AN ANALYSIS OF THE RELATION OF SPECIFIC TISSUE DAMAGE TO ACCESSORY LIMB PRODUCTION IN THE AXOLOTL, AMBYSTOMA MEXICANUM

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ABSTRACT

AN ANALYSIS OF THE RELATION OF SPECIFIC TISSUE DAMAGE TO ACCESSORY LIMB PRODUCTION IN THE AXOLOTL, AMBYSTOMA MEXICANUM

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The present study has demonstrated that accessory limb parts can be produced in the axolotl through manipulation of limb tissues by various operational techniques. In this investigation, the limb tissues which appeared to be the most influential in the production of accessory parts were the skin and cartilage. Skin must be injured to provide a wound epithelium otherwise induction will not occur. Injured cartilage appears to be necessary, since accessory parts arise at the site of cartilage damage, provided a wound epidermis is available. Analysis shows that limbs with separated cartilage form significantly more accessories than limbs with broken cartilage. In addition, more formations appear on the hindlimbs than the forelimbs. Muscle does not have an observable influence on accessory production. If nerves are cut, there is no perceptible increase in the induction of accessory parts, although the structures which do result seems to be better developed.

There appears to be a tendency for the inhibition of accessory development in the axolotl. Although the early stages of many of the experiments showed much "blastema-like" activity, the later stages showed practically none. A histological investigation failed to reveal the cause of the inhibition.

The accessories produced, in general, varied from small mounds of cartilage to complete limb duplication. The most frequent response, however, was the formation of digit-like structures.

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In a moment of insanity, this author decided to write a thesis. With the help of some axolotls, lots of pencils, more paper, and much grief, the herculean task was undertaken. The mind staggers at the epic proportions of such a task, requiring the strength of Atlas, the courage of Danial, and the patience of Job. Yet, against all odds, the battle was fought and won, the task completed, and a thesis written. Now all that remains is the cold, critical eye of history which is capable of dealing a death blow without remorse, awarding winning acclaim, or at best, capable of granting a begrudging approval. Regardless of this, however, the weighty tome which follows is guaranteed to make an impression upon the mind of man, provided that it is used as a club. It has a fairly good heft.

Many and numerous are the thanks which cascade from the author and land, wet and sloppy, upon those individuals who have contributed toward the production of this work. To the axolotl gang which fought me at every turn, I offer my best wishes for a speedy recovery and the respect of one skilled infighter for another.

Sincere thanks are offered up to Dr. John Shaver and Dr. Benjamin Banta, who in the face of adversity, and really no other choice, took the bull by the horns and consented to serve as my committee. Such bravery in the face of awesome odds is seldom seen. I offer my profuse thanks.

Special thanks and regards are directed toward the man responsible for it all. Dr. Charles S. Thornton, I could not begin to thank enough for allowing me the opportunity of academic achievement. To some measure, I tried. Long were the hours spent in his scientific abode, cleansing dishware and offering sacrifices to the gods Mexicanum, Maculatum, and Opacum, so that all his goods might shine and function more efficiently.

I fully recognize that the opportunity to achieve is not a gift lightly given. Thus, it was not a gift that was lightly taken. To merely say that I learned and benefited much from this experience would be quite meaningless. I can only hope that, for you Sir, it was not all in vain.

My warmest regards are saved for last. With unabashed emotion and frank admiration, I would place these in her hands who worked at my side unquestioningly, and who granted to me that most valuable of all gifts, the prize of true friendship. This work is as much hers as mine.

Thus, I would dedicate this thesis to Laura, with Love.

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INTRODUCTION

Supernumerary limbs and tails have been found in amphibians naturally and have been induced experimentally in the laboratory. Some of the natural causes of super-regeneration include bites from other animals (Przibram, 1921; Brunst, 1937, 1961); injury to the limb skeleton (Tornier, 1898); extremes of temperature (Schmalhausen, 1925; Voitkevich, 1958a); malnutrition (Schmalhausen, 1925); and a possible genetic mechanism (Bishop, 1947).

A variety of experimental methods has been used to induce the formation of supernumerary structures, including injury to the developing limb buds (Tornier, 1905; Puppe, 1925; Swett, 1926; Cooper, 1965); mechanical injury of the limb skeleton (Tornier, 1897; Studitsky, 1948); application of a ligature (Della Valle, 1913; Kazancev, 1930; Nassonov, 1930, 1936c); and transection of the spinal cord (Fraisse, 1885; Barfurth, 1901; Godlewski, 1904; Terri, 1931).

Other experimental methods include the deviation of limb nerves to the surface of the body (Locatelli, 1925, 1929; Guyénot and Schotté, 1926; Polezhajev, 1933; Guyénot, et al., 1948; Bodemer, 1958, 1959, 1960); x-irradiation (Brunst, 1950a, 1950b, 1952; Brunst and Figge, 1951); ultraviolet irradiation (Rieck, 1954; Butler and Blum, 1955, 1963); skin transplantation (Glade, 1957; Droin, 1959); limb transplantation (Carpenter, 1932, Lecamp, 1935; Yntema, 1962); epidermal cap transplants (Thornton and Thornton, 1965); the injection of carcinogenic substances (Breedis, 1952); tissue implants which undergo cytolysis (Nassonov, 1936c, 1937, 1938a,

1938b, 1938c; Fedotov, 1946; Ruben, 1957a, 1960b; Ruben and Frothingham, 1958; Ruben and Stevens, 1963b; Stevens, et al., 1965; Dent and Benson, 1966; Carlson, 1967; Carlson and Morgan, 1967); and inert celloidin implants (Balinsky, 1927 as cited by Glick, 1931; Ruben, 1957b).

Super-regeneration was observed in the laboratory by Przibram (1921) to occur in axolotls (Siredon pisciforme) and newts (Triton cristatus) as the result of bites which produced complicated wounds. Brunst (1937) noted that adult newts feed on small amphibian larvae, and in cases where tadpole limbs or tails were damaged, super-regeneration of these structures was often the result. These observations led Brunst to state that most cases of super-regeneration occurring in nature were caused by damage due to bites (1961).

Damage to developing limb buds has been cited as a cause of limb duplication in various amphibians. Tornier (1905) made single longitudinal cuts in the posterior limb buds of Pelobates fuscus tadpoles, which in some cases were deep enough to split the pelvic girdle. Where the damage was severe, contraction of tail muscles separated the two halves and regeneration resulted from both surfaces. The growth of the regenerate was found to be directly proportional to the size of the wound. Puppe (1925) achieved the same results by making a transverse incision in the posterior limb buds of Rana temporaria tadpoles. Cooper (1965) produced duplicates and triplicates by accidentally cutting the anterior limb buds of R.catesbeiana. Swett (1926) concluded that if disturbances of any kind were

applied to limb buds before they become differentiated, duplications would result.

Nassonov (1936c) states that in the majority of cases duplications and multiple formations result from wounds in which the skeleton is injured. Tornier (1898) showed that a case of forelimb triplication in R. esculenta could be traced to a broken scapula. Studitsky (1948) reported supernumerary formation when he removed the proximal part of the humerus in an axolotl. Della Valle (1913) fractured the femur of a European newt and applied a silk ligature to the fracture site. The ligature was not tight enough to hinder limb circulation, although it did prevent the bone from knitting. Duplicates formed on both sides of the ligature. This experiment was repeated in the axolotl by Kazancev (1930) and Nassonov (1930).

The application of the ligature was shown by Kazancev (1930) to result in the complete disintegration of the dermis, muscle, and cartilaginous skeleton of the limb directly beneath the ligature. The skin alone remained whole. As a consequence, the part of the limb distal to the ligature bent at an angle and was dragged around by the animal. Kazancev further notes that while the limb was constricted the cartilage did not regenerate. However, if the ligature was released during the development of an additional part, the wound healed and the cartilage regenerated, but further development of the duplicate ceased. Under these conditions, then, accessory development depends upon the separation of the cartilage. Nassonov

(1930) reached the same conclusion, and later (1936c) suggested that probably an important cause in the development of accessory parts was the presence of a destroyed part of the skeleton which contained cartilage.

Further evidence supporting this contention was supplied by Nassonov (1936c). He found that repeated flexing of an axolotls' limb prevented the cartilage from healing. Using 12 animals, he repeatedly bent the limbs twice a day for two weeks. Although the innervation and the circulation of the limb were not disrupted, some damage probably took place. Several days after the cessation of the bending, two animals showed regenerative activity which resulted in the development of duplicate leg parts. In experiments where no attempts were made to prevent the skeleton from healing, 70 newt limbs were fractured in the humerus or femur and the skin above the fractured area broken. In these cases, no additional formations were observed. Nassonov suggests that when additional formations develop after repeated bendings at the point of fracture, the healing of the injured tissues is retarded and enough time is allowed for decomposition products to act on the surrounding tissues and stimulate regenerative processes. When the limb is fractured once, healing is rapid and the products of decomposition disappear quickly.

Tornier (1897) produced duplications in the newt by fracturing or inflicting a wound on the femur. However, in each case, he amputated the distal part of the limb. In the ligaturing experiments of Della Valle (1913), Kazancev (1930), and Nassonov (1930), it was found that the for-

mation of duplicates was facilitated by amputating the tips of the limbs. Nassonov (1936c) demonstrated that such duplicates must develop before the wound heals and the fractured parts of the skeleton reknit. He suggested that these accessory structures formed because amputation of the limb tip hastens the onset of the regenerative processes at the point of fracture.

Nassonov (1930) also found that ligaturing alone, without prior skeletal damage or amputation, was sufficient to produce duplication either proximal to the ligature, or on both sides. In one case, he observed two duplicates arising from the same spot on the proximal side.

It is possible to initiate the development of supernumerary tails, through damage to the tail skeleton. Fraisse (1885), Barfurth (1901) and Terri (1931) demonstrated in the newt that a split spinal axis could result in the establishment of two or more separate foci from which tail regeneration could occur. Godlewski (1904) cut a square piece from the axis of the tail through the spinal cord and obtained secondary tails at both the anterior and posterior cut surfaces. In the above cases, the tail is cut in such a way so that the spinal cord is exposed only at the posterior and anterior surfaces, but not at the dorsal or ventral surfaces. Thus, regeneration at the exposed surfaces is related to the presence of the spinal cord (Godlewski, 1904).

