes over. Normal vascularization inables respiration to be aerobic, and pH returns to normal in the stump. This first occurs in the most proximal region of the regenerate and proceeds distally as morphogenesis advances. Needham (1952) stated that differentiation is not simultaneous but proceeds from the stump outward distally. Also different tissues do not differentiate at the same rate.

The pluripotency of dedifferentiated cells mentioned previously will now be discussed. Much evidence such as Steen's (1958) investigation has shown no pluripotency in cells. However, the accepted theory of origin of blastema cells states that they are of local origin at the

stump. This presupposes that all tissue types must be present at the stump, if there is no pluripotency. However, this has not been shown to be the case. Weiss (1939) and others removed certain bones from the stump, yet got formation of bone in the distal regenerates. If the ulna was removed and there was an amputation through the lower arm, there arose an ulna starting at that amputation and proceeding distally. The ulna was not regenerated in the stump. Removal of the humerus gave similar results. With the humerus removed, there was bone formation in the regenerate with no skeletal elements present in the stump. Additional evidence in favor of pluripotency was the half-limb experiments of Weiss (1925a, b, 1926; rev. by Rose 1964) and Goss (1957a). The limbs were split longitudinally, making anterior and posterior halves and then amputated. If all the posterior half except for the skin was removed, the distal regenerate from the half stump was complete in all tissues. From these examples, it could be concluded that some cells possess a pluripotency that can be marshalled if an abnormality occurs in the stump. Newth (1958) demonstrated that there could be considerable pluripotency. The inner tissues from a undifferentiated blastema was removed, minced, and stuffed back into the blastema. The regenerated limb had only a few distal abnormalities. It was then proposed that the fate of undifferentiated cells may be determined by their position in the blastema at the time differentiation began.

Differentiation of tissue finally reached the farthest distal level in creating the digits. One of the first histological events to occur in the shovel-shaped regenerates was the formation of procartilaginous prongs. The remaining events include growth and enlargement. The

next phase is concerned with these final steps and control of morphogenesis in regeneration.

Controlling Factors

There are several factors which control regeneration at different times. The role of the epidermal cap and the blastema in controlling regeneration have already been mentioned. The following are factors which also exert a control.

Nerves

Mention has been made of the necessity of the nerves to be in contact with the epidermal cap. Todd (1823) was one of the first to recognize that denervation resulted in no regeneration. It was also shown that an intact reflex pathway was not needed for regeneration. Singer (1943) demonstrated that the sensory supply was sufficient to cause regeneration. Also Kamrin and Singer (1959) transplanted spinal ganglion into denervated limbs and induced regeneration.

Singer (1942, 43, 45, 46a, 47a, 47b, 49) made many thorough investigations into neural control of regeneration and illucidated many of the principles of neural control. Singer is probably considered the authority on effects of nerves on regeneration. He found that the sensory supply of nerves were sufficient alone to induce regeneration. Singer (1946b) did quantitative investigations of nerve fibers in the stump. He used this material for his nerve fiber threshold theory. He proposed that there exists a range of nerve fiber number in the stump, below which regeneration can not occur. If the fiber number of the stump falls above the range, regeneration occurs. If the fiber

number falls within the range, regeneration may or may not occur. Singer noted that the reason that the sensory supply was effective alone in inducing regeneration was that it exceeded the threshold range. Of the three pathways, (sensory, motor, and sympathetic), the sensory supply alone was the only one that surpassed the threshold range. Rzehak and Singer (1966) used this quantitative method to explain why some animals do not regenerate. Such animals as Mus and adult Rana do not regenerate possibly, because the number of nerve fibers in the stump fall below the threshold of regeneration. Therefore, this shows that regeneration requirement is not specific with regard to which innervation, only nerve fiber number.

Schotté and Butler (1941, 1944), Singer and Craven (1948), and Butler and Schotté (1949) denervated limbs at different levels and discovered when the nerve's influence was expressed. The investigators found that the nerves were needed until the blastema resembled an embryonic bud. Once the blastema started differentiating, it was liberated from nervous control, and it could regenerate even if denervated. According to Goss (1969), the nerves are not needed during wound healing or dedifferentiation. The nerves are needed during a period when old structure is being lost, when tissues are becoming cells, and a mass of cells is accumulating to form blastema. Butler and Schotté (1949) thought that nerves were involved indirectly or directly in transmission of morphogenetic messages to the undifferentiated cells. Goss (1969) felt that nerves in some way checked the regressive tendencies of limbs, perhaps by directly or indirectly initiating formation of a blastema. After blastema formation, denervation will only result in the fact that growth in volume does not occur. In later phases of regener-

ation, it is required for quantitative growth. The nerve's early function as an agent to promote blastema formation is most important.

Singer (1959b, 60) tried to find how neural influence was mediated. The prime suspect was the neurohormone, acetylcholine, Singer (1959b, 1960) as well as Rose (1964) infused inhibitors of acetylcholine, atropine and procaine hydrochloride, into stumps and delayed and blocked regeneration. Singer infused acetylcholine into a denervated stump, yet regeneration was not induced as expected. Therefore, it was concluded that interfering with neural mechanism by inhibiting acetylcholine may block regeneration but the trophic effect of nerves may be more than just acetylcholine.

One of the striking exceptions to the requirement of nerves is aneurogenic limbs. Yntema (1959) removed the neural tube of larval salamanders which would have given rise to nerves which supplied the limbs. It was necessary to parabiotically join this subject to a normal one for its survival. The aneurogenic limb was amputated and it regenerated. Steen and Thornton (1963) using the same technique, attempted to find what part of the limb was nerve dependent. The skin was found to be the part that was addicted to nerve influence. From the aneurogenic experiments it was deduced that once nerves invade the limb, this structure forever depended on them to regenerate.

It has been known for a long time that s supernumery limb can be created by deviating a nerve to a site on or near the limb and wounding this area. It has been mentioned by Thornton (1959), Singer (1952) and others. The nerves appear to induce an apical cap under which a blastema forms.

Singer (1954) deviated the sciatic nerve of an adult frog into the forelimb, amputated, and the limb regenerated. This could be interpreted as a means of inducing regeneration by increasing the fiber number in the stump above the threshold level needed for regeneration.

Therefore, Rose (1962) proposed a step-wise control for regeneration in which nerves influence the epidermis which then promotes blastema formation. Even though there are many pitfalls to this theory, most evidence demonstrates that the trophic effect of nerves is greatest on the wound epidermis, as seen in the supernumary limb investigations.

Hormones

The endocrine glands exert a control over regeneration. Schotté (1926) produced evidence that the pituitary was necessary for regeneration. It was thought that the lack of growth promoting hormone (STH) causes the inhibition. Schotté and Hilfer (1957) demonstrated by replacement therapy that adrenocorticotropic hormone (ACTH) was the cause of nonregeneration. The real importance of the pituitary lies in its relationship with the adrenal cortex. Selye's general adaptation syndrome is involved in this axis of control. An animal under stress secretes glucocorticoids, such as cortisone, from the adrenal cortex to adapt to the distressing conditions. It was demonstrated that a hypophysectomized and adrenalectomized (destroyed by drugs) subject when given cortisone treatments after amputation would regenerate. Goss (1969) stated that cortisone in mammals inhibits synthesis of collagen fibers needed for scar formation. In the salamanders, it may prevent precocial scar or dermis formation. This is supported by Hall and Schotté (1951), and Schotté and Hall (1952), who noted that dermal fibers formed pre-

cociously in hypophysectomized subjects which prevented blastema formation and regeneration. The above investigators and Schotté and Hilfer (1957) demonstrated that the pituitary, via the adrenals is needed only in the early stages of regeneration, while wound healing is proceeding. Without the pituitary, the tissue balance is shifted to differentiation much too early and the blastema is not formed. There exists an axis of control from pituitary to adrenal cortex to cortisone to inhibition of dermis. The importance of cortisone or adrenal cortex is shown in the work of Schotté and Wilber (1958) and Schotté (1953). Adrenal glands were transplanted into adult frogs. Their limbs were amputated, and those that had the extra adrenals could regenerate those limb.

The thyroid gland must be normal during regeneration or the limb is retarded. Richardson (1940, 45) and Schotté and Washburn (1954) noted that early removal of the hormone, thyroxine, inhibited dedifferentiation resulting in a stunted abnormal limb. Late removal interfered with growth. Hay (1956) found that excess thyroxine interfered with growth and differentiation.

Inhibitors

There are many items which have inhibitory effects on regeneration. These inhibitors serve as excellent experimental tools to study the processes of regeneration. Several have already been mentioned.

X-radiation

Butler (1931, 33, 35) discovered that X-radiation of 1000-10,000r applied locally to a wound prevented regeneration. X-radiation is effective only if the cells on the wound surface are irradiated. Irradiation of cells a few millimeters above and below the amputation

level results in no inhibition. It has been thought that X-radiation might destroy the mitotic mechanism or cause failure to transmit morphogenetic information. The effects of X-radiation is permanent, but Ross, Quastler, and Rose (1955) induced regeneration by supplying an irradiated stump with unirradiated epidermis.

