

A Staging System for Forelimb Regeneration in the Axolotl, *Ambystoma mexicanum*¹

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ABSTRACT A staging system has been devised for normal regeneration from the upper arm in the mature axolotl. It consists of seven externally definable stages: (1) Wound healing (WH); (2) Dedifferentiation (DD); (3) Early bud (EB); (4) Medium bud (MB); (5) Late bud (LB); (6) Palette (Pal), and (7) Digital outgrowth (DO). Serial histological sections of 38 regenerating limbs were used to correlate gross stages with microscopic events in the regenerative process.

For decades the axolotl (*Ambystoma mexicanum*) has been a favorite subject for research on limb regeneration, particularly experimental studies on morphogenesis. Despite the widespread use of this species in such studies, no generally used system of staging normal forelimb regeneration is available. Recent work in this laboratory involving both the descriptive (Grim and Carlson, '74) and experimental (Tank, unpublished) analysis of morphogenesis has underscored the need for a uniform system of staging normally regenerating axolotl forelimbs according to external morphology.

Two main criteria were followed in devising a staging system for normal limb regeneration from the upper arm (stylopodium) of the axolotl. (1) The stages must be readily recognizable by examination of external morphological features in the living animal, and (2) they should be as nearly equivalent as possible (both grossly and histologically) to already published staging systems for regeneration in the newt, *Notophthalmus* (= *Triturus*) *viridescens* (Singer, '52; Iten and Bryant, '73). One partial staging system for limb regeneration in young axolotls has been proposed by Faber ('59), but it covers only part of the outgrowth phase rather than the entire spectrum of the regenerative process.

MATERIALS AND METHODS

This study was carried out on 10-12 month old axolotls (*Ambystoma mexicanum*) raised from a single clutch of eggs from the standard strain maintained at the Hubrecht Laboratory. The animals ranged from 156-182 mm in total length and were sexually mature. After anesthetization in 1:1000 MS 222 (Sandoz) or 1:1000 ethyl m-aminobenzoate methanesulfonate (Eastman), both forearms were amputated with scissors through the distal third of the upper arm. After initial retraction of soft tissues, any protruding segments of the humerus were trimmed. Overlap of stump skin onto the fresh amputation surface was minimal except for one case (in which there was surprisingly little deviation from normal in the overall progress of regeneration). After the humerus was trimmed, the fresh amputation surfaces were flat, but in large axolotls further retraction of soft tissues during the first few days after amputation commonly causes a certain degree of relative protrusion of bone beneath the wound epidermis. Following limb amputation, the animals were placed in individual containers, maintained in the dark interior

¹Supported by NIH training grant 5-T01-GM 00312 (PWT), the Muscular Dystrophy Association (BMC) and a Rackham Grant and NIH-HD 08150 (TGC).

of a Freas "815" incubator at a constant temperature of 21°C and fed three times weekly with beef liver.

For the initial establishment of gross stages, both forelimbs of 15 animals were examined daily under anesthesia for 41 days and at irregular intervals from 44-83 days. Daily anesthetization of the animals did not affect the morphology of the regenerate, since it did not differ from that of scores of other animals, including littermates of these, which were only anesthetized once or twice. In fact, *Ambystoma* larvae kept under continuous anesthesia with MS 222 for up to 15 days developed normal gross limb morphology and muscle patterns (Carlson, '72). Detailed records and camera lucida drawings were made in conjunction with these observations.

In another series of 38 animals both limbs were amputated as before. At daily intervals up to 26 days and at two day intervals from 28-50 days, the regenerating limbs were photographed and then fixed in Bouin's for histological study. After fixation and decalcification with 5% HNO₃ in 70% ethanol, the limbs were serially sectioned at 7 μ and stained with Ehrlich's hematoxylin and eosin.