Weiss (1925) has indicated that the plane of amputation may play

a role in the initiation of accessory structures. If the plane is oblique in relation to the longitudinal axis of the limb, or tail, super-regeneration may occur. In the newt, Triton cristatus, cross sectional and longitudinal amputations performed simultaneously result in two wound surfaces and two independant blastemas. Regeneration from the cross section was almost normal, but regeneration from the longitudinal section never replaced the amputated portion and only resulted in a series of digits forming close together.

Temperature has been cited as being the most important of the factors which can cause disturbances in the normal development of limbs (Voitkevich, 1958a). Duplications in the development of the hind limbs of the frog were observed in tadpoles whose metamorphosis had been considerably delayed, and particularly in specimens developing in a large direct supply of cold spring water. The formation of additional limbs on animals collected in nature could not be correlated with any traumas of the leg anlage (Voitkevich, 1958b).

Schmalhausen (1925) has shown that abnormally high temperature, as well as malnutrition, retards development of the post-axial portions of the limbs in the axolotl, resulting in some cases in fusion of the tarsal or carpal bones.

A possible genetic basis of limb anomalies in Ambystoma tigrinum found in nature was suggested by Bishop (1947). Examples of limb duplication consistently demonstrated an absence of mirror-image symmetry,

the addition of extra digits laterally, and the limitation of the phenomenon to the hind limb. The anomalies were observed to carry over into the second generation of the population being studied. Bishop suggests that a specific developmental mechanism other than the splitting of the growth center may be the cause.

Support for a possible genetic mechanism of limb duplications may be found in the work of Voitkevich (1958a). He observed that the majority of duplications in the pond frog were in the right rear limb. Out of 304 individual cases, 9 occurred in the left limb, and in all of these cases, there was an increased amount of material on the right side as well. He suggests that the disturbances may have begun on the right side initially, and he states (1964) that the region forming the girdle of the hind limbs demonstrates a condition of high morphogenetic instability, with its predominance on the right side of the body.

The most reliable method of experimentally producing a supernumerary limb on an adult is to deviate nerves to the surface of the body near the base of a limb (Rose, 1964). Locatelli (1925, 1929) diverted the femoral nerve to different places on the body near the base of the leg. Additional extremities on the back were obtained mainly by diverting the same nerve through the muscles of the back to the surface of the skin where its end was fixed. During these operations, tissues were destroyed and in some cases, the skeleton injured. Locatelli (1929) suggested that the nerve had a specific morphogenetic effect. Guyénot

and Schotté (1926) extended Locatelli's work and demonstrated that the nerve was non-specific in its effect on the initiation of a supernumerary structure. The deviation of nerves to specific areas results in structures typical of that area and independent of the nerve inducing them.

After a nerve has been deviated, it is necessary to inflict a surface wound before any induction will take place. (Polezhajev, 1933; and Bodemer, 1958). The induction begins, as in early limb regeneration, with the formation of an apical epidermal cap at the distal end of the nerve (Thornton, 1954). Depending on the experimental procedure, Guyénot, et al., (1948) produced accessory structures in the newt ranging from digits to the formation of a supernumerary arm at the shoulder level. Kiortsis (1953) found an area in the shoulder region which would give rise to two limbs when a nerve is deviated to it.

Brachial nerves deviated to the chest do not normally induce the formation of supernumerary limbs. Bodemer (1959), however, noticed that an epidermal thickening and some cellular accumulation did take place. If various tissues from another adult newt are added, supernumeraries will form. Those tissues which were best able to initiate the accessory structure include liver, lung, and bone. Newt liver implants were used by Bodemer (1960) to enhance the influence of nerves. He also demonstrated the importance of the quantity of nerve fibers in development of nerve-induced supernumeraries. By deviating limb nerves of different fiber content to traumatized muscle at the base of the forelimb, a correlation

was found between nerve fiber number and the capacity to induce a growth reaction. Implants of liver around the end of the smallest deviated nerve, resulted in a growth response which was three times greater than that obtained with the nerve alone (Bodemer, 1960).

Thornton (1954) demonstrated that the denervation of a limb may give rise to supernumerary structures. In Ambystoma opacum, supernumerary structures were produced through the aberrant regeneration of the cut brachial nerves to the site of the denervation operation in the shoulder region.

The stimulating effects of x-irradiation upon the induction of supernumerary limbs was discovered by Brunst (1950a) as the result of an experimental error. In an experiment originally designed to explore the influence of local x-irradiation on the developing limbs of the young axolotl, Brunst found that a gradation of x-ray intensity from 100 roentgens to 3000 roentgens produced effects ranging from a slight inhibition of growth at low levels to complete inhibition at the higher levels. However, in several cases, he observed a stimulation of growth resulting in the production of supernumerary limbs. The irradiation had been directed locally at the lumbo-sacral region in 30 day old axolotls. Due either to an improper orientation of the animal before irradiation, or to movement of the animal during irradiation, the lumbo-sacral region in several animals did not receive all the intended dosage. Instead, the

limb girdle region was on the unirradiated periphery. In these cases the irradiated area underwent a degeneration followed by a secondary stimulating effect in the unirradiated boundary zone expressed by greatly increased mitotic activity of its cells. This proliferation apparently produces the supernumerary limbs. Brunst suggests that this secondary stimulating effect is a result of disintegration products or "necrohormones" released during degeneration. It is significant that supernumerary limbs were produced only when the limb buds were in the unirradiated boundary or "zone of stimulation". Thus, the potentiality of limb formation was not suppressed, but was actually stimulated by the x-irradiation (Brunst, 1950a).

The development of secondary tails by x-irradiation stimulation was observed by Brunst (1950b). Brunst and Figge (1951) extended this study in axolotls. In larvae 10 days after hatching, they found that the development of the new structure from the zone of stimulation depended upon the strength of the dose. The development of the additional tail was always in the dorsal, more proximal region of the original tail, while the growth of the original structure was completely arrested.

Stimulation by x-irradiation is characterized by two distinct periods of activity (Brunst, 1952). The acute "primary reaction" beginning from 23-28 days after irradiation and lasting for 30-40 days, is a period of cellular multiplication in the zone of stimulation. The temporary stimulating effect acts as a stimulus to new development. In the development of

secondary tails, the formation of the first cartilaginous elements occur during this period. The secondary reaction begins with the development of the new structure. The skeleton of the supernumerary tail is composed entirely of cartilage which develops in the zone of stimulation in connection with the neural tube and is independent of the original cartilaginous skeleton. Thus, Brunst (1952) concludes that the irradiation produces a new type of development which differs markedly from the formal type of development.

It is interesting to note that the necrohormone theory proposed by Brunst (1952) is similar to Nassonov's suggestion (1930) that disintegration products might explain the stimulation of regenerative processes.

The irradiation of developing urodele limb buds with ultraviolet light was shown by Rieck (1954) to produce accessory structures. Larvae of Ambystoma opacum and A. maculatum in stages 29-43 were subjected to ultraviolet light localized to the limb bud. It was found that the developing limbs responded to the irradiation in a variety of ways depending upon the developmental stage of the limb. Duplications occurred at a relatively low frequency at stages 37-41. At earlier stages, complete inhibition of limbs occurred when damage was extensive, and, in cases where development was not arrested, complete limbs developed. At later stages, suppresion of discrete parts distal to the elbow was observed in most cases. The majority of accessory structures consisted of "spikes" growing from

the forearm, while the remaining types varied from extra digits on the hand to an accessory hand or digit on the forearm. These duplications appeared in 46 out of 190 cases.

The frequency and type of duplications produced in differentiated limbs of Ambystoma were demonstrated by Butler and Blum (1955) to be dependent upon the level of irradiation. Out of 150 cases of irradiation at the elbow, 110 showed accessory growths, the majority of which (51%) were spikes or extra digits (45%). Irradiation of either the area proximal or distal to the elbow produced no accessory growths, while irradiation of the shoulder region produced two cases of complete limb duplication out of 170 cases.

Regression is noted in limbs subjected to ultraviolet irradiation (Rieck, 1954; and Butler and Blum 1955, 1963). Butler and Blum (1955) found that the extent of regression as well as the incidence of accessory structures was dependent upon photorecovery, since both are reduced by illumination with visible light for a few days after irradiation. Butler and Blum (1963) found that supernumerary limbs arise with the highest frequency at points of articulation of the skeletal structures. They also noted that after irradiation, the epidermis sloughs off and is replaced by a wound epidermis with no basement membrane.

Regression and subsequent cytolysis lead to dedifferentiation of tissues which provides cells which can form a blastema. The nerves in the irradiated area branch and the parent nerves in the area of the supernu-

merary anastomose. As a result, the developing blastema is well provided with large nerve trunks. It is suggested that the variation in structures observed after ultraviolet irradiation is dependent upon the variation in regression rates (Butler and Blum, 1963).

The transplantation of skin between the forelimbs of Triturus viridescens has been observed to result in the formation of accessory structures. Glade (1957) obtained extra digit formation in 2 out of 9 cases of skin transplantation between forelimbs and subsequent amputation. Droin (1959) observed 5 cases of duplication out of 16 after exchanging skin between the forelimbs of Triton without changing the orientation of the skin. As a result of Kiortsis's observation (1953) that a region exists in the shoulder which gives rise to two limbs if a nerve is deviated to it, Droin (1959) transplanted skin from this region to the forelimb. After amputation, 6 of the 14 regenerates resulted in double limbs.

Rose (personal communication) has indicated that supernumerary limbs can be produced by changing the orientation of a section of limb skin by 90 degrees with respect to the limb.

Carpenter (1932) demonstrated that developing limbs of Ambystoma punctatum larvae will give rise to supernumerary limbs upon transplantation to a heterotopic site. The limbs from larvae of stages 29-46 were transplanted to the flank and out of 68 operations, 22 yielded duplications. Limbs from stage 41 larvae, which are distinct appendages showing digits,

produced 7 of these duplicates. Out of 120 cases of limb transplantation from larvae at stage 46 up to metamorphosis, nine duplications occurred. The limbs at these stages were differentiated and functioning.