Beryllium

Needham (1941) discovered that salts of beryllium had a powerful inhibitory effects on regeneration. Beryllium nitrate was most effective when applied to the surface of the amputation stump immediately (or not more than 6 hours) after amputation. This was the basis of Needham's proposal of the secretion of a wound hormone by the stump cells. This wound hormone may trigger the entire regeneration response soon after amputation. Scheuing and Singer (1957) infused beryllium into early regenerate and there was much destruction of bone, fibrous connective tissue, and muscle. In normal limbs, there was no reaction to the infusions. It was during these experiments that the epidermis was seen to help phagocytize and dispose of tissues.

Ultra-Violet light

Butler and Blum (1955) found that ultra-violet light slows cell division, may cause regression, and induction, and induction of accessory limb at point of irradiation. Thorton (1958) using ultra-violet light at 2537 Å wavelength prevented regeneration for at lease 30 days. It was found that if the apical epidermal cap is present in enough layers, the outer layers will block the rays and regeneration will proceed slowly.

Chemicals

Mitotic inhibitors such as colchicine and others will halt regeneration when infused into the early regenerate. Chemicals that block various hormones or neural hormones are detrimental to regeneration.

Denervation

It has been mentioned previously that denervation prevents regeneration. The forelimb is innervated by the third, fourth, and fifth spinal nerves. Singer (1942, 43, 45) states that to denervate the limb adequately, the spinal ganglion of the above nerves must be removed. This is the only way that the limb can be totally denervated for a considerable time, since the peripheral nerves grow back very quickly.

Control of Morphogenesis

The factors controlling morphogenesis are quite numerous and not very well understood. Therefore, there have been many theories proposed to explain the mode of action of the different factors. There is no evidence for an exclusive force that is responsible for control, instead, several factors have been shown to exert some influence.

The morphogenetic message which transcribes the replacement of a given area resides only in those cells in and around that area of the body. Guyenot et al (1948) demonstrated this and discovered the morphogenetic map for the forelimb. By diverting a nerve from the brachial plexus to various sites on and around the limb and injuring that site, a supernumary limb decreased as the injury was moved farther from the limb. A supernumary limb could be induced outside the morphogenetic

limb area by transplanting a full thickness of limb skin and innervating it. In this way, the morphogenetic area of the limb was determined, and it was demonstrated that the morphogenetic message resides permanently within the cells of an area. Removal of cells from an area did not result in a loss of morphogenetic information. This demonstrated that nerves and epidermis are apparently without morphogenetic specificity, but they are necessary for morphogenesis. The nerves serve as a permissive agent, not an instructive one. The nature of expression resides in the underlying mesodermal tissues.

The production of these supernumery limbs is a replacement response which differs from regeneration. This differs from normal regeneration in that a replacement response has been triggered even though there was no structural loss. The morphogenetic message has been caused in some way to transcribe a new part. Rose (1964) reviewed the other ways supernumery limbs can be induced. Supernumery limbs can be induced by X-radiation, ultra-violet radiation, subdermal insertions of foreign tissue, and ligatures. The most recent method is the subdermal injections of various foreign tissues and objects. Breedis (1952) inserted carcinogenic substances which induced some supernumery limbs, and Ruben (1960) found that frog kidney was the most effective substance for the induction of supernumaries. Ruben (1960) proposed that the induction of supernumary limbs was due to chronic irritation. The foreign implants may traumatize the host tissue and cause release of cytolytic enzymes. The enzymes start dermal dissolution. The epidermis and mesodermal elements then have intimate contact which is a requirement for regeneration. The morphogenetic message is then caused to be transcribed.

Nerve requirement in supernumery limb induction is much reduced as compared to normal regeneration.

Control of pattern in the limb can originate from a small disc of the organized stump tissue. Guyenot (1927) reported that a blastema, too young to maintain itself if transplanted, will continue to regenerate when transplanted, if a disc of organized stump tissue of no more than one millimeter thickness remains. Without the disc, the host site will control the pattern of the regenerate.

From deletion experiments mentioned previously, it was discovered that the stump of blastema could compensate for missing structures in the stump. The half limb experiments of Weiss (1939), Swett (1928), and Goss (1957a 1957b) illustrate this also. The stump was split into anterior and posterior halves, and certain parts of the halves were removed, after the hand was amputated. If an entire half was removed, the blastema which arose from the intact half would produce only half of the distal structures. If only the mesodermal portion of a half was removed leaving the skin intact, a whole blastema was formed on the half stump. From the above evidence, Weiss (1924b; rev. by Rose 1964) proposed a field nature of morphogenetic control. This theory proposed that control of regeneration is in longitudinal directions and there exists fields or territories which defines an anatomical region within which a complete structure can be reorganized from a fraction of the whole. The forelimb seems to possess two fields - anterior and posterior. Neither field can make up for the absence of the other, but part of each field can reproduce its structures distally from the amputation surface.

Rose (1964) and Goss (1956) further investigated these longitudinal

lines of control. Amputated limbs were parabiotically joined. First, the stumps were parallel to each other, two blastemas were formed, and the regenerate had some fixed inner parts. By increasing the angle between them to 90°, more of the central parts failed to form. It was proposed that control operates along lines parallel to main axis of the limb. Where the lines of control of the two stumps crossed, there was interference and structures at the intersections failed to form.

There was a possible correlation between lines of control and electrical polarity. Monroy (1941 rev. by Rose 1964) found that in all growing differentiating structures potential differences exist between the distal and proximal. Umanski et al (1951; rev. by Rose 1964) found that the electrical resistance is much less along the distoproximal axis than 90° to it.

Weiss (1939) states that regeneration is an unsymmetrical and highly polarized process. It can occur distally, but not proximally, lateromedially, or dorsoventrally. Also, Butler (1955) and Needham (1942) state that no matter the polarity, only structures that lie distal to the surface can regenerate. This was strikingly shown by Butler (1951, 1955). A hand was amputated and the stump was inserted into an incision on the body wall. This was allowed to heal and become innervated. The limb was cut through the middle creating two stumps on the same side, one normal and one abnormal that had reversed distal-proximal axis. The abnormal stump regenerated a short segment of the humerus, another radius and ulna, and another wrist even though these structures were already present proximally in the stump.

It can be stated that only a few lines of control have to be pre-

sent to explain the field nature of morphogenetic control. Once a few lines of control are established, the pattern spreads.

Recently, several investigators have proposed theories on the actual physical nature of transmission of morphogenetic information. Becker (1960, 1961a, 1961b) proposed that the nervous system was involved in transmission of morphogenetic data. Nerves maintain an electrical potential difference with the gradient of negativity with a distal high point. The potential difference is responsible for the movement of charged particles. Becker (1961b) demonstrated that cutting the nerve caused the potential difference to vanish. It was also shown that the amputation stump of the frog did not have the gradient of negativity. This was correlated with inability of the adult frog to regenerate. Therefore, nerves may be directly responsible for transmission of morphogenetic information by generating a potential difference that furnishes power for movement of intercellular co-ordinators.

Rose (1964) reviewed scattered evidence for a theory that control of differentiation may be inhibitory. The compounds responsible for inhibition were histones. Histones are compounds that when closely associated with nucleotide segments of DNA prevent production of their RNA. There are many types of histones. It was hypothesized that histones from distal regions inhibit DNA or genes more proximal to them. In amputation, these distal histones are removed and genes which control morphogenesis are no longer inhibited and their messages are transcribed.

MATERIALS AND METHODS

Description of Species

Ambystoma texanum, the small-mouthed salamander, is a member of the family Ambystomidae of the order Caudata. Smith (1961) states that this species of salamander inhabits woodlands, prairies, and pastured areas in Illinois as far north as Henry and Kankakee counties. Distribution in the United States comprises most of the Ohio and Mississippi River Valleys. In February and March, the A. texanum migrate from the woodlands to any standing body of water to breed and deposit their egg masses. The eggs incubate and hatch in a few days with metamorphosis of larvae occurring in late May through July.

Ambystoma texanum is a brownish-black salamander covered with lichen-like patches of silver-grey. They possess an extremely slimy epidermis which is shed about every week in the laboratory. The trunk of the body is marked by several costal grooves that create from 13-15 folds. The salamander has a narrow head, small mouth, and a wide neck. The limbs are short and stocky with the forelimb having four digits and the hindlimb with five.

Collection of Experimental Subjects

Forty (40) small-mouthed salamanders (Ambystoma texanum) were taken from a temporary pond two miles southwest of the Eastern Illinois University campus, Charleston, Illinois (NW¼, SW¼, Sec. 22, R. 9 E, T. 12 N., Coles County, Illinois). Collecting trips were made to the site at

night during a period from March 15-20, 1968. Daylight trips were not productive since the species was nocturnal. The salamanders had migrated from the woodlands to the pond to breed, following a previous period of warm and rainy days. The subjects were captured by the use of flashlights, dip nets, and bare hands.

Even though the salamanders were slow, awkward, and sluggish on land they proved just the opposite in the water. The slimy surface of their bodies in contact with water made them difficult to hold. Under the water, their powerful tails propelled them rapidly in serpent-like movements.