RESULTS

Seven readily distinguishable stages in

TABLE 1

Newt Iten and Bryant ('73)	Axolotl Present study	Axolotl Faber ('59)
WH (Wound healing)	WH (Wound healing)	—
EDD (Early dedifferentiation)	DD (Dedifferentiation)	—
LDD (Late dedifferentiation)	—	—
MEB (Moderate early bud)	EB (Early bud)	I
EB (Early bud)	—	—
MB (Medium bud)	MB (Medium bud)	II
LB (Late bud)	LB (Late bud)	III
Pal (Palette)	Pal (Palette)	IV, V
ED (Early digits — earliest)	—	—
ED (Early digits — late)	DO (Digital outgrowth)	—
MD (Medium digits)	—	—
LD (Late digits)	—	—

TABLE 2

Summary of key gross observations used in defining stages of regeneration

Stage	
WH	Begins with amputation; ends with rounding of the edges of the stump.
DD	From rounding of the edges of the stump until the first evidence of blastemal outgrowth.
EB	From first blastemal outgrowth to formation of a symmetrically contoured, cone-shaped regenerate.
MB	Starts with a symmetrical cone which curves markedly in a posterodorsal direction. Ends with the first indication of flattening of the cone.
LB	Begins with flattening of the dorsally curved cone and ends with the appearance of the first digital primordium.
Pal	The period beginning with the gross appearance of the first and ending with the appearance of the fourth digital primordium.
DO	The period from appearance of the fourth digital primordium to stabilization of the mature regenerate.