In the toad, Alytes obstetricans, Lecamp (1935) grafted old limb buds and young limbs to a heterotopic, non-limb field and obtained supernumerary limbs from the base of the graft. If a young limb is transplanted to a limb field, three limbs result.

The transplantation of aneurogenic limbs to normally innervated limb stumps of Ambystoma punctatum was shown to produce duplicates in 29 out of 397 cases (Yntema, 1962). However, similar orthotopic transplants from aneurogenic to aneurogenic, normal to aneurogenic, and normal to normal resulted in no accessory structures. Heterotopic transplants of aneurogenic limbs to the flank of a normal host also produced supernumeraries in 20 out of 46 cases, whereas a reverse transplant of normal to aneurogenic produced none.

Yntema suggests that on the basis of the orthotopic transplants, duplications may result from an ingrowth of nerve fibers and that the aneurogenic or sparsely innervated tissues respond excessively to this stimulus. However, he points out that the series of heterotropic transplants do not support this theory since nerve fibers are acquired quite slowly in a heterotopic position. Thus, he concludes, either very few nerve fibers can promote duplication, or its cause must be sought elsewhere.

It is interesting to note that in 38 cases of normal heterotopic limb transplants, Yntema (1962) observed no duplications. Although Yntema indicates that these observations are of a preliminary nature, it is clear that his results do not coincide with those of Carpenter (1932). Further investigation in this area is needed.

Thornton and Thornton (1965) have reported that accessory limbs result when epidermal apical caps are transplanted to the base of a blastema. In Ambystoma talpoideum and A. mexicanum, limbs were amputated and allowed to form a mound blastema. A small section of epidermis equivalent in area to an apical cap was then removed from the preaxial surface of the base of the blastema. The apical cap of the blastema was removed and after cleaning, autoplastically grafted into the wound at the base of the blastema. Out of 51 cases, 27 produced duplicate parts. In the remaining 24 cases, the accessory cap was suppressed. The cases of duplications in mexicanum were only digits, while those positive cases in talpoideum all consisted of several limb segments. The authors concluded that the transplanted epidermal cap provides the stimulus for blastemal cell accumulation in the accessory regenerates, although not necessarily the stimulus for their morphogenetic response.

Carcinogenic substances are capable of producing accessory structures. Breedis (1952) injected the following carcinogenics subcutaneously into the proximal forearm of the newt: coal tar, methylcholanthrene (MCA),

benzpyrene (BP), acetylaminofluorene (AAF), scarlet red (SR), vaseline, beryllium hydroxide, and fragments of 2 amphibian neoplasms (frog kidney adenocarcinoma and newt sarcoma). For controls, he damaged forearms thermally with steel heated to 500 degrees C., or fractured the humerus after making an incision in the skin. He found that coal tar, MCA, BP, AAF, and SR alone or in combination produced accessory structures in 11 to 40 percent of the cases; vaseline in 5 percent; beryllium hydroxide in 33 percent; and R. pipiens kidney carcinoma in 4 percent (3 out of 75 cases). The newt sarcoma and the controls yielded none. The coal tar and carcinogenics produced the most complex growths earlier while the vaseline and carcinoma produced the simplest structures at a later time. The accessory structures varied from small digit-like structures to full duplications and all developed from the site of injection. If the materials were injected into the hind limb or the back, nothing resulted. However, when injected into the tail, secondary tails were produced in 6 out of 12 cases.

Breedis (1952) indicates that the destruction of tissues was not related to accessory formation in this instance. He observed that in the MCA series where tissue damage was high, accessory induction was low, while in the coal tar series, where induction was high, damage to tissues was low. Whether or not Breedis equates his observations of tissue damage to the necrohormone theory of Brunst (1952) remains uncertain. However,

any parallel which is drawn between the two studies should be done in light of the differing experimental circumstances.

The production of supernumerary limbs by means of tissue implantation has been extensively studied. Nassonov (1934a,b) found that macerated or desiccated regeneration buds, from 7 to 15 days old, if implanted subcutaneously in the limb of an axolotl, would occasionally induce the development of an accessory structure. He noted that if the implant was situated next to the insertion wound, the structure was produced at the wound site. Otherwise, no growth was produced. The type of structure which develops depends upon the level of insertion into the limb. The duplications characteristically contained limb components which were distal to the point of implantation. Out of 300 cases of implants of desiccated blastemas, less than 5 percent yielded positive results (Nassonov, 1934b).

Nassonov (1934b) suggested that a substance was produced by the decomposition of tissues which acted on the muscles and perichondrium after the formation of a wound epithelium and induced the proliferation of their cells.

Cartilage was also found to induce accessory formations. Nassonov (1935) observed that if cartilage were undamaged, after implantation, nothing resulted. However, if the cartilage is macerated previously, it is broken down by phagocytic activity. This is followed by a break-

down in the dermis and the surrounding musculature. The wound epithelum of the incision thickens and enlargens to incorporate the accumulating cells. Nassonov compared the cartilage to an organizer setting up a series of processes which result in new formations. He postulated the existence of two organizing substances found in cartilage, coriocide which breaks down the dermis and chondrogen which participates in the formation of a cartilaginous skeleton (1936a). Of normal axolotl implants, 13 out of 74 produced duplications. Altering cartilage by boiling, immersion in alcohol or ether destroys its inducing capabilities while cooling or drying causes a considerable drop in induction (Nassonov, 1936a; Kuzmina, 1940). Cartilage from almost any part of the axolotl will induce structures to form in a low percentage of the cases if transplanted heterotopically to any part of the axolotl except the head or back. These areas will not support accessory formations. Limb cartilage from adult frogs produced 7 out of 52 structures when implanted in axolotl limbs, while implants of axolotl cartilage in frogs produced nothing. Cartilage from the lizard (Lacerta agilis) and from rabbit ribs both failed to produce any structures (Nassonov, 1936b). The implantation of epithelial, osseous, and muscular tissue all failed to provoke accessory growth (Nassonov, 1937), even though very slight responses were obtained from lung, small intestines, and gills (Nassonov, 1938a,b,c).

Acid hydrolysates of axolotl cartilage have been shown to be extremely good in inducing accessory structures, as well as enzymatic (cathepsin and trypsin) hydrolysates, although the percentage and developmental stage of the new formations were lower in the latter case (Fedotov, 1946).

The importance of superficial wounding in the production of supernumerary limbs was demonstrated by Ruben and Frothingham (1958). They determined that the presence of a distal insertion wound close to the implant greatly facilitates the ability of the limb to respond to a growth stimulus. They also suggested that the implant may serve to prevent dermal replacement as well as extend the dermis-free boundary beneath the insertion wound.

For implantation in urodeles, normal frog kidney (R. pipiens) is used most frequently since it is more active in inducing accessory structures than urodele tissues (Ruben and Stevens, 1963b) or other frog tissues (Ruben, 1960a). Extremely high percentages of induction can be obtained with frog kidney (Carlson and Morgan, 1963, 1964, 1967; Stevens et al., 1965). Von Hahn, et al., (1964) suggests that the high potential acid phosphatase activity of kidney may be one reason for its inducing power.

The inducing ability of normal frog kidney can be destroyed by boiling (Stevens, et al., 1965; Carlson and Morgan, 1963, 1967) and

lowered by freezing (Stevens, et al., 1965), while lyophilization has no effect at all (Carlson and Morgan, 1963, 1967).

Innervation is necessary to insure a response in the limbs (Ruben and Frothingham, 1958; Bodemer, 1959). The delayed denervation of implanted limbs of larval and adult urodeles results in the formation of cartilaginous nodules in the larval limbs (Ruben and Armer, 1958). This happens normally in the adult but not the larva (Ruben, 1957a). It is suggested that the delayed withdrawal of innervation may remove the growth stimulus to the blastemal cells which are "chondrogenetically" oriented in respect to their developmental potential (Ruben, 1959). A similar occurence in the adult may be due to a pattern of innervation less than in the larva.

Ruben (1960b) proposed an "immunobiological" model to explain the production of implant-induced supernumerary limbs. In this model, he suggests that the implant induces a foreign-body response in the host which begins destroying the implant. The cytolytic by-products of this reaction act upon the peripheral nerve supply which, in turn, has the role of initiating dedifferentiation in the limb tissue. The dermis is then dissociated, an epithelial plate forms and blastema cells aggregate beneath it. The implant-induced truama must continue to act upon neural elements for at least two weeks in larval limbs and no longer in adults. Unfortunately, evidence supporting this model is lacking. Although it is evident that an inflamatory reaction accompanies the im-

plantation of foreign tissue (Carlson, 1967), a specific immune response has not been shown. In addition, Ruben and Stevens (1961a,b) have demonstrated there is no dosage effect when one or more implants are used, and there is no systemic effect. If an immune mechanism is at work, both a dosage and systemic effect would have been observed. No effect upon innervation could be found either (Ruben, 1963) since no increase of nerves was found in implanted limbs. It is suggested that perhaps cytolysis activates the normal host tissues to be hypersensitive to the normal nerve complement, or the effect of cytolysis is additive to that of the nerve. Ruben and Stevens (1963b) conclude that implant-induced trauma alone cannot account for accessory induction. It is postulated that growth promoting quality intrinsic to the implant may explain the varying effectiveness of implants from different sources. Carlson and Morgan (1967) arrive at a similar conclusion.

X-irradiation has no effect upon the ability of implants to induce growth (Carlson, 1964). Irradiated frog kidney was implanted into the forelimbs of the adult newt and in 16 out of 16 cases induced accessory growth, whereas control limbs with unirradiated kidney produced 14 out of 15 structures. If unirradiated implants are placed in irradiated forelimbs no growths are observed.

Frog renal adenocarcinoma implants are capable of inducing accessory limbs in larval as well as adult urodeles (Ruben, 1955, 1956, 1957a).

They are capable of initiating limb formation without undergoing extensive cytolysis. They do, however, induce dissociation, aggregation, and growth in adjacent tissues which are able to support regeneration. It is suggested that these cancerous cells can accomplish, without cytolysis, what normal tissues only do after cytolysis. They are not as effective as normal kidney implants in promoting accessory growths, however.