The final collection trip brought few subjects, for as quickly as the salamanders had appeared, they disappeared in a mass exodus back to their woodland niches, leaving the eggs to incubate.

Laboratory Habitat

The salamanders were originally housed in glass laboratory dishes with paper towels and approximately 2-3 centimeters in diameter. The containers were arranged in tiers of three with a plate of glass over the top one. The salamanders were housed two to four per container, since little aggressive behavior was observed. The temperature ranged from 20°-30° C and the humidity varied. Photoperiods varied from 12-16 hours a day.

Continual contact with water caused much shedding of the skin, and might have interfered with regeneration. Therefore, a closer approximation of their woodland habitat was constructed. Wet paper toweling and a small amount of water were placed in the containers. Several dif-

ferent species of mosses, lichens, and ferns were placed on the toweling. Subjects seemed predisposed to hide under the plants, many often huddling together. Periodically, a fine mist of water was sprayed into the containers to maintain humidity. The laboratory dishes were satisfactory in maintaining high humidity, while also allowing for the passage of air.

The toweling was changed and the containers cleaned each week, or whenever mold or fungi started to grow. The plants were drained of any excess water during the cleaning. The mosses required complete replacement every two to four weeks because of decomposition. New mosses were obtained from dried samples which were rehydrated. The salamanders were placed into a water bath and agitated about to cleanse them of debris from plant decomposition, excretory products, and shedding skin.

Identification

Variations in certain bodily features, such as, different ground colors ranging from olive brown to black, variations in pattern of grey flecks scattered over the dark color on the back, sides, and abdomen, relative sizes, and other morphological differences were used to distinguish individual subjects, which were then given identification numbers.

Feeding

Feeding the salamanders proved to be a problem. The subjects did not feed for the first two months of captivity. Smith (1961) stated that their natural diet consisted of small invertebrates such as worms, slugs, and small arthropods. However, the subjects did not eat these items when placed in the containers. Neither did they feed on bits of

lean beef. It was decided to force feed the salamanders even though they appeared to contain large amounts of fat.

Ambystoma tarranum were force fed using the following method. They were grasped around the anterior part of the body to prevent them from squirming away. The jaws had to be forced open with forceps. This was difficult because of their small mouth and powerful jaw muscles. Therefore, in opening the jaws, care must be taken to avoid injury to the animals. Each subject received small bits of lean beef moistened in water every week when operative procedures did not interfer. The salamanders were never fed immediately following or preceeding operations.

The salamanders were stimulated to swallow by spraying water on them and gently holding their dorsal side down. After swallowing had been observed, the subjects were placed in the container and again sprayed with water, for continuous handling resulted in much dehydration from the body surface.

Later these techniques were supplemented. Strained beef liver was placed in a 3 cc syringe with a trimmed nozzle to fit the size of the salamander's mouth. The syringe was placed down the pharynx of each subject, and the strained meat subsequently was injected.

Operation Procedures

The following procedures and techniques were followed in all operations performed.

Preoperative phase

Subjects were placed in a glass petri dish filled to 3/4 its volume with Amphibian Ringer's solution and several crystals of chloro-

tone as anaesthetic. The salamanders remained in the covered petri dish for ½-1 hour depending upon when the anaesthetic took effect. This anaesthetic condition was determined by gently squeezing the limbs and tail with forceps from time to time until little response could be ellicited. Operations were undertaken while the subjects were not quite under the effects of the anaesthesia, rather than risking the toxic effects of over-exposure to the chlorotone.

Operative phase

Anaesthetized salamanders were transferred to a second petri dish filled to 3/4 its volume with Amphibian Ringer's solution and a lesser amount of chlorotone. A layer of toweling was placed on the bottom of the petri dish to prevent the subject from slipping. The Amphibian Ringer's solution was used to maintain isotonic conditions. The petri dish was placed on the stage of a binocular dissection microscope.

Instruments and materials used in the operations were not sterilized. Equipment was always washed and dried before and after use, but no additional aseptic procedures were followed. This technique proved quite adequate since infections were unnoticed.

The amputation procedure was as follows: The subject was oriented under the dissecting microscope and the forelimb was held steady with watchmaker forceps. Iridectomy scissors were utilized for the amputation. The limb of the salamander was severed by a slow deliberate stroke at the level of the distal 1/3 of the upper forelimb. Therefore, the elbow was included in the discarded portion. The stump was trimmed of any protruding cartilage, muscles, or skin, and when necessary, the amputated forelimb was placed in Bouin's fixative for future histological

analyses.

Reaction to amputation varied from minor bleeding to thrashing about. When reactions to the amputation had subsided, bleeding was stopped by gently clamping the stump with the hemostat or forceps, giving just enough pressure to stop the issuance. Every minute the pressure was released to observe the flow of blood. Finally, when there was a complete cessation of blood, the subjects were left in the operative dish for 10-15 minutes to decrease the chance of hemorrhage.

Postoperative phase

The salamanders were then placed into a paper towel lined postoperative petri dish filled to 3/4 its volume with only Amphibian Ringer's solution. The amputation surface was always kept submerged in the Ringer's solution. The subjects remained in this petri dish for an hour or until they started responding.

Originally, the subjects were returned to their housing containers, but in this situation, several salamanders died. It was thought that the housing container environment might not allow for the diffusion of the excess chlorotone. Therefore, the subjects were immersed into a laboratory dish with toweling and about 3-4 centimeters of tap water for 24 hours in an effort to allow the chlorotone to diffuse out and allow for more recovery time. Following this period, the subjects were placed back into their containers. There were no more deaths from the operation after this innovation.

Histological Analysis

For histological analysis, the regenerating limbs were reamputated

through the stump and immediately placed in Bouin's fixative for at least 24 hours or longer. Following the techniques of Humason (1967), the limbs were placed in several baths of 70% alcohol and lithium carbonate to remove excess picric acid. The limbs were dehydrated, cleared in xylene, placed in two paraffin baths and finally embedded in Tissuemat (56° C).

The limbs were longitudinally sectioned at 10 microns, and complete serial sections of the limbs were placed on slides. The sections were stained using Heidenhain's Iron Hemotoxylin. Slides were placed in a 4% iron alum mordant overnight' stained in Hematoxylin 24 hours; destained in 2% iron alum. Destaining of the sections was followed under a microscope using low power.

Morphological Observations

Ambystoma texanum were observed every 2-4 days for a period of 60 days or longer to study external gross morphological changes in the regenerating limb. Each subject had its own experimental history chart that included the number of the specimen, identification characteristics, operation or treatment given, morphological observation, the date observed, and the number of days since amputation.

Subjects were taken separately and placed in a dry petri dish which rested on the stage of a binocular dissecting microscope. The regenerated limb was observed first under the lowest magnification (7X). Wound-healing and early stages of regeneration were observed from a lateral aspect. The petri dish was tilted at an angle of approximately 60° to make possible a lateral view of the salamander. Later stages of regeneration could be viewed entirely from the dorsal surface and with highest magnification (30X). This entire procedure was difficult for the salamander

moved occasionally, especially when the petri dish was tilted.

The morphological observations were mostly qualitative and subjective in nature. These observations consisted of (1) differences between stump and regenerate (that portion of the limb that is regenerating), (2) progressive changes in the regenerate, and (3) comparison of regenerated and normal limbs.

RESULTS

Under the experimental conditions, a total of 59 completely regenerated forelimbs were obtained from the amputations. There was approximately an equal number of right and left regenerates, and certain limbs were amputated repeatedly giving rise to several regenerates over the period of the experiment. A structure was considered a completely regenerated forelimb when and if the limb which arose possessed elongated digits.

A normal unamputated forelimb has four digits with the middle two digits longer than the outer two. From the above total of 59, 85% of the regenerated forelimbs possessed four digits with the middle digits longer than the outer ones. Nine of the limbs (15%) displayed digital abnormalities, and of these, eight forelimbs had 3 digits and one forelimb possessed two. Abnormalities were created by either the fusion or deletion of digits. Every one of the thirty subjects produced a regenerate, except for subject #5. This subject produced a blastemal cone of 1 millimeter three times, but these turned black and did not advance any farther.

The number of regenerates might have been greater, but many prospective regenerates, had to be terminated at various early stages for histological analysis. These, therefore, did not reach the criteria of a complete regenerate and could not be added to the total.

Twenty-two of the regenerates were observed every 2-4 days over a 60 day period to study the outwardly morphological changes as described in the materials and methods section.

Several stages in regeneration were selected, defined, and the time of inception and duration of the stages were determined for each of the 22 limbs. Table 1 lists the stages, the range of values of duration of each stage, average duration, and average day of inception of each stage. Some stages seemed to blend imperceptably into the next stage, but an effort was made to select stages with several different criteria to make determination better.

The stages included:

1. Wound closure and healing - This occurred from amputation until a bulge of ½ millimeter appeared on the wound surface.

2. Blastema Formation (½-1 millimeter) - The clear bulging area increased to 1 millimeter and the distal part of the stump swelled laterally.