Dent and Benson (1966) implanted trunk and tail parts of larval newts into the epaxial trunk musculature of adult newts. In two cases, limb-like projections similar to the aborted regenerates characteristic of Xenopus appeared between 60-90 days.

Balls and Ruben (1964) reported 4 out of 30 cases of accessory cartilaginous nodule formation in Xenopus laevis forelimbs which had been implanted with normal Xenopus kidney.

Balinsky (1927, as reported by Glick, 1931) reported a case of induction after implanting celloidin in the body wall of Triton embryos. From this, he suggested that the implants were acting in a nonspecific manner. Ruben (1957b) also reported 1 case out of 24 in which a supernumerary structure was formed after implanting celloidin in the forelimb of Taricha granulosa. In all other cases, the implants were rejected. Ruben suggested from this that any stimulus which is capable of traumatizing a limb field for a time, and which does not destroy limb in-

nervation, will induce supernumerary formations. Since these are isolated cases, however, their significance is lessened.

Many different methods have been employed in the production of supernumerary limbs and tails. It is significant, however, to note that tissue damage is required in all cases. Although tissue damage alone can initiate the development of an accessory structure, no attempt has been made to clarify the relationship which must exist between the two. The present investigation, therefore, was undertaken to study the effect of selective tissue damage on the development and formation of accessory limbs.

By selectively damaging the different tissues of the limb, alone or in combination, a better understanding may be gained of their roles in the initiation of accessory morphogenesis.

METHODS AND MATERIALS

This investigation consisted of a series of 36 different experiments designed to study the formation of accessory structures in the axolotl through manipulation of limb tissues by various operational techniques. Larvae of the Mexican axolotl, Ambystoma mexicanum, were obtained for experimental animals from several successive hatches of adult axolotls maintained in this laboratory for breeding purposes. After hatching, the small larvae were fed brine shrimp until they had attained a size large enough to permit their being fed small pieces of beef liver by hand. At this point, the larvae were isolated into small plastic dishes, fed beef liver three times a week, and maintained at a laboratory temperature ranging from 20 to 30 degrees C. At the time of operation, the larvae ranged in length from 5 to 7 cm. (snout to tail) and in age, from 7 months to 1 year old.

TECHNIQUES

To attain consistency in the various techniques employed in this experimental study, those methods which were used repeatedly were standardized.

As a rule, those operations involving the mechanical destruction of tissues were carried out on the dorsal surface of both the upper arm of the forelimb and the shank of the hind limb. There are two ex-

ceptions to this rule, which will be noted later. Generally, all four limbs of an animal were used. The experimental animals were anesthetized prior to the operations in a 1:2,000 solution of MS-222 (tricaine mathanesulphanate, Sandoz), and all operations were carried out with the aid of a binocular microscope and No. 5 watchmaker forceps sharpened to a fine point (for a summary of the operations, see Table 1, p. 33).

Where the removal of skin was involved, a piece of skin (approximately 1/2 cm. x 1/5 cm.) was carefully cut from the dorsal surface of the limb without damaging the underlying tissue. This can be accomplished fairly easily, especially in younger animals.

When cartilage was fractured, blunt forceps were used to insure that there would be no damage to the soft limb tissues. All cases of cartilage fracture involved both the humerus of the forelimb and the femur of the hindlimb. The same portions of the limb skeletons were involved when cartilage was separated. Both the humerus and femur, in the axolotl, consist of main shafts which fit at either end into proximal and distal articulating heads. The main shaft can be easily pulled from either articulating head, especially in younger axolotls. This can be accomplished without removing any skin for gaining access into the interior of the limb. If done carefully, there is almost no damage to the surrounding tissues. As a result of such action, the continuity of the

cartilage is interrupted in a manner analagous to that of a cartilage break, except that no cartilage is broken. In all cases of cartilage separation, the humerus and femur were separated from their distal articulating head.

In several of the experimental series, the distal portions of the limbs were amputated after various operations had been performed on the proximal portions. This was attempted as a result of the observations of Tornier (1897), Della Valle (1913), Kazancev (1930), and Nassonov (1930) who noted that the formation of limb duplicates was facilitated, in the case of extensive limb trauma, when the distal part of the limb was amputated. Amputations were performed at the mid radius-ulna and mid tibia-fibula. Since the soft limb tissues retract slightly after amputation, the protruding limb cartilages were trimmed to the level of the surrounding tissues so as to insure an even amputation surface.

THE OPERATIONS

S SERIES

In 28 animals, skin was removed from the dorsal surface of all forelimbs and hindlimbs. The skinned area was then used to gain access into the interior of the limb where the limb nerves were severed. Of the 112 cases, 32 limbs (SN) underwent no further injury and care was taken not to damage either the limb muscle or cartilage. In 60

limbs, the muscles were also severed, although the cartilage was not damaged. The distal portions of 20 of these limbs were amputated (SMN-AMP), while in the remaining 40 limbs (SMN), nothing further was done. In the last 20 limbs (SMCN), in addition to the removal of the skin and the severed nerves, the muscles of the posterior-dorsal and ventral regions of the limb were torn and the limb cartilages fractured. In all of the experimental groups mentioned above, the total number of cases was divided equally between forelimbs and hindlimbs. Thus, in any given group, the number of forelimbs always equalled the number of hindlimbs.

BCE SERIES

In both forelimbs and hindlimbs of 13 animals, the cartilage of the humerus or femur were fractured using blunt forceps without breaking the skin. From a total of 52 limbs, 20 were amputated through their distal parts (BC-AMP). The remaining 32 limbs (BC) received no further operation. Both groups contained equal numbers of fore and hindlimbs.

The cartilages in the limbs of an additional 32 animals were fractured, and skin was also removed from the dorsal part of the upper fore- and hindlimbs either at the time of the operation or at 5, 8, or 11 days later. Each of these experimental groups had 32 cases equally divided into fore- and hindlimbs for a total of 128 limbs (BCE, BC-5E, BC-8E, and BC-11E). In 5 additional animals, the cartilage was broken and the

skin removed at the same time; however, these 20 limbs were amputated through the forearm and foreleg (BCE-AMP).

In 23 animals, portions of the cartilaginous limb skeletons were removed. Thus, following the removal of the limb skin from 92 limbs, the proximal one-half of the humerus or femur was extirpated in 32 cases (BCE-Prox. C). The cartilage removed included the proximal articulating head. In 32 other cases (BCE-Mid. C), the mid-portion of the humerus or femur was extirpated, while in the 28 remaining cases (BCE-Dist. C), the distal one-half of the limb cartilage, including the distal articulating head, was removed.

In 9 animals, the skin was removed from the dorsal area of all the limbs and, with the aid of sharpened forceps, the cartilage of the humerus or femur was completely macerated to produce a cartilaginous, pulpy mass. In these 36 cases (MAS-CE), as in all cases in the BCE series, care was taken not to sever the limb nerves or damage the limb muscle. However, those cases involving an extirpation of either the proximal or distal articulating head could not have been accomplished without damaging some muscle, since these structures are also the sites of attachment of various limb muscles. Undoubtedly, these attachments were torn during the operation. All of the groups in this BCE series contained equal numbers of forelimbs and hindlimbs.

BCEN SERIES

All four limbs in 32 animals were used in this series for a total of 128 experimental limbs. In all cases, the cartilage of the humerus or femur was broken, and either at the time of fracture, or at 5, 8 or 11 days later, the skin on the dorsal surface in the area of fracture was removed and at the same time, the limb nerves were cut. Each experimental group was comprised of 32 limbs (BCEN, BC-5EN, BC-8EN, and BC-11EN), divided equally into forelimbs and hindlimbs.

BCEM SERIES

In 128 limbs, from 32 animals, the femur or humerus was fractured. At the time of the fracture, 5, 8, or 11 days later, the skin on the dorsal surface in the area of fracture was removed and the muscles of the posterior-dorsal and ventral areas of the limb torn apart. Care was taken not to sever any limb nerves. Each group consisted of 32 limbs (BCEM, BC-5EM, BC-8EM and BC-11EM) so that the numbers of fore- and hindlimbs in each case were equal.

DENERVATION SERIES

In this series, only the forelimbs of experimental animals were used because of the difficulty in completely denervating the hind limbs. The skin in the area of the posterior border of the scapula was torn slightly with sharpened forceps. The third, fourth, and fifth brachial

nerves are then located slightly caudad from the posterior border of the scapula and severed at the point where they begin to converge to enter the limb. After denervation, the humerus was fractured in all 76 forelimbs of the 38 animals. 20 limbs were maintained in this state of denervation without further trauma (BC-DEN). In 20 additional limbs (BCE-DEN), the skin from the dorsal surface of the forelimbs was removed after the cartilage had been fractured. Two remaining groups with 18 limbs each had the skin on the dorsal surface of the limb removed at 8 days and 11 days (BC-DEN-8E and BC-DEN-11E) after the denervation and cartilage fracture.

SEPARATION SERIES

This series is characterized by a separation of the humerus or femur from their distal articulating heads. This technique was applied to all four limbs in 52 animals for a total of 208 experimental limbs. In 28 cases (SEP-C), the limb cartilage was merely separated without damage to the skin or other tissues. In 60 cases, a piece of skin was removed from the dorsal surface of the limbs in the area of articulation between the upper and lower limb. In these cases the distal end of the cartilage came to protrude out of the limb. Care was taken not to damage any muscle or nerves. Of this group of 60 limbs, 32 were amputated through the forearm and forelegs (SEP-CE-AMP), while the remaining 28 were left unamputated (SEP-CE).

The operation described above, including the removal of skin and the separation of cartilage, was augmented in 60 cases by severing the muscles of the limb which had their attachments on the posterior border of the distal articulating head. 28 of the limbs in this experimental group were not amputated (SEP-CEM), while 32 were subjected to amputation through the forearm and forelegs (SEP-CEM-AMP).

In a final 60 limbs, in addition to the removal of skin and the separation of the cartilage, the limb nerves were severed in 28 cases (SEP-CEN) with no further tissue damage, and in 32 cases (SEP-C-MASDT) all the tissue on the dorsal half of the limb in the skinless area was macerated (muscle, nerves, blood vessels and connective tissue).

All groups in this experimental series contained equal numbers of fore- and hindlimbs.

IMPLANTATION SERIES

The forelimbs of 12 larger axolotls (10mm snout to tail) received an implant of either limb muscle or a piece of cartilaginous humerus. All implants were made into a subdermal pocket of the dorsal fore-arm. Cartilage was inserted in 12 forelimbs and muscle in 12 forelimbs. The implants were placed so that their distal edges were next to the proximal border of the insertion wound. After implantation, the animals were placed in the cold for one hour to discourage movement and allow

wound healing to occur.

HISTOLOGY

For the present study, a separate series of experimental animals provided limbs for histological examination. Limbs were taken at intervals of 3 days, preserved in Bouin's fluid, and sectioned at 10 microns parallel to the long axis. Sections were stained with hematoxylin and eosin for general histological features.

Table 1.--The operations performed and the tissues which were damaged.

OPERATIONS	TISSUES DAMAGED			
	skin	muscle	nerves	cartilage
S Series				
SN	X		X	
SMN	X	X	X	
SMN-AMP	X	X	X	
SMCN	X	X	X	X
BCE Series				
BC				X
BC-AMP				X
BCE	X			X
BC-5E	X			X
BC-8E	X			X
BC-11E	X			X
BCE-AMP	X			X
BCE-Prox.C	X	X		X
BCE-Mid.C	X			X
BCE-Dist.C	X	X		X
MAS-CE	X			X
BCEN Series				
BCEN	X		X	X
BC-5EN	X		X	X
BC-8EN	X		X	X
BC-11EN	X		X	X
BCEM Series				
BCEM	X	X		X
BC-5EM	X	X		X
BC-8EM	X	X		X
BC-11EM	X	X		X
DEN Series				
BC-DEN			X	X
BCE-DEN	X		X	X
BC-DEN-8E	X		X	X
BC-DEN-11E	X		X	X
SEP Series				
SEP-C				X
SEP-CE	X			X
SEP-CE-AMP	X			X
SEP-CEM	X	X		X
SEP-CEM-AMP	X	X		X
SEP-CEN	X		X	X
SEP-C-MAS-DT	X	X	X	X
Implant Series				
Cart. Imp.	X			
Muscle Imp.	X			

RESULTS

S. Series

No morphogenetic response was observed in the SN group as a result of skinning on the dorsal surface and local denervation. These 32 limbs were observed for 35 days, after which the experiment was repeated. Even in those animals which underwent the operation twice, no morphogenetic activity was observed. Healing of the skinned area was rapid, and resulted in a wound epithelium several cell layers thick. This epithelium was replaced in about 7-10 days by normal limb skin.

The SMN group, on the other hand, showed a slight morphogenetic response . (See table 2). After skinning and severing the nerves in 40 limbs, the muscles of the posterior-dorsal and ventral portion of the upper fore-and hind limbs were also severed. The torn muscles contract and are enveloped by the rapidly growing wound epithelium. By 2 days, the muscle ends are contracted into compact masses at the proximal and distal borders of the damaged area and present the appearance of 2 small wounds covered by a thick wound epithelium, not unlike a very early regeneration blastema. The distal parts of all the limbs did not respond to sensory stimuli while the limb parts proximal to the wounded area remained sensitive. The limbs eventually regained their sensitivity and ability to move. The blastema-like mounds at either end of the wounded region remained from 6 to 10 days and gradually disappeared. One

limb, the right front of SMN # 6, however, continued to show the development of a small bud on the pre-axial surface of the humerus. The bud turned laterally and eventually developed three digit-like outgrowths. This limb was fixed at 50 days post-operation for histological examination.

In addition, 20 limbs operated on in the same manner as the SMN group had their distal tips amputated through the mid radio-ulnar and tibia-fibular cartilages (SMN-AMP). Instead of normal wound healing at the amputation surfaces, however, 8 of the original 10 forelimbs underwent a disintegration process characterized by a loss of all the limb tissue from the level of the wound distally to the amputation surface. Beginning on the second day after amputation, the skin was lost, followed by the other soft limb tissues until only the cartilage remained. By the eighth day, the protruding cartilage had disintegrated up to the level of the wound on the upper arm. Cartilage protruded in some limbs as long as 13 days after amputation. In limbs unaffected by this process, normal healing occurred, and by 8 to 11 days, a blastema had formed at the amputation surface. The wound site on the upper limb was characterized in these limbs by small epithelial aggregations. One experimental animal died at 17 days, bringing the total of limbs lost to 10. At 25 days, all surviving limbs were in the process of regenerating, but showed no activity at the proximal wound site.

In 20 limbs of the SMCN group, the skinning and local denervation was followed by a severing of the posterior-dorsal and a fracture of the cartilage. The distal limb was then connected to the proximal limb at the wound site by only skin and the muscles of the pre-axial humerus or femur. The trauma proved too great in 2 cases, as the parts of these 2 limbs distal to the wound site were lost. Healing of the wound occurred within a day, supported by a wound epithelium which thickened and assumed the appearance of a small mound by 10 days. From 10 to 20 days, the small mounds gave the appearance of growing in size, similar to a regeneration blastema. The similarity to blastemata lasted until around the 24th day when the blastema-like mounds began to recede and diminish in size. By 30 days, the mounds had almost disappeared. One forelimb, however, produced a digit-like structure on the pre-axial surface of the humerus. The accessory structure began, interestingly enough, at the point where the original limb cartilage had been fractured. This limb was fixed 38 days for histological examination.

BCE SERIES

Using blunt forceps, the humerus and femur in 32 limbs were fractured without breaking the skin. Care was taken to avoid as much internal damage as possible. This control group (BC) was included to determine if damage to the cartilage alone can induce accessory limbs. None developed,

but injury to the cartilage was noted to have some effect on the surrounding tissues. A marked thickening of the epidermis was noted over the fracture site from 6 to 12 days after the operation. This thickening lasted for about 20 to 25 days after which it decreased and disappeared. From 8 to 11 days, pigment cells were observed to accumulate in the area over the fracture. At about this same time, a slight hemorrhage appeared in some limbs at the fracture site, causing a reddening of the area. No dermal breakdown was noted at any time. The cartilage re-knitted rapidly, and while the majority of the forelimbs healed without leaving any visible trace of a cartilage fracture, the hind limbs on the other hand, almost always healed crookedly at the point of fracture (see figure 1a). This may well be one reason why, in this series, the majority of accessory structures was found on hindlimbs

After 20 additional limbs had been prepared by fracturing the cartilage, their distal tips were amputated (figure 1b). By 7 days, all the limbs had developed small blastemas at their amputation planes, and by 18 days, most of the limbs displayed regenerates with 2-4 digits. No accessory limbs were observed in this group (BC-AMP).

In the 32 limbs of the BCE group, after the humerus and femur were broken, a section of skin was removed from over the damaged area. Care was taken not to damage the musculature. The epithelium was replaced by the first day after operation, and at 8 to 10 days later on many

limbs a small mound appeared directly above the fracture site. Many of these mounds looked similar to a 10 day regeneration blastema. By 15-17 days, the majority of the mounds had begun to diminish in size, although several remained the same size and appeared unchanged. The mounds were most prevalent over fracture sites on those limbs which did not heal correctly. In these limbs, the fracture had divided the cartilage into proximal and distal segments which did not assume their correct orientation with each other upon healing. As a result, the cartilage segments fused at an angle. The mounds observed to remain the longest were those situated at the distal ends of the proximal cartilage segment. By 20 to 22 days, most of the mounds had either disappeared or were in the process of shrinking. Four limbs were observed, however, which still maintained mounds of blastema size. One of these mounds on a hindlimb, developed into a small digit by 26 days while all the other mounds disappeared with the exception of one. The one remaining mound was still present at 39 days on the posterior surface of an upper arm. In the course of the experiment, one animal died and one additional limb was lost. Thus out of 27 limbs, a small digit and a cartilaginous mound were observed.

It is perhaps significant that the mounds appeared at the fracture site and seemed to diminish or disappear with the healing of the cartilage. This may lend some support to the observation of Kazancev (1930) that the development of accessory formations depend upon the separation of cartilage.

The result of other experiments performed as a part of this study also tend to agree with this observation. These will be noted when they are discussed.

The upper limb cartilages of 96 limbs were fractured at the same time. These limbs were then divided into 3 groups of 32 limbs apiece. From the first group, BC-5E, the skin over the fracture site was removed 5 days after the cartilage had been broken. In the second and third groups, BC-8E and BC-11E, the limb skin was removed 8 days and 11 days respectively after the time of fracture. The limbs in none of these groups formed any accessory structures. However, to a certain degree, they all showed the formation and eventual disappearance of mounds.

The BC-5E group displayed blastema-like mounds at 12 days after fracture and 7 days after removal of the skin. These lasted up to 20 days after skinning,at which time they began to disappear.

The BC-8E group displayed mounds at 12 days after fracture and 4 days after skinning which lasted until around 13 days after skinning (21 days after fracture).

Limbs from the BC-11E group showed the formation of only a few mounds which were small in size and which disappeared by 9 to 10 days after skinning (20-21 days after fracture).

It is interesting to note that the incidence of mounds was highest in the BCE groups and lowest in the BC-11E group. The mounds first appeared at 10-12 days after fracturing in all groups, yet their times of

appearance from the time of skinning all differ.

20 limbs were experimentally altered using the method described for the BCE group. In addition, the limbs had their distal parts amputated in the manner previously discussed. This group of limbs (BCE-AMP) showed normal regeneration from the amputation surface with blastemas developing at 7-10 days after amputation and digits appearing in one case as early as 15 days. Mounds were observed to develop at the fracture site in 7 limbs at about 10 days. By 18 days, only one mound remained which developed gradually into a digit. The rest had either disappeared, leaving an area of thickened epidermis, or were in the process of diminishing. Of 20 limbs, then, only one showed induction of an accessory structure.

The BCE-AMP group did not show an increase in incidence of accessory structures over the BCE group, although it might be expected to occur (Della Valle, 1913; Kazancev, 1930; Nassonov, 1930). It should be pointed out that the 2 groups contain different numbers of experimental cases, so that no real comparison can be made on this basis.