3. Blastema Cone Formation (1-2 millimeters) - The elongation of 1 millimeter bulge created a cone-like structure of 2 millimeters. The cone started to form a right angle with the stump.

4. Paddle-Shaped Regenerate - This is initiated during or after right angle formation or from 1½ to 2½ millimeters. It was created by elongation and flattening dorsoventrally of distal regenerate. The tip of the regenerate is able to flex or bend at an angle.

5. Foot Stage-Digits Budding Out - From the first indication of cartilaginous columns of the digits the epidermis indented to form the digits.

6. Foot Stage-Digits Equal and Extending - Digits elongate and grow outward. There is webbing between digits.

7. Foot Stage - Middle Digits longer - Two middle digits grow out

or elongate farther than the outer two. The webbing between digits are absent. There is the creation of separate phalanges and digits curving down.

The following is a descriptive morphological account of regeneration as observed from 22 case histories. The events occurred in the majority of subjects approximately in the order given.

The amputation surface appeared inactive for the first week after injury. The surface sometimes had a marked depression caused by trimming of muscle and bone. The stump slowly started to swell laterally. The wound surface was covered by transparent cells allowing clear view of the severed muscle and bone underneath the wound epithelium. The darkly pigmented epidermis that bordered the wound appeared to be transformed slowly into clear tissue. The silver-grey pigment cells seemed to disappear leaving a narrow ring of black background pigment. These black pigment cells also started to disappear slowly. This transformation was more strikingly shown in a subject that had a piece of normal epidermis extending like a peninsula over the wound area. During healing, the junction of the piece of epidermis to normal epidermis dissolved first, and slowly the entire piece was transformed into clear tissue identical to wound epithelium. This transformation of pigmented tissue to transparent tissue was one of the first changes noticed.

The quiescence of the wound surface ended as the depression disappeared, swelling increased, and a blastema started to bulge out of the surface. Through the clear overlying wound-epithelium cells, a whitish opaque, often translucent wave spread under the surface occluding the ends of muscle and bone. Many subjects had black aggregates or scabs

TABLE I

RANGE OF DURATION, AVERAGE DURATION, AND DAY OF INCEPTION OF VARIOUS STAGES OF FORELIMB REGENERATION OF 22 AMBYSTOMA TEXANUM

Stage	Range of Duration	Average Duration	Average Day of Inception
Wound Closure and Healing	6-10 days	7.5 days	0th
Blastema Formation (½-1 mm)	7-21 days	12.0 days	7.5th
Blastema Cone (1-2 mm)	5-13 days	9.0 days	19.0th
Paddle Shaped Regenerate	4-10 days	6.3 days	28.6th
Foot Stage: Digits Budding	4-11 days	5.8 days	35.0th
Foot Stage: Digits Extending	5-14 days	8.6 days	40.7th
Foot Stage: Middle Digits Longer	---------	--------	49.0th

over the tip of blastemas which slowly disappeared. The blastema elongated past 1 millimeter and was termed a blastemal cone. The cone appeared grayish and opaque near the proximal part, and clear and vascular at the distal tip. A wave of increasing darkness spread down the regenerate cone. It was black at the proximal border with a transitional grey zone ahead which blended into a clear vascular area at the distal extent. The blastema which became oriented at a 180° to the stump, soon started to form an obtuse angle, finally approximating a right or 90° angle. The front of the dark wave moved at an acute angle to the long axis of the regenerate. Therefore, the wave didn't reach the various distal portions at the same time.

The blastemal cone was transformed into a paddle-shaped regenerate by a distal flattening dorsoventrally and creation of a flexing tip. The tip was still clear and vascular, but proximally from the site of flexing the zone of blackness had moved down. Next, digital cartilaginous columns appeared and looked clear and whitish as opposed to the opaque grey granular tissue in between them. These cartilaginous columns were outlined and intertwined with blood vessels networks and black pigment granules. The epidermis indented separating the digital buds. As the digits grew outward, they were joined to each other by webbing which was clear, avascular, and wrinkled.

During this time, the zone of dark pigment cells passed through the digital plane at an angle to the long axis of the limb. It occluded the outermost or 4th digit and then progressed inwardly. The webbing between the digits disintegrated as the digits elongated. The middle two digits became longer than the outermost digits. The silver-grey pigment

patches, which were the first structure to disappear, finally had re-invaded over the black pigment layer making it difficult to discern the site of amputation.

The last changes to be seen which occurred quite slowly were increase in volume of the forelimb, elongation of digits, and growth of phalanges.

Figures #1-#8 illustrate the slow gradual rate at which growth in volume of forelimb and elongation of digits proceeded once the digits were formed. The figures show comparisons between a normal limb and a 13 month regenerate (subject #26), a normal limb and a 9 month regenerate (subject #14), a 7 month regenerate and a 13 month regenerate (subjects #20 and #21), and a 4 month regenerate and a 13 month regenerate (subject #16), a normal limb and a 9 month regenerate with only 3 digits (subject #13), a 13 month regenerate and a 2 month regenerate severed at the shoulder (subject #18), and two non-regenerating limbs (subject #15).

Subject #5 has 2 non-regenerating amputated limbs which abortively stopped during blastema formation. Subject #13 had a digital abnormality. It appeared to have the two middle digits fused into a large single one. This is presented as a representative of the 15% abnormal digital regenerates which appeared during the study. Subject #14 demonstrated the size differences in a 9 month regenerate and a normal limb. Note the width of the wrist in the normal limb as opposed to the wrist width in the regenerate. In subject #16, the digits on the regenerating right limb were still partially clear and vascular. There was a slight constriction at the site of amputation and some webbing between the toes were still present. Subject #18 had digits on the regenerate that were

Figure #1: Ambystoma texanum (Subject #26) has a right forelimb that is normal (unamputated) and a left limb that is a 13 month regenerate.

Figure #2: Ambystoma texanum (Subject #14) has a right forelimb which is normal (unamputated) and a left forelimb which is a 9 month regenerate.

PLATE 1.