In 92 limbs, portions of the cartilaginous limb skeleton were removed. After a section of skin was removed from the dorsal surface of the upper limb, the proximal one-half of the humerus or femur including the proximal articulating head was extirpated in 32 cases (BCE-Prox. C). In 32 other cases, the mid-portion of the humerus or femur was removed (BCE-Mid. C) while in the remaining 28 cases, the distal one-half of the

limb cartilage, including the distal articulating head, was removed (BCE-Dist. C). In all cases, the removal of the cartilage resulted in a drastic shortening of the upper parts of the limbs. After 40 days, no accessory limbs developed. There was also a complete lack of mound formation. The only effect noted, other than the reduction in limb length, was the formation of a thick wound epidermis at the wound site.

These observations are at odds with the report by Studitsky (1948) of a case of accessory limb induction after removal of the proximal one-half of the humerus in the axolotl. The difference may lie in technique, however, since in his experiment, Studitsky not only removed the proximal portion of the humerus, but also part of shoulder girdle. As a result, the accessory structure developed from the proximal end of the segment of humerus remaining in the limb, apparently before a cartilaginous matrix could develop between the regenerating shoulder girdle and the end of the remaining humoral segment. In addition, he does not state his method of surface wounding. One can only conclude, then, that more study is needed.

In the last group of the BCE series, after a section of skin was removed from 36 limbs, the humerus or femur was macerated to a pulp. Care was taken to keep damage to the muscles and nerves to a minimum. This group (MAS-CE) showed the formation of mounds in 2 cases at 12 days. By 26 days, however, one had disappeared and the other was still present as a small mound after 50 days.

BCEN Series

The operation previously described for the BCE group was performed on 32 limbs. At the same time, however, the limb nerves were severed. Mounds began appearing in this group (BCEN) around 10-12 days. By 15 days, some of the mounds assumed blastema proportions and by 20 days, they appeared simular to cone-stage blastemas. Between 20 and 28 days, though, the majority of the mounds began to diminish and only 3 remained at 28 days. These all developed on hindlimbs into digit-like protrusions. In this group, one animal died and one additional limb was lost. As a result, out of 27 cases, 3 showed induction of accessory limb parts.

In 96 limbs, the cartilage was fractured without damaging the soft tissues. In the first group of 32 limbs (BC-5EN) skin was removed and the limb nerves severed after the cartilage had been broken. The same operation was performed 8 and 11 days after fracture in 2 consecutive groups of 32 limbs (BC-8EN and BC-11EN). No accessory formations were observed in these cases. The incidence of mound formation in the BC-5EN group was lower than the BCEN group and lower still in the BC-8EN and BC-11EN groups. The most typical wound site formation was a thickened epidermis.

BCEM Series

The 32 limbs comprising the BCEM groups were fractured and skin was

removed in the manner previously described. The muscles of the posterior-dorsal and ventral limb were then carefully macerated in order to avoid damaging the limb nerves. When severed, the muscles contract to either end of the damaged area and soon are covered by the rapidly growing wound epithelium. These compact masses of musculature resembled mounds except that they disappeared in 6-10 days. After this, mounds with their characteristic blastema-like shape begin to appear. In this group, the mounds remained for about 16 to 18 days and then gradually began to disappear. By 22 days, only 3 structures remained, two of which developed into digit-like structures while the third remained a mound. All of these structures developed at the site of fracture on hindlimbs. During the course of the experiment, 2 limbs were lost as a result of extensive tissue damage, thus out of 30 limbs, 3 cases of induction were observed.

In 96 additional animals, the cartilages were broken. The skin was then removed and the limb muscles torn at intervals of 5, 8, and 11 days after the cartilage fracture. Each of these groups, BC-5EM, BC-8EM, and BC-11EM respectively, contained 32 limbs.

Of the BC-5EM group, 2 animals died, leaving 24 experimental limbs. The mounds appeared from about 10-14 days after skinning and remained until about 18 days at which time they began to diminish. At 25 days after skinning and 30 days after fracture, 2 mounds remained. These appeared

almost like digits, but at 30 days after skinning they both began to shrink and were soon lost.

The BC-8EM group showed a low induction of mounds. These mounds were initiated from 7 to 10 days and by 18 days they were gone.

The BC-11EM group, on the other hand, developed some mounds from about 9 to 12 days after skinning (20-23 days after fracture). Several had the appearance at 15 days after skinning of good regeneration blastemas. By 20 days after skinning and 31 days after fracture, however, these also disappeared.

Denervation Series

For this series, only forelimbs were used. After denervation at the level of the scapula, the humerus was fractured in 76 limbs. One group of 20 limbs, BC-DEN, was maintained in this condition. A second group of 20 limbs, BCE-DEN, had a section of skin removed from the area over the fracture. The two remaining groups, BC-DEN-8E and BC-DEN-11E, had skin removed from their limbs 8 and 11 days after the denervation was performed.

These groups were observed for 40 days and none showed signs of accessory limb induction. Regression occurred in some of the limbs, although it was not extensive.

Separation Series

This series involved separating the shaft of the humerus, or femur, from the distal articulating head. The effect achieved is similar to a cartilage fracture although no cartilage is actually broken. In addition, this method permits a longer separation of cartilage and apparently as a result, better induction.

A group of 20 limbs served as a control. The humerus, or femur, of each of these limbs was separated from the distal articulating head. This can be accomplished without breaking the skin and with very little damage to the other tissues of the limb. This group, SEP-C, was observed for 40 days, during which no accessory limb induction was noted.

After separation of the cartilage, the distal ends of the humerus or femur protruded into the dermis of the skin, but did not penetrate (see figure 1c). A cap of thickened epidermis formed on the skin at the point where the cartilage protruded. This was maintained as long as the cartilage remained in that position. It is interesting to note that the hindlimbs more readily responded to cartilage separation than the forelimbs. When the humerus was separated from the distal articulating head, there was a tendency for the entire forelimb to straighten out and the cartilages to reunite. On the other hand, the separated cartilages of the hindlimbs tend to remain separate. It is not suprising, then,that the majority of structures induced in this series was found on the hindlimbs.

After the cartilage had been separated in 60 limbs, skin was removed from their dorsal surfaces in the area of articulation between the upper and lower limb. As a result, the distal end of the cartilage came to protrude out of the limb. Of those 60 limbs, 28 underwent no further operation (SEP-CE). The remaining 32, (SEP-CE-AMP) underwent amputation through the forearm.

The exposed cartilage of the SEP-CE and SEP-CE-AMP group began to disintegrate immediately. The action was more pronounced in the hindlimbs than in the forelimbs and,depending on the amount of cartilage exposed, lasted from 4 to 18 or 20 days. While the cartilage is in the process of breaking down, the wound epithelium covers the exposed tissues and surrounds the base of the projecting cartilage. If the cartilage remains exposed for any length of time, the wound epithelium masses about its base and continues to multiply. Eventually, when enough cartilage has disintegrated, the epithelium can grow over the exposed end. As a result, the distal ends of the cartilage are in intimate contact with a wound epithelium in a physical relationship which could be termed a "pseudo-amputation plane" (Figure 1c).

Many limbs in the SEP-CE group at 16 days showed an epithelial mound situated at the distal end of the cartilage. These mounds appeared to be composed of mesenchymal cells, resulting possibly from the break down of cartilage. By 20-22 days, the majority of the mounds had disappeared,

but 2 remained. These developed into digit-like structures projecting from a forelimb and a hind limb. Thus, out of 28 limbs, 2 showed evidence of induction.

The limbs of the SEP-CE-AMP group at 14 days showed 21 "blastema-like" accessory mounds on 32 limbs. Of these, only 6 were located on forelimbs. Also at 14 days, the amputated forelimb stumps showed good 2-digit stages. At 18 days, only 7 accessory mounds remained. Of these, 2 disappeared later. At 35 days, one forelimb showed a small digit developing from the point where the cartilage had protruded. The other 4 were situated on hindlimbs and also were developing from the point where the cartilage had protruded. One of these remained only as a mound. The other three were in the shape of a small forearm with a single digit. Thus, out of 32 limbs, 5 showed evidence of accessory limb induction.

It is sifnificant that in the SEP-CE-AMP group, only those accessory mounds which had been large blastemata at 14 days survived to the 18th day. The 18 day forearm regenerates generally displayed the form of a complete limb. Perhaps, then, if an accessory is to develop it must do so before the normal regenerate has completed its morphogenesis. Nassonov (1936c) points out the necessity of an accessory structure developing before the wound heals or the fractured skeleton reknits. In the case of the SEP-CE group, it was found that if the cartilage remained exposed for too long, or for a very short time, induction did not occur.

The operation described for the SEP-CE group was carried out in 60 additional limbs. However, the trauma was augmented by severing the muscles which had their attachments on the posterior border of the distal articulating head. Of the 60 limbs, 32 underwent amputation of the forearm (SEP-CEM-AMP) while the remaining 28 were left unamputated (SEP-CEM).

In the SEP-CEM experimental group, the number of mounds which developed by 16 days was smaller than the number found with the SEP-CE group. They are also less well developed. By 22 days, only 2 small accessory mounds remained and these developed into small digit-like structures both situated on forelimbs. In this group, the protruding cartilage tended to be more pronounced in the hindlimbs. As a result, the exposure time was longer, leaving less time for the development of accessory mounds in the hindlimbs. During the experiment, one animal died, and 3 other limbs were lost. As a result, of 21 experimental limbs, 2 showed induction of accessory digits.

Accessory mounds began developing on the limbs of the SEP-CEM-AMP group at about 11 days. At 14 days, 21 small accessory mounds were located on the 32 limbs of the group. Of these, 7 were located on forelimbs. At 18 days, a 3-4 digit regenerate had developed on the amputated forelimb stump, and only 3 accessory mounds remained, all on hindlimbs. By 35 days,

one accessory mound remained , one developed into a long digit, and the third developed a forearm with 2 digits.

It is difficult to draw a conclusion concerning the effects of amputation on this group because of the large difference in experimental numbers between it and the SEP-CEM group.