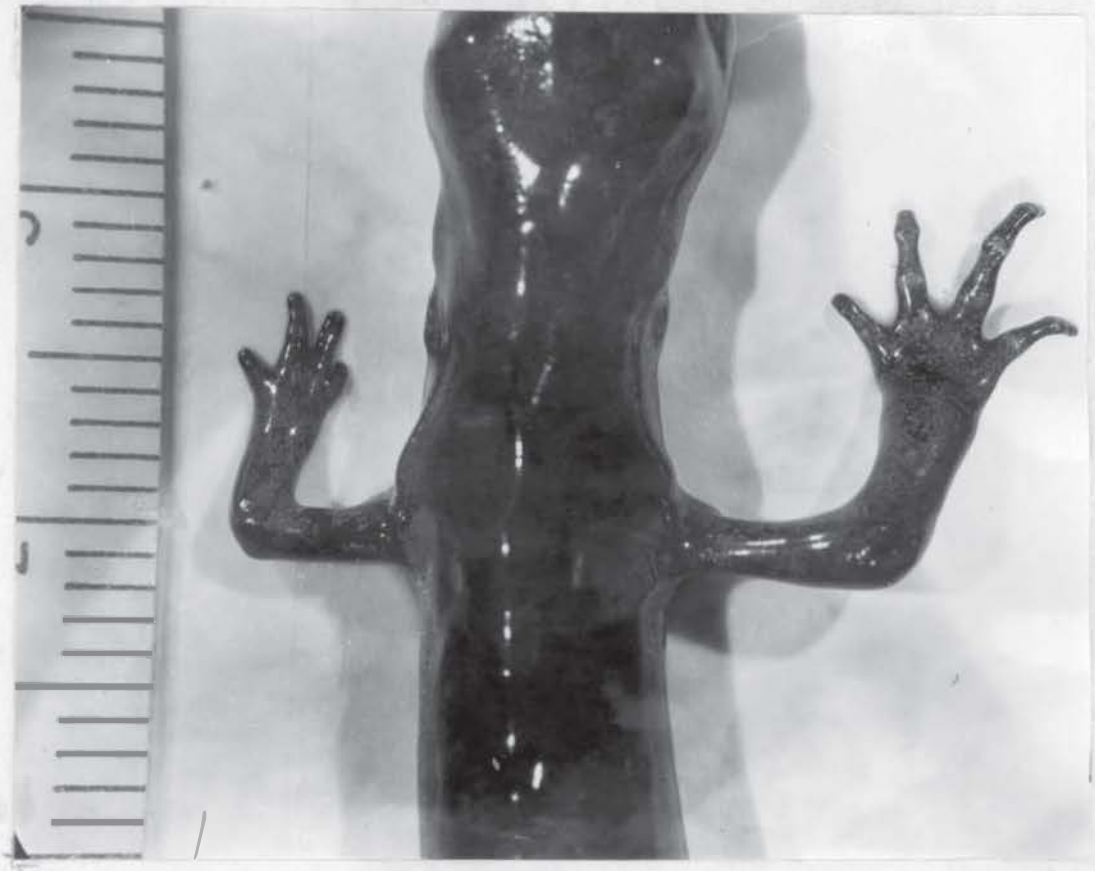

Figure 1

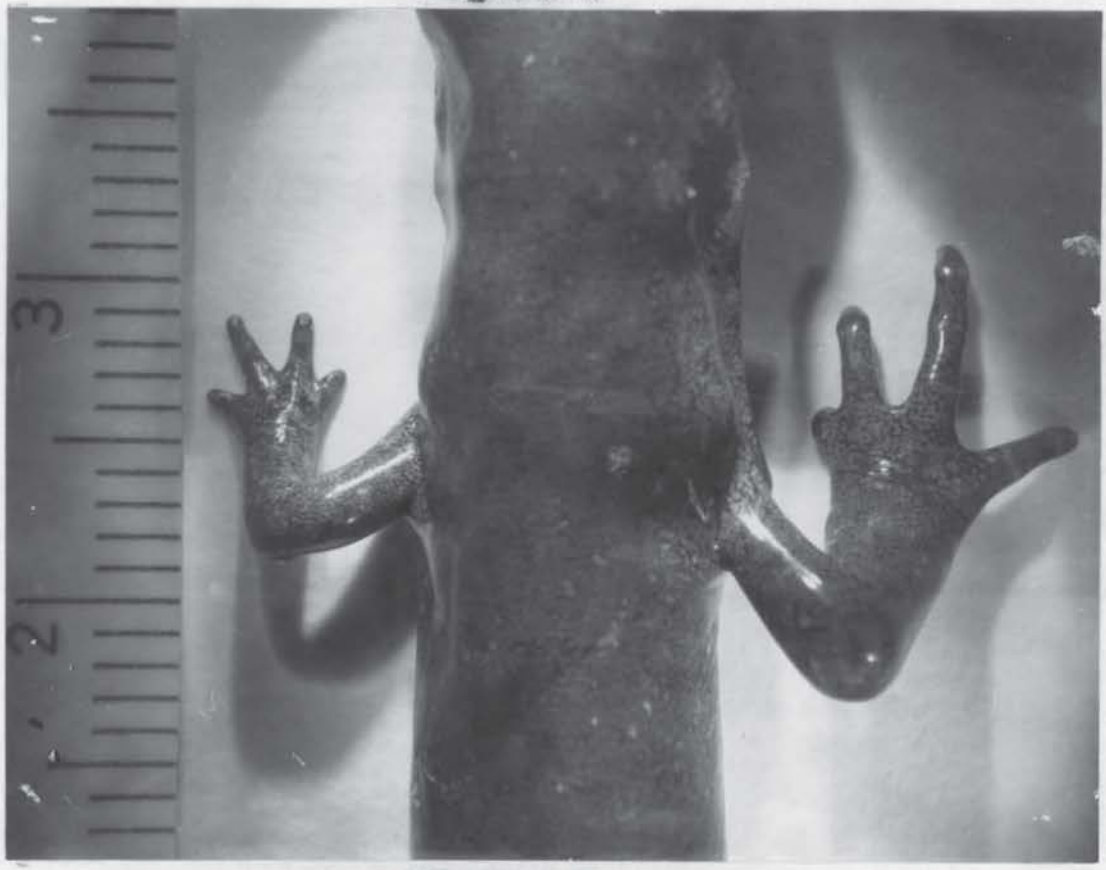

Figure 2

Figure #3: Ambystoma tigrinum (Subject #20) has a right forelimb that is a 7 month regenerate and a left forelimb that is a 13 month regenerate.

Figure #4: Ambystoma tigrinum (Subject #21) has a right forelimb that is a 7 month regenerate and a left forelimb that is a 13 month regenerate.

PLATE 2

Figure 3

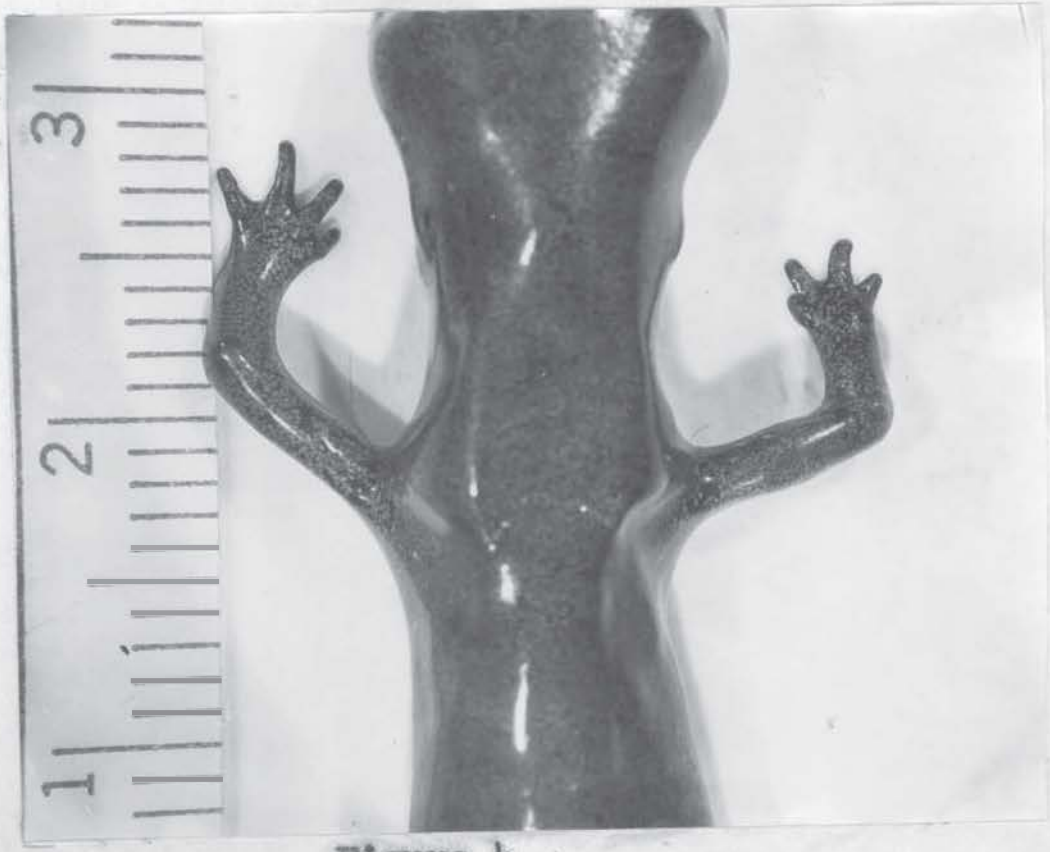

Figure 4

Figure #5: Ambystoma texanum (Subject #16) has a right forelimb that is a 4 month regenerate and a left forelimb that is a 13 month regenerate.

Figure #6: Ambystoma texanum (Subject #13) has a right forelimb that is normal (unamputated) and a left forelimb that is a 9 month regenerate, but has only 3 digits caused by fusion of middle 2 digits.

PLATE 3

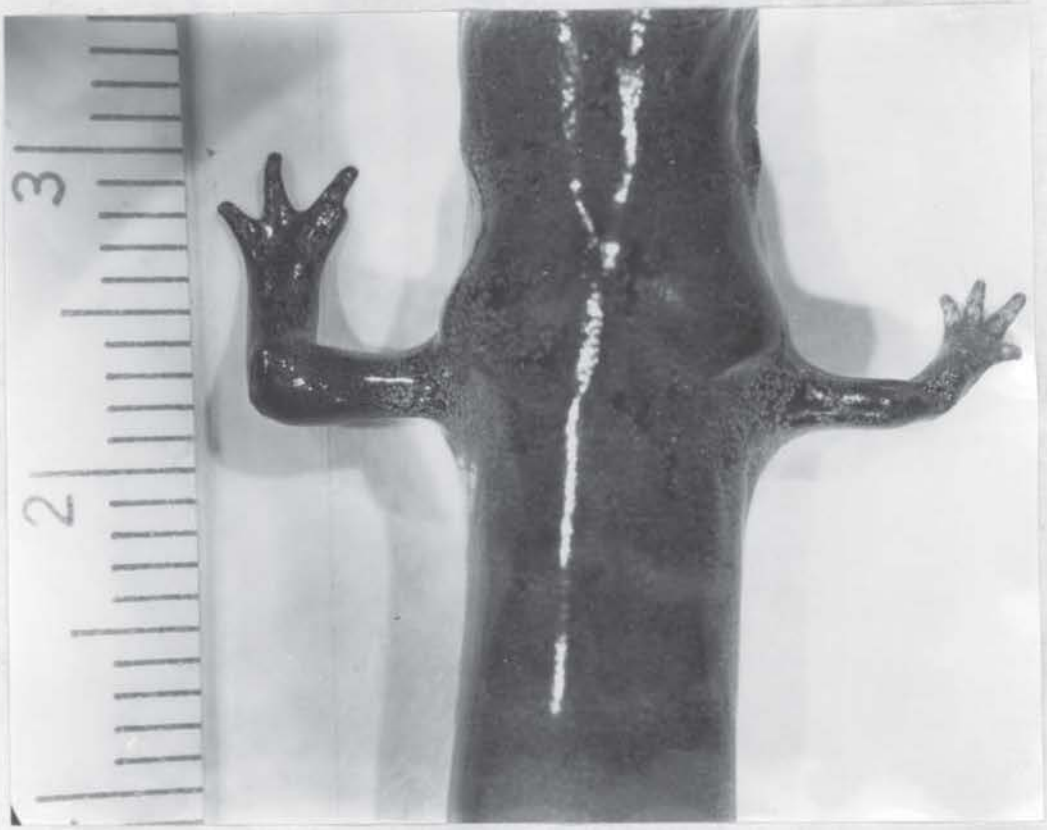

Figure 5

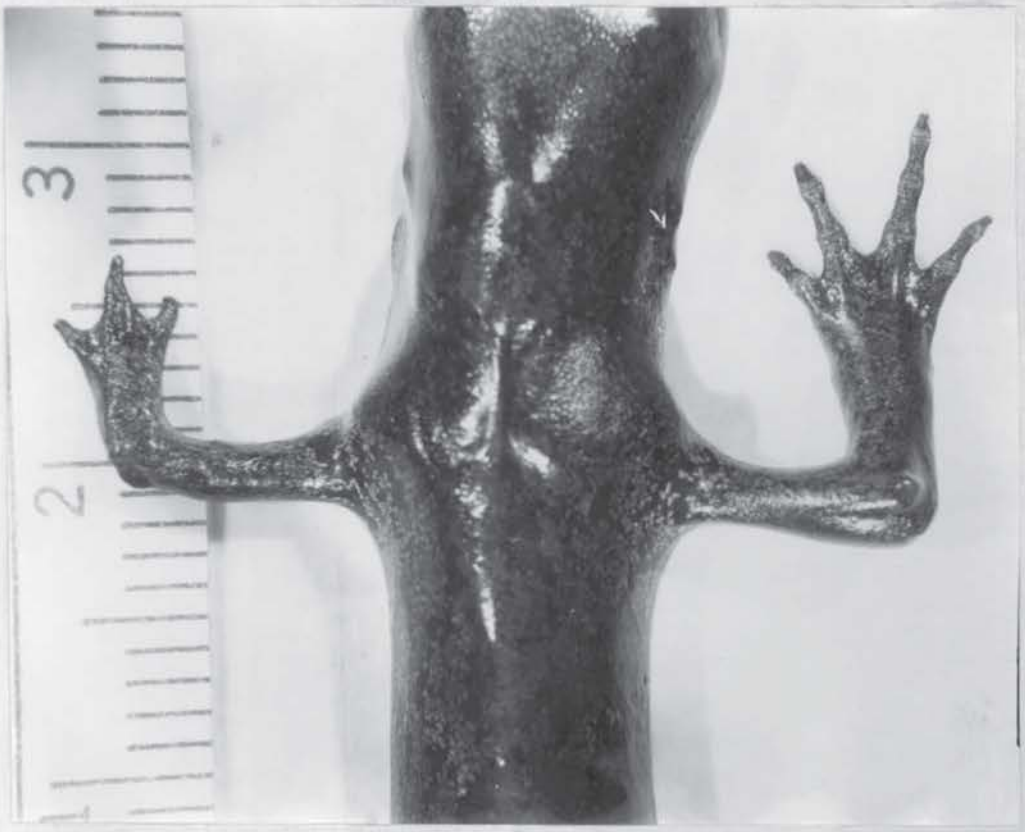

Figure 6

Figure #7: Ambystoma texanum (Subject #18) has a right forelimb that is a 2 month regenerate, but was amputated at the shoulder, and a left forelimb that is a 13 month regenerate amputated at the elbow.

Figure #8: Ambystoma texanum (Subject #5) has a right forelimb that is non-regenerating (amputated 9 months previously) and a left forelimb that is non-regenerating (amputated 3 months previously).

PLATE 4

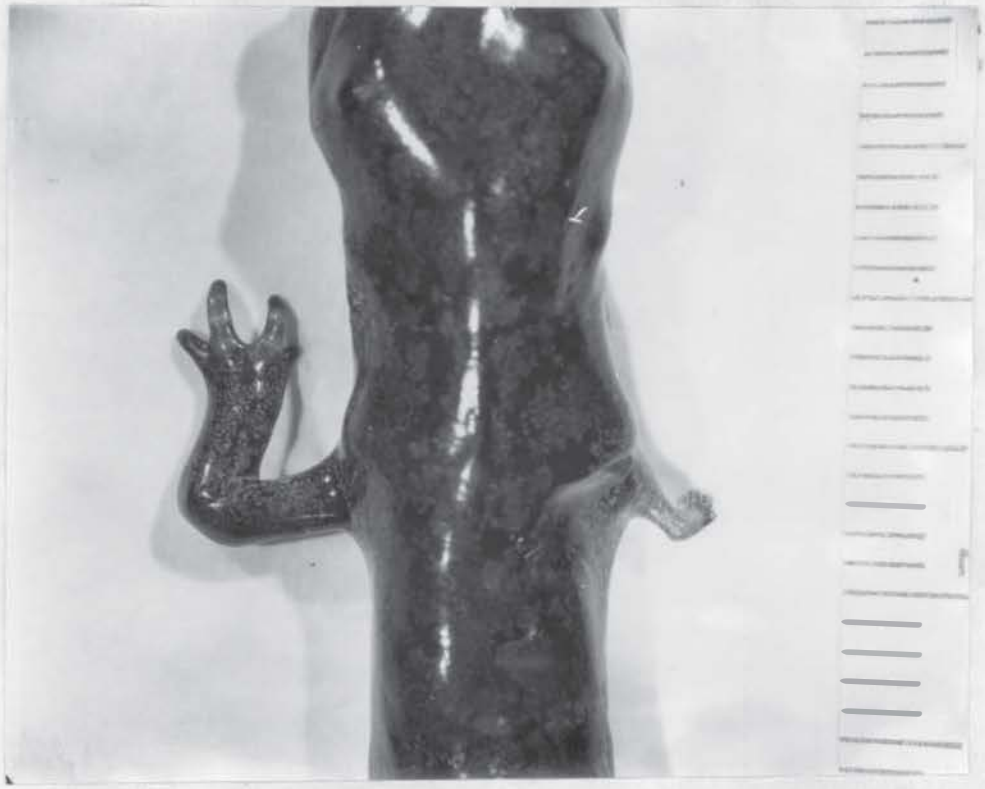

Figure 7

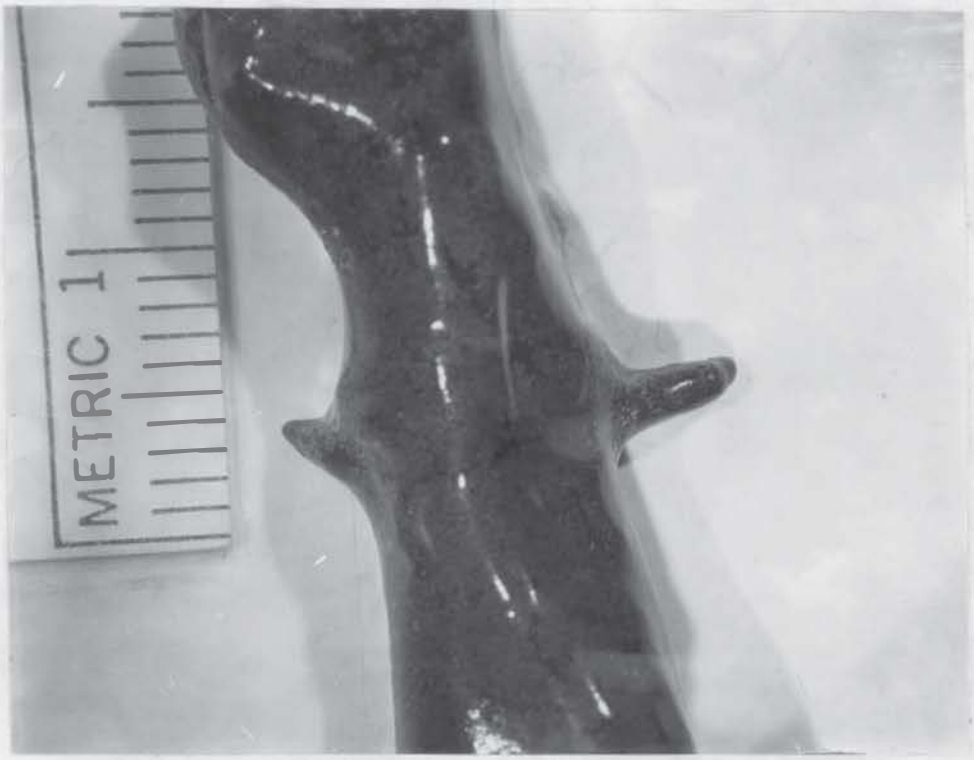

Figure 8

still in the budding stage. The limb was much smaller, because it was severed at the shoulder instead of the wrist. Subject #20 demonstrated a slight difference between a 7 and a 13 month regenerate. Subject #21 showed a much greater difference between a 7 and a 13 month regenerate.