In 28 limbs, in addition to the separation of cartilage and removal of skin, the limb nerves were severed (SEP-CEN). Cartilage protruded in some animals until the 15th day. By 18 days, only 3 limbs gave evidence of possible accessory limbs. These were all located on hindlimbs, at 25 days, and were represented by long spikes. By 42 days, one structure had diminished in size to a small digit-like structure, but the remaining 2 supernumeraries had differentiated into limbs, one with 3 digits, the other with only one. In the course of the experiment, two forelimbs were lost. Thus, from a total of 26 experimental limbs, 3 cases of accessory limbs induction were observed.

The final group of 32 limbs in the separation series was that in which all the tissues of the dorsal half of the limb had been macerated, after the cartilage had been separated (SEP-C-MAS-DT). Cartilage protruded from these limbs until 13-15 days after the operation. By 18 days, only 3 accessory mounds were observed and 2 of these disappeared by the 25th day. The one remaining mound was large and situated on a hindlimb. However, by 35 days, this had diminished to only a small mound.

Implantation Series

Both forearms of 12 large axolotls received implants of either limb muscle or cartilage from humerus. The implants were placed so that their distal edges were next to the distal insertion wound. Of the 12 cases of muscle implants, no activity was observed after 40 days, by which time, most of the implants had disappeared. In the 12 cases of cartilage implantation, one accessory digit was obtained. By 4 days after implantation, all the insertion wounds had been obscured by accumulations of epithelial cells. In some cases, the epithelial thickening extended over the whole area of the implant. By 9 days, the accumulations had decreased slightly except in one case, which showed an increase in the epithelial mass at the wound site. At 17 days after implantation, a prominent epidermal lobe extending distally from the insertion wound was visible. By 25 days, the lobe had increased in size and had turned in a vertical direction. By 35 days, it had the appearance of a digit extending from the mid-forearm.

The data collected in the present study was subjected to a chi-square analysis and is presented in Table 3. The implant series of limbs was not included in the analysis because of the small number of experimental cases.

The chi-square method of analysis will indicate within the limits of the experiment whether or not the experimental results can be attributed to random chance.

As Table 3 indicates, the X^2 value obtained was 23.344. However, the probability of the X^2 value being equal to or greater than 23.344 when the df=5, is less than .001. Thus, it can be concluded that considerable influence is present, other than chance, which is responsible for the experimental results. Further, by comparing the observed and expected responses in each of the experimental series, it can be shown that certain series are more influencial than others in determining the overall X^2 value. Table 4 illustrates that the BCE and the SEP series have the most influence on the appearance of accessory limb parts. The BCE series exerts a negative influence while the SEP series exerts a quite strong positive influence. The significance of this will be discussed later.

Chi-square analysis can also demonstrate that there is a significant difference between the contribution of the forelimbs and hindlimbs to accessory limb production. The contribution of the fore- and hindlimbs to the production of accessory limb parts is summarized in Tables 5 and 7, while the significance of their contribution is analyzed by the chi-square method in Table 6. It is quite apparent that in this study the hindlimbs have a much greater tendancy to form accessory limb parts than the forelimbs.

Table 2.-- Summary of experimental results.

OPERATIONS	Total no. of limbs	No. of limbs lost	No. of surviving cases	No. of accessory structures	Type of structure: mound	digit	fore-limb
S Series							
SN	32	-	32	-			
SMN	40	-	40	1			1
SMN-AMP	20	10	10	-			
SMNC	20	6	14	1		1	
BCE Series							
BC	32	-	32	-			
BC-AMP	20	-	20	-			
BCE	32	5	27	2	1	1	
BC-5E	32	-	32	-			
BC-8E	32	-	32	-			
BC-11E	32	-	32	-			
BCE-AMP	20	-	20	1		1	
BCE-Prox.C	32	-	32	-			
BCE-Mid.C	32	-	32	-			
BCE-Dist.C	28	-	28	-			
MAS-CE	36	-	36	1	1		
BCEN Series							
BCEN	32	5	27	3		3	
BC-5EN	32	-	32	-			
BC-8EN	32	-	32	-			
BC-11EN	32	4	28	-			
BCEM Series							
BCEM	32	2	30	3	1	2	
BC-5EM	32	8	24	-			
BC-8EM	32	-	32	-			
BC-11EM	32	-	32	-			
DEN Series							
BC-DEN	20	-	20	-			
BCE-DEN	20	5	15	-			
BC-DEN-8E	18	2	16	-			
BC-DEN-11E	18	4	14	-			
SEP Series							
SEP-C	28	-	28	-			
SEP-CE	28	-	28	2		2	
SEP-CE-AMP	32	-	32	5	1	1	3
SEP-CEM	28	7	21	2		2	
SEP-CEM-AMP	32	-	32	3	1	1	1
SEP-CEN	28	4	24	3		1	2
SEP-C-MAS-DT	32	-	32	1	1		
Implant Series							
Cart. Imp.	12	-	12	1		1	
Muscle Imp.	12	-	12	-			
TOTALS -	1004	62	942	29	6	16	7

Table 3.-- Chi-square analysis of the appearance of accessory limb parts in the axolotl as the result of selective tissue damage.

Experimental Series	Positive Responses	Negative Responses	Total No. of Cases
S	2	94	96
BCE	4	319	323
BCEN	3	116	119
BCEM	3	115	118
SEP	16	181	197
DEN	0	65	65
TOTALS -	28	890	918

$$X^2 = \frac{(O-E)^2}{E} \quad , \quad X^2 = 23.34 \quad , \quad df = 5 \quad , \quad P(X^2 \geq 23.34) \ll .001$$

* Note that the implant series of limbs was omitted due to lack of data.

Table 4.-- A comparison of accessory development in the forelimbs and hindlimbs of the axolotl.

	Mound	Digit	Limb	TOTAL
Forelimbs	1	6	1	8
Hindlimbs	5	10	6	21
TOTAL	6	16	7	29

* Note that the Implant series of experimental limbs is included in the above table.

Table 5.-- Chi-square analysis of the contributions of the forelimbs and hindlimbs to the production of accessory parts.

	Positive Responses		Negative Responses		Total No. of cases
	Observed	(O-E)	Observed	(O-E)	
Forelimbs	7	-7.945	483	+7.949	490
Hindlimbs	21	+7.946	407	-7.942	428
	28		890		918

$X^2 = 9.345$, df = 1 , $P(X^2 \geq 9.345) < .01$ but $> .001$

* Note that the forelimbs of the Implant series are not included in the above analysis.

Table 6.-- Percentages of induction of accessory limb structures.

Operations	No. of Accessory Structures	No. of Surviving Cases	Percent of Surviving Cases	% of Fore-Limbs	% of Hind-Limbs
SMN	1	40	2.5	5.0	-
SMCN	1	14	7.1	14.3	-
BCE	2	27	7.4	7.1	7.1
BCE-AMP	1	20	5.0	-	10.0
MAS-CE	1	36	2.8	-	5.6
BCEN	3	27	11.1	-	23.1
BCEM	3	30	10.0	-	21.4
SEP-CE	2	28	7.1	7.1	7.1
SEP-CE-AMP	5	32	15.6	6.3	25.0
SEP-CEM	2	21	9.1	20.0	-
SEP-CEM-AMP	3	32	9.4	-	18.8
SEP-CEN	3	24	11.5	-	25.0
SEP-C-MAS-DT	1	32	3.1	-	6.3
Cart. Imp.	1	12	8.3	8.3	-
TOTAL -	29	375			

DISCUSSION

In the axolotl, accessory limbs may develop as the result of damage to the tissues of the limb. Evidence obtained in this investigation suggests that tissue injury and cartilage may play important roles in this process.

The phagocytic and histolytic activities of the wound epidermis in amphibian limb regeneration (see review by Singer and Salpeter, 1961) as well as its ability to influence the aggregation of mesenchymatous cells to form the regeneration blastema (Thornton, 1960, 1965; Steen and Thornton, 1963; and Thornton and Thornton, 1965) have been well established. For the present study, histological and experimental evidence indicates that if an accessory limb is to arise as the result of injury to the limb tissues, a wound must be present. In those cases where limbs were damaged internally, without injury to the skin, no accessory development was observed. However, in similar operations in which skin was also damaged, accessory limbs were produced. Furthermore, if the wound is not made at the same time that the cartilage is injured, accessory induction will not take place. The evidence further suggests that in this investigation, the production of an accessory limb may be dependent upon the presence of injured or broken cartilage. Out of a total of 29 cases of accessory induction, 28 cases resulted from experiments involving injury to the cartilage. However, as the statistical analysis demonstrates, in this

study, the production of accessory limb parts is not as dependent upon cartilage damage alone as it is dependent upon the type of damage. The BCE series shows a tendency to form fewer accessory limb parts than would be expected by chance. Whereas the SEP series shows a remarkable tendency to form more accessory structures than would be expected. Thus, it is apparent that breaking the cartilage is not enough to produce accessory morphogenesis. Indeed, the appearance of accessory structures seems to be slightly inhibited when cartilage is fractured. On the other hand, a separation of the cartilage produces a significant increase in the number of accessory parts which would normally be expected. Similar evidence was obtained by Nassonov (1930, 1936c) and Kazancev (1930) showing that cartilage must be separated, after having been fractured, if accessory limbs are to appear. Nassonov (1935, 1936a), Kuzmina (1940), and Fedotov (1946) further stress the importance of injured or broken cartilage in the production of accessory limb parts.

Accessory induction appeared to be related to the formation of a "blastema-like" mound at the site of injury on a limb. These mounds are characteristically composed of a thick wound epidermis and a core of mesenchymatous cells. In those cases which involved a separation of the cartilage, mounds appeared directly over the exposed end of the cartilage. In cases of cartilage fracture, the mounds arose over the fractured area. Mounds showed 3 different types of behavior (1). The most typical behavior was gradual disappearance by 12 to 14 days after

their appearance. (2). In a few limbs, the mounds remained as such and developed a cartilaginous core. (3). Lastly, they developed into typical regenerates-the accessory limb.