The amputation site was difficult to observe because of the almost normal redistribution of melanocytes and silver-grey pigments. In some instances a slight constriction or lack of silver-grey pigment indicated the site of amputation.

Regenerating structures representing each of the stages described previously in the text and Table I were reamputated sectioned, stained, and examined. Each stage had four or more different serial sections on which observations were made. In addition, several examples of abnormal digital structures, normal forelimbs, and regenerates several months old were also prepared.

The slides of limbs in the wound healing and closure phase showed much cellular debris and destruction. The wound epithelium flowed over and filled any depressions on the surface. Many of the sections of limbs showed that the wound epidermis had invaded with tongue-like projections into the stump. In the later part of this phase, the wound epithelium was from 8-12 cells thick. The wound epidermis was in direct contact with the cut ends of muscle and bone. There was no basal membrane or dermis layer under the epidermis, nor was there the characteristic mucous glands of amphibians present. The area is not very well vascularized. Connective tissue on the wound area was mainly composed of sparce cells as opposed to fibers.

The dedifferentiational process had not appeared to start completely.

The muscle tissue did not seem to be affected. Slight changes were occurring to the bone and cartilage. The matrix had started to expand and dissolve leaving the bones hollow. Osteocytes and chrondrocytes appear unimprisoned, large, oval and active.

The analysis of blastemal (½-1 millimeter) stage showed similiarity to the previous stage. The wound epithelium of 8-12 layers thick was still in direct contact to mesodermal tissues. There was no dermis or mucous glands present in the regenerate. Many mitotic figures were observed in the wound epidermis. The bone was still being dissolved by means of loss of matrix and liberation of osteocytes.

The dedifferentiational forces were in full control by this time. Muscular tissue appeared to dedifferentiate. First, striations were lost and the fibers separated into long single cells. By observing cross sections of muscles, it was demonstrated that the muscle covering, the perimysium, was dissolved, which released individual fibers to be dispersed out. The undifferentiating muscle fibers still retained their orientation with each other in a certain direction.

With the above action, undifferentiated mesenchymal cells started to accumulate until their mass caused an outward swelling. The cells were quite stellate with many cytoplastic processes coming off the nuclei. There were many mitotic figures in the blastema of ½ millimeter. Cells within the tip were made more compact.

With increase in size to a blastemal cone of 1-2 millimeters, a new stage was observed. There still was no dermis or mucous glands under the 8-12 layers of wound epidermis. The undifferentiated cells were compressed into the tip and they were quite dense. There was mitotic activity everywhere, in mesenchymal cells, epidermis, and

chondrocytes. Vascular networks are being set up in the proximal part of the regenerate. The tip of the regenerate was starting to form a right angle to stump. Muscle fibers were still disintegrating as described previously. Undifferentiated cells were aggregating in the proximal part of the regenerate. These appeared to be redifferentiating the elbow (distalhead of humerus and proximal heads of radius and ulna.) However, these structures seemed fused together. True perichondrium separating the cartilaginous structures had not formed. The chondrocytes seemed to accumulate more to the edge than the center of the structures. The matrix of cartilage had not as yet formed in the regenerate. Since mitotic activity was still observed in the presumptive chondrocyte, it would be premature for the matrix to form.

In the next stage, the paddle shaped regenerate, the balance was swinging toward differentiation. Vascular networks, dermis, and mucous glands occurred down to the middle of the radius and ulna, but not farther distally. Undifferentiated mesenchymal cells are still much in evidence.

Perichondrium at junction of humerus, radius, and ulna was formed and the elbow joint was completed. Radius and ulna were completely separated by perichondrium. Matrix in humerus and radius and ulna was just starting to be well formed. The metacarpals, carpals and digits were just starting to form. Chondrocytes accumulated in the tip to form the previous structures. However, the structures associated with the wrist seemed to flow together, because the perichondrium was not formed at this time. Muscles seemed to be differentiating as single nuclei fibers grouped together side by side. There was still no

striations in the muscle fibers in the regenerate.

The foot stage (digits budding out) had more differentiation. Dermis and mucous glands occur down to the wrist. There was vascular network even to the top of the digits. At the tip of the digits the epithelium was 8-10 layers in thickness. There was only a few undifferentiated mesenchymal cells present near the digits. Muscle fibers acquired striation in the forearm, but fibers were unstriated in the digits. Long single nuclei fibers in bundles were observed in digits. Matrix of cartilage was complete in the humerus and increased as one goes distally. A majority of the chondrocytes were on the periphery of the structures with the centers being clearer. Perichondrium was forming in those structures which compose the wrist. The radius and ulna appeared separate from the wrist, yet the digits appeared fused to carpals and metacarpals.

As the digits started extending outward, the cartilage matrix became more evident in the radius and ulna. The perichondrium of the cartilaginous structures of the wrist became more differentiated and the carpals and metacarpals appeared separate and not fused. Some of the cartilaginous structures in the wrist appeared to have a considerable amount of matrix. Muscular fibers in the wrist demonstrated striation and perimysium development. There were few undifferentiated cells present, with a few exceptions near the tip of digits. Dermis and mucous glands were present in all locations except for the tip of digits.

The final stage of middle digital elongation in addition to regenerates of several months were almost identical to normal limbs.

Digital muscles had striations. Dermis and mucous glands occurred even in the tip of digits. The epithelial layers then numbered 4-8. Separate phalanges were established by the differentiation of their perichondrium. The matrix of the digits increased until they appeared like other proximal structures. The main differences in the normal limb and regenerates of several months were the size and length of the wrist and digits.

In addition to these analyses, one of the non-regenerating blastemas of #5 was observed. It was a blastemal cone of 1½ millimeters long. It appeared much like other representatives of this stage, except for the fact that the cone and tip of the cone were heavily vascularized as opposed to the sparce vascularization in others. There was a space between the epithelium and the blastemal cells, and there appeared to be a basement membrane on the epidermis.

DISCUSSION

The experimental evidence including 59 completely regenerated forelimbs, many regenerative stages, and the fact that only one out of thirty subjects failed to produce regenerates clearly demonstrates that adult Ambystoma texanum do possess the power to regenerate forelimbs under the experimental conditions. Reference to adult A. texanum forelimb regeneration was not found in a survey of literature. Therefore, this probably established for the first time that forelimb regeneration occurs in the adult of this species. This ascertains that another species (A. texanum) in the Ambystomidae family can regenerate forelimbs in the adult form. The power of regeneration is equal on both sides of the animal and limbs are capable of repeated regeneration. The criteria for a completely regenerated forelimb did not specify the number of digits present so that limbs with digital abnormalities could be counted in the total.

From the morphological observations and histological analysis, the events or stages occurring during the regenerative process were similar to those reported in the literature. A sequence of stages was selected and defined for the purpose of timing regeneration. These stages were chosen so that there were several different characteristics that defined them. However, there was some subjectivity involved in deciding when one stage was transformed into another.

Since the experimental subjects differed in weight, size, and age, it was necessary to arrive at average time of inception and duration of each stage. Therefore, the times for these regenerative stages were average ones for the species as a whole. With the individual variations,

value of duration could be expected to fall with the ranges in Table I.

The wound closure and healing stage lasted for about a week. During this time, the stump appeared lifeless and inactive. The apparent lifelessness was shattered by the blastemal phases. This could include both blastemal formation and blastemal cone formation which lasted 3 weeks. It was during this phase that growth in volume of the regenerate started and proceeded at a very rapid, almost logarithmic increase. At the end of the 4th week, there was ample evidence of regeneration. A rapidly growing blastemal cone of 2 millimeters has erupted from the stump. At this point, however, the cone was still primitive, without form. The remaining stages which occurred during the second month were ones of differentiation. Less than a week was required to transform the cone structure into a paddle-shaped regenerate. The form of the regenerate was starting to be determined. From this stage onward, the formation of foot and digits were of critical importance. The formation of foot and digits occurred at a rapid rate. The appearance of cartilaginous columns budding out of the foot take another 6 days and marked another stage. The extension of digits out of the foot required another week. Therefore, at the end of 40 days, there existed a limb structure that met the requirements for a completely regenerated forelimb. At the 49th day, the middle digits started to grow at a more rapid rate. Observation was continued through the 60th day, but no new changes were seen. The regenerate was complete functionally as well as morphologically. Functionally the regenerate or stump was utilized almost at once. Even early cone stages could be used to move or support the animal.

Once the digits had elongated, the rate of growth of the forelimb volume and length of digits was maintained at a very slow rate. From

Figures #1-#8 the difference in size between regenerates and normal limbs and between younger and older regenerates can be observed. It is questionable if a regenerate would ever match a normal limb as to size, volume, and length of digits. The study showed that a regenerate of 13 months was still dwarfed in size and volume as compared to the normal limb. The older regenerate always was a fraction larger than its younger partner (subjects #20 and #21). The rate of growth in volume of forelimb and growth in length of digits may not be related to regeneration, as much as they are related to slow maturation. At the end of two months, the regeneration process seemed to have completed its mission. It had replaced the structural parts totally, but their regrowth in normal volume was left to a slow maturational process. This should not be viewed as an inadequacy in regeneration, since functionally, the limb had been completely restored. The above discussion is meant to add insight into the limits of regeneration.

The 15% of the complete regenerates which displayed an abnormal number of digits represented morphological mistakes made in late stages of regeneration. For some reason, the digits did not bud out or there was fusion of the digits. It was this type of abnormality caused by nature that drew attention to regeneration by investigators in the late 19th century.

The reason for some of the events observed during morphological observation were as follows: The amputation surface appeared quiet, because the only events occurring were wound closure, necrosis, and accumulation of cellular debris. The depression of the surface was caused by trimming of muscle and bone, plus contraction of muscle.

The swelling laterally of the stump was probably due to necrosis, accumulation of fluids, or increase in cells due to dedifferentiation and mitosis. The wound surface was clear and transparent because the dense opaque dermal layer was absent. The surface was covered with several layers of clear epidermal cells.

The dedifferentiation of dark melanocytes and silver grey pigment cells resulted in loss of color, and these structures were transformed into clear tissue. Since these pigment cells were so highly specialized, they might have been the first to show the effects of dedifferentiation. Swelling of the blastema outward might have been due to the mitotic activity of the dedifferentiated cells.

The white opaque layer that spread under the surface was probably due to the dedifferentiational process and accumulation of primitive cells. Elongation of the blastema to 2 millimeters was due again to mitotic increase of cells and concentration of these cells under the wound epidermis. The wave of darkness that passed down the regenerate was the re-establishment of melanocytes over the surface. It occurred first in the proximal parts and gradually moved distally. Therefore, there was a gradation of color from black to grey to clear.

The transformation of the orientation of the cone from 180° to the stump to a 90° angle was probably due to the differentiation of the elbow (creation of distal head of humerus and proximal heads of radius and ulna). The 2 millimeter cone soon has the dorsoventral axis determined and was transformed into a paddle-shaped regenerate. The paddle shaped regenerate has dorsal-ventral flattening at the distal end, creating a flexing tip. This flexing tip was probably created by

differentiation of the wrist. (creation of carpals and metacarpals).

Cartilaginous columns budded out of the stump. Digits had a complex vascular network around them to supply the needs of the growing tip.

At the tip, there were two events occurring simultaneously, cellular growth and cellular death. They work in concert to give form to the foot. The digits were involved with cellular growth. The webbing between the digits was in the midst of cellular death. The tissue was avascular, opaque, and wrinkled. Soon the webbing completely dissolved, the digits elongated, and the middle digit became longer. The silver-grey pigments were slow to reinvade the limbs, but soon they had covered the entire limb.

From the histological investigations, the stump was a site of much cellular destruction and disorder. The wound epithelium quickly sealed the surface to protect it. The disruption in the bone and cartilage might have been due to the crushing effect of the scissors. This might also have caused the disruption of the matrix. The 8-12 layers of wound epithelium agreed with the value of 12 or more layers that Singer (1959) reported in the literature. The tongue-like projections of wound epithelium which seemed to extend back into the stump might have been the type of evidence that caused Rose (1948b), Hay (1952), and Scheuing and Singer (1957) to propose considerable epidermal contributions to the blastema. It was felt that these tongue-like projections were caused by amputational depressions, and that these were filled with invading epidermis. This could demonstrate that these tongue-like projections were the source of phagocytolytic cells which Scheuing and

Singer (1957) proposed. Also, the absence of a dermal layer or basement membrane was apparent. This agreed with literature sitings that a dermis was correlated with non-regeneration. Because of the amputation, the blood supply was curtailed, and the area had few blood vessels. The mucous glands that characterized amphibian skin was not present below the wound epithelium. In this first stage, dedifferentiational forces had not yet taken effect.

It was during the next stage (blastemal formation, ½-1 millimeters) that the effect of the dedifferentiation were observed. Matrix of bone and cartilage dissolved releasing the chondrocytes. The perichondrium seemed to remain intact. Muscular tissue started dedifferentiating by destruction of the perimysium which caused separate fibers to be dispersed outward. Striations were lost from the fibers. The individual fibers remained close to one another. Undifferentiated mesenchymal cells cause the ½ millimeter swelling. However, the density of these cells was not great, so there were many cytoplasmic projections. Mitotic activity was observed in the mesenchymal cells as well as the epithelium which had to increase as the blastema increased.

The mesenchymal cells became compressed in the tip as the size increased past 1 millimeter. This caused the cells to be less stellate. Mitotic figures were seen in all places of the regenerating tip. There was a suggestion in the proximal part that differentiation was progressing. Mesenchymal cells were aggregating together to form what appeared to be the elbow. The distal head of the humerus and proximal heads of the radius and ulna seemed to be created by presumptive chondrocytes. These structures appeared to flow together since a perichondrium had

not differentiated. The formation of this joint might have been the cause of the change from 180° to 90° orientation of the regenerate to the stump. The chondrocytes were more prevalent on the periphery of the structures. The matrix had just started to form, but many of the chondrocytes were still undergoing mitosis. The observation of mesenchymal cells verified the views of Schotté (1940) and Hay (1958, 1959, 1962) and Butler (1935) that there were no differences in the mesenchymal cells to indicate tissue of origin.

During the next stage, paddle shaped regenerate, there existed a regenerative structure that was equally divided between differentiation and dedifferentiation. Differentiation was seen down to the middle of the radius and ulna. Vascular networks, dermis, and mucous glands were created down to the above level.

The perichondrium of the structures of the elbow was established, creating a functional joint. Matrix in these structures increased trapping the chondrocytes. Aggregation of mesenchymal cells in the distal portion created the future wrist. The perichondrium was not as yet differentiated so the structures seemed fused together. Muscles started to differentiate in the manner described by DeHann (1956). There was elongation of undifferentiated cells into a long column of cytoplasm with one nuclei. The muscle still had no striations, since it was still primitive.

The foot stages (digits budding out) showed much differentiation. Those highly differentiated structures, dermis, and mucous glands could be found down to the wrist. The vascular network was in the digits to supply needed materials for growth. Because of the rapid differentia-

tion, few of the mesenchymal cells remained. Further differentiation brought striations and aggregation of single fibers to muscles in the forelimb. Matrix of cartilage was progressing distally as well as the perichondrium growth. This process was creating more mature and functioning cartilaginous structures.

In the final stages there was a complete differentiation of the regenerate. Dermis and mucous glands occurred through the entire regenerage. Regenerages of several months appeared almost identical to the normal limb. The non-regenerating blastema of subject #5 was analysed to discover why it did not regenerate. It, unlike other regenerates of the blastemal stage had heavy vascularization in the tip. This, as well as the space between the epidermis might be the cause of non-regeneration. For some reason, the mesenchymal cells were not differentiating.

The study verified the findings of Needham (1952) that differentiation was not simultaneous but proceeded from ths stump distally. This produced a regenerate that at a certain time had differentiation in the proximal part as well as undifferentiated cells in the tip. Needham (1952) stated that different tissues do not differentiate at the same rate. This study also demonstrated this. Cartilaginous structures were observed to differentiate before any other type of tissue. It appeared that cartilage should differentiate first since it would give form to the regenerate. The axes of the limb are determined by the orientation of the humerus, radius, and ulna. Differentiating muscle needed something on which to attach while it was forming.

During the period of the study, numerous regenerates ranging from a few days to 13 months old, were observed during their formation.

The regenerative process was usually complete after 1½ to 2 months. The remaining period of time was one of slow maturation and growth in volume. Even though the limb was replaced functionally, it never equaled the size of the normal limb. If regeneration rate were to be graphed, it would appear as follows. It would be slow if not static at first and then suddenly the rate would appear logarithmic. Then at the end of two months the rate would slow down to an almost static rate.

However, for the survivial of the species, even a miniature limb can be very important. Ambystoma texanum is well suited to regeneration that takes a period of one to two months. The species spends most of its time hidden under logs, moss, and leaves, and has time to wait for replacement of structure to occur.

SUMMARY

1. The present investigation was an initial general descriptive study of forelimb regeneration in the adult Ambystoma texanum.

2. Subjects had forelimbs severed just above the elbow and progress of regeneration was observed.

3. It was ascertained that adult Ambystoma texanum did possess the power to regenerate forelimbs under the experimental conditions.

4. From the amputations, 59 completely regenerated forelimbs and many early regenerative stages were observed to form. 15% of these regenerates had digital abnormalities.

5. Twenty-two regenerates were observed every 2-4 days for a period of 60 days for the purpose of making morphological observations and timing regeneration.

6. Several stages of regeneration were described and the average time for each was listed in Table I.

7. It was discovered that, on the average, after 40 days a completed regenerate with elongating digits was formed.

8. From Figures #1-#8, it was proposed that after 2 months regenerative processes might no longer be functioning. After this replacement was due to slow maturation and growth.

9. Serial sections of regenerates at different stages were obtained and analysed.

10. Regenerative events were identical to those reported in the literature concerning other species.

11. Cartilage was the first type of tissue to differentiate. Differ-

entiation does not occur simultaneously, but rather starts proximally and proceeds distally.

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