It may be significant that accessory structures showed consistent development from specific points on the limb. When cartilage was fractured, the two broken ends would frequently heal asymmetrically (see Figure 1a). This was true more often in the hindlimbs than in the forelimbs, where re-fusion of cartilage ends quickly occurred. Mounds would form over the injured site and if the limb straightened and cartilage re-fused end to end, the mounds would disappear in most cases. However, when accessory development was observed in limbs with fractured cartilage, it always occurred at the bend in the limb where asymmetric re-fusion took place. In those experiments involving the seperation of cartilage, accessory development consistently appeared at the point where the cartilage protruded from the limb. This point (see fig. 1c) on the limb is somewhat analogous to an amputation surface, since a shaft of cartilage is present which is covered on one end by a wound epidermis and there are no distal structures in line with the proximal stump. Although the original distal structures of the limb are still present, they are removed from the point of cartilage protrusion and, indeed, at right angle to it. In these cases, the forelimbs once more had a tendency to straighten out while the hindlimbs tended to remain as they were. In limbs which had straightened, no accessory develop-

ment was observed. For this reason, hindlimbs tended to show more accessory structures than the forelimbs (see Table 5, p. 54). In addition, statistical analysis of accessory formation on forelimbs and hindlimbs (see Table 6, p. 54) indicates that the forelimbs exert a significantly negative influence on accessory formation while the hindlimbs exert an influence which is significantly positive. Part of this influence undoubtedly lies in the tendency for the forelimbs to straighten while the hindlimbs tend to remain asymmetric.

These results may be interpreted in light of Rose's work on the polarized inhibitory control of regeneration (Rose, 1952, 1957, 1962, 1964). In Tubularia, Rose (1957) found that if pieces of the stem were too small to form a complete hydranth, they would, upon isolation, form the distal part of the hydranth instead. In Lineus, Tucker (1959) found that supernatant fractions of centrifuged hemogerates of heads or of any anterior region would prevent head regeneration from occurring in regions lying posterior to the level from which the supernatant was obtained. They would not, however, prevent regeneration from regions anterior to the donor level. Rose (1962) suggested that in these cases, there is an inhibition of distal parts by already existing distal parts. The accessory limbs induced in the present experiments all developed from a distal limb point, analogous to an amputation surface, due to the deflection of the broken and separated distal segments of limb skeleton. Thus, in these cases there were no formed limb structures directly distal to the proximal limb segment. Rose (1962) emphasises that

polarized control operates in straight lines and thus, according to his theory, the duplicated distal limb segment could not influence the proximal stump.

Della Valle (1913), Nassonov (1930), and Kazancev (1930) have reported similar results. Their application of a ligature to a limb resulted in a loss of structure of the internal tissues at the level of application. Accordingly, the limb became bent at this point and was dragged along by the animal. Gradually, accessory blastemata formed on the proximal as well as distal sides of the ligature. Although these authors did not point this out, it should be noted that there is no direct connection between proximal and distal limb parts and thus no inhibition. However, as Kazancev (1930) has noted, if the ligature is removed during the early formation of the accessory limbs, and the cartilage allowed to heal, development of the accessory stops and occasionally regression occurs. By repeatedly bending the limb, Nassonov (1930) showed that accessory development could be produced if the cartilage is not allowed to heal. Again, however, repeated bending causes a disruption of the continuity of the cartilage and a loss of connection to distal parts.

The evidence obtained in the present study suggests that accessory structures can be formed if the cartilage is prevented from healing. In the cases involving cartilage fracture, there was a negative influence on the formation of accessory parts. From an investigation of histologically

prepared slides, the impression was gained that the low induction of accessory parts was due to the cartilage healing. However, in those series involving a separation of the cartilage, it appeared that the resulting disunion of cartilage segments prevented their healing and thus allowed for the possibility of more accessory formations. Further research is required, however, before a definate relationship can be provided.

Tornier (1898) fractured the scapula of R. esculenta and the resulting contraction of the muscles connected to both halves caused the scapula to split. Two amputation surfaces were thus formed from which there was corresponding regeneration.

If the elbow region of the limb is irradiated with ultraviolet light, supernumerary limbs can result. Irradiation causes a loss of skin and an immense amount of regression. As a result, Butler and Blum (1955, 1963) noted that limbs became bent and misshapen. Interestingly enough, the accessory limbs were produced from the elbow region which during regression became abnormally bent. If the mid fore-arm or upper arm is irradiated, extreme regression occurs, but the limbs never lose their morphological pattern. In these cases, no supernumeraries resulted.

Thornton (1953) observed that if a limb is denervated and its cartilage fractured, regression results. If maintained in a denervated condition until the distal structures are lost, re-innervation results in complete regeneration, without amputation. However, if the distal

elements are only partially regressed and still present on the limb when reinnervation occurs, regeneration fails. Only the repair of tissues takes place. It would seem that in this case, the distal structures which were present, even though abnormal, influenced the limb and prevented regeneration.

The character of the accessory structures obtained in the present study appeared to obey Faber's (1960) rule of distal transformation. In general, digits were more often formed than any other structure (see Table 5, page 54). The amount of material available for the development of an accessory structure may have been a limiting factor in accessory limb morphogenesis. Thus, according to Faber (1965), when the amount of material available to a blastema is small, the blastema will tend to form only the most distal skeletal elements of the limb, principally digits. If more material is made available,(de Both, 1965) more proximal skeletal elements are formed.

In conclusion, therefore, it would seem that a mechanical trauma which injures limb skin and cartilage, bringing about a separation in the latter, will in up to 25 percent of the cases (hindlimbs) induce accessory limb structures, provided that the distal limb segment is deflected. The broken end of the cartilage in the proximal limb segment in such cases, forms a distal point unencombered by a directly apposed distal limb segment. From this point, as though it were an amputation surface, a regenerate forms which develops into the accessory structure.

SUMMARY

The present study has demonstrated that accessory limb parts can be produced in the axolotl through manipulation of limb tissues by various operational techniques. In this investigation, the limb tissues which appeared to be the most influential in the production of accessory parts were the skin and cartilage. Skin must be injured to provide a wound epithelium otherwise induction will not occur. Injured cartilage appears to be necessary, since accessory parts arise at the site of cartilage damage, provided a wound epidermis is available. Analysis shows that limbs with separated cartilage form significantly more accessories than limbs with broken cartilage. In addition,more formations appear on the hindlimbs than the forelimbs. Muscle does not have an observable influence on accessory production. If nerves are cut, there is no perceptible increase in the induction of accessory parts, although the structures which do result seems to be better developed.

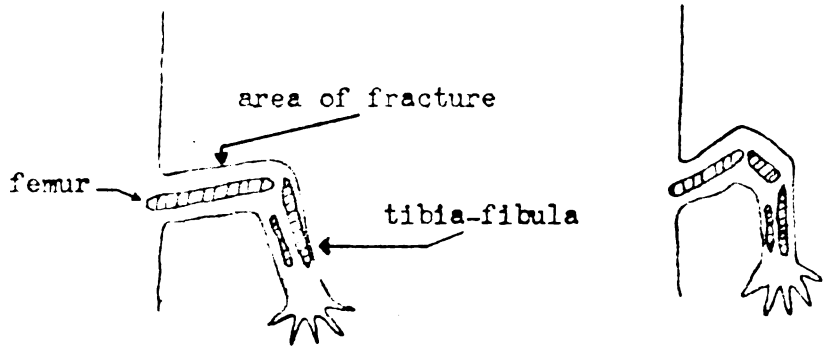
There appears to be a tendency for the inhibition of accessory development in the axolotl. Although the early stages of many of the experiments showed much "blastema-like" activity, the later stages showed practically none. A histological investigation failed to reveal the cause of the inhibition.

The accessories produced, in general, varied from small mounds of cartilage to complete limb duplication. The most frequent response, however, was the formation of digit-like structures.

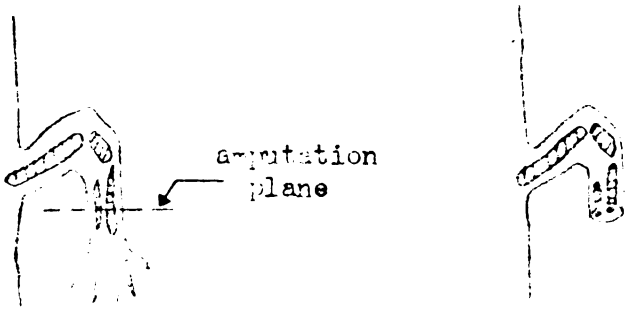
Figure 1a.-- Diagrammatic drawing of a limb showing the relationship of the cartilage before and after the cartilage has been fractured.

Figure 1b.-- Diagrammatic drawing of a limb in which the cartilage has been fractured prior to amputation through the midforearm or foreleg.

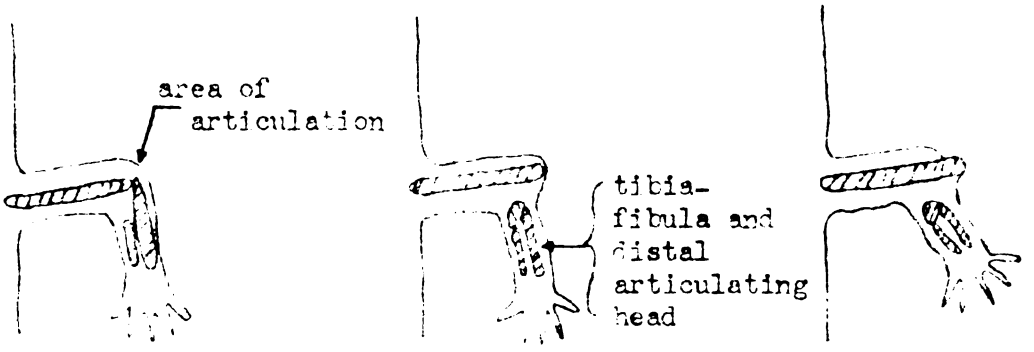
Figure 1c.-- Diagrammatic drawing of a limb before and after separation of the cartilage at the joint and illustrating the relationship of the distal end of the separated cartilage with the rest of the limb.



1a.) Before Fracture After Fracture



1b.) Pre-amputation Post-amputation



1c.) Before Separation at Joint After Separation Pseudo-amputation Surface

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