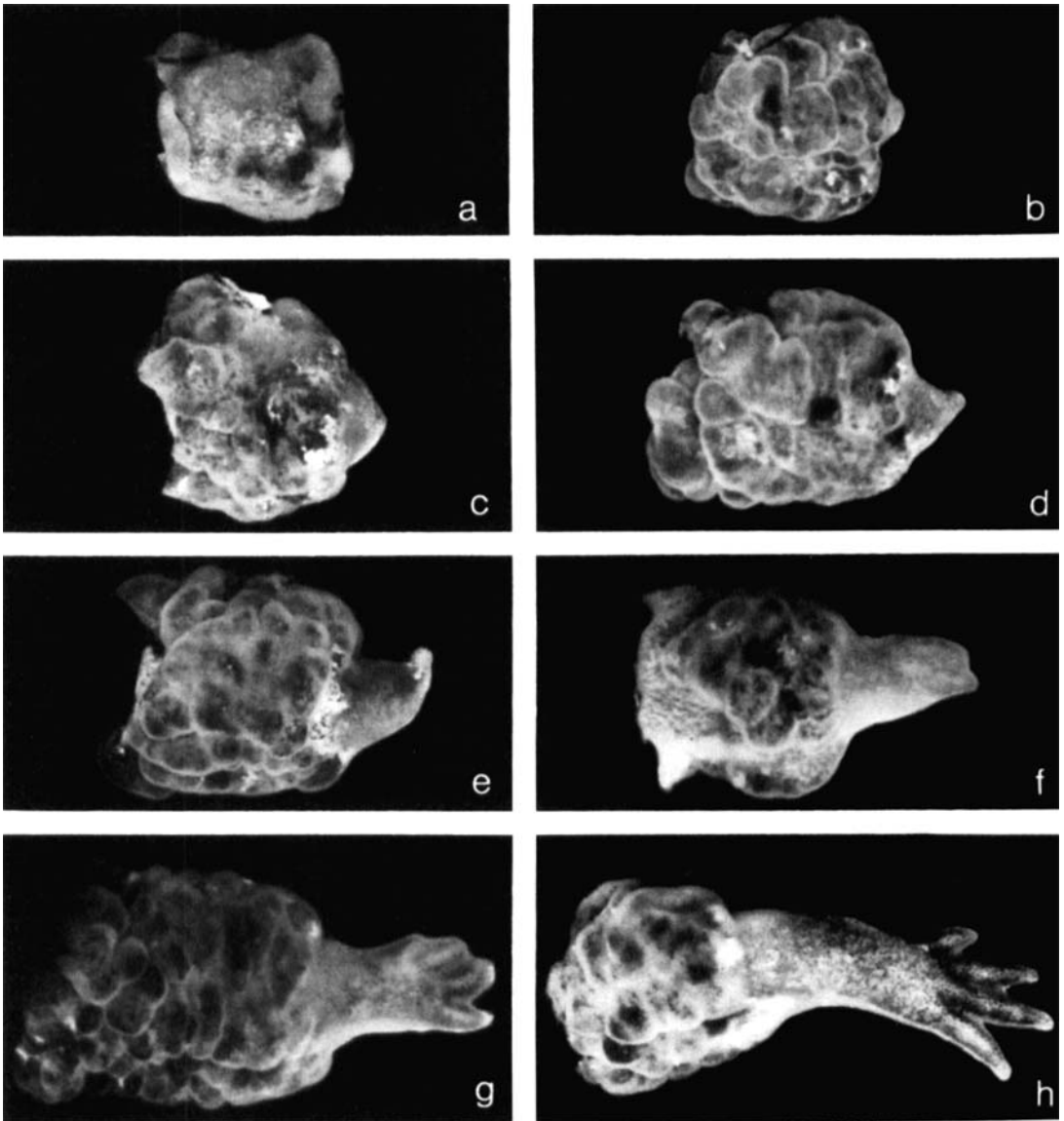


Fig. 1 Gross appearance of forelimb regenerates representative of the stages that have been defined. The orientation of all limbs is the same. All are left limbs, dorsal side is upward. $\times 6$. a. Wound healing — day 3. b. Dedifferentiation — day 8. The distal projection here is bone, not a blastema. c. Early bud — day 15. d. Medium bud — day 17. e. Late bud — day 20. Note the prominent apical epidermal lobe on the tip of the blastema. f. Palette — day 28. g. Digital outgrowth — day 32. h. Digital outgrowth — day 37.

the limb regeneration process were defined for the axolotl. Some incorporated more than one of the Iten and Bryant stages for the newt because during certain periods subtle differences that can be

detected in the newt are not clearly recognizable in the axolotl. A side by side comparison of our staging system with those of Iten and Bryant ('73) and Faber ('59) is presented in table 1. A summary of the key

external characteristics of the designated stages is presented in table 2.

Stage of wound healing (WH) —
0-5 days after amputation)

External appearance (fig. 1a). Within 24 hours the amputation surface is covered by a thin wound epidermis which remains quite transparent during this stage. Occasional protruding sharp edges of bone may not be covered by an epithelium for several days. Foci of deep hemorrhage and vascular stasis are frequently seen beneath the wound epidermis. After the first day, necrotic portions of the distal end of the humerus are easily recognized by their white appearance. Areas of stump skin may become mildly to moderately edematous, but this reaction is quite variable. At the end of this period, there is a conspicuous rounding of the edges of the limb stump.

Histology (fig. 2). The wound epidermis is generally thin, but its thickness is variable. Nevertheless it may possess up to ten poorly defined layers of cells. Leydig cells are present throughout the wound epidermis, and occasional mitotic figures are seen near its edge. The surface layer of cells is squamous, and patches of the basal layer show a tendency to be organized into a regular cuboidal arrangement. Within a few days the wound epidermis is filled with tissue debris, representing the epidermal

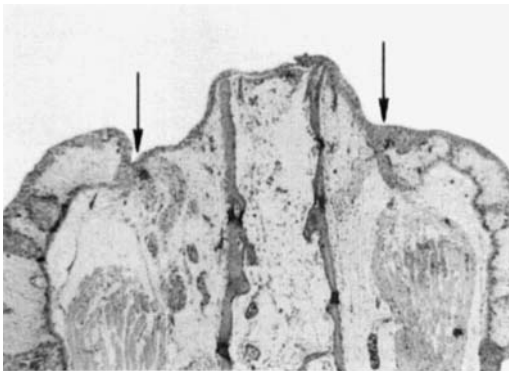


Fig. 2 One-day regenerate in *wound healing* stage. The amputation surface is covered by a thin wound epithelium (between arrows). H & E. $\times 14.25$.

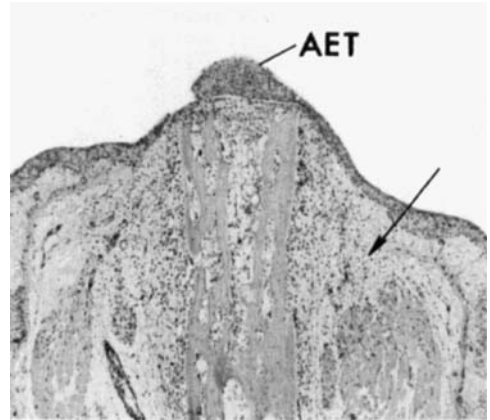


Fig. 3 Ten-day regenerate in late *dedifferentiation* stage. An apical epidermal thickening (AET) is prominent. The dedifferentiative reaction has removed mature muscle sarcoplasm from the distal parts of the limb stump (arrow). H & E. $\times 16.50$.

phagocytic reaction (reviewed by Singer and Salpeter, '61). Beneath the wound epidermis is a thin eosinophilic membrane, possibly of fibrin.

The underlying tissues are characterized by terminal necrosis in bone and nerve and removal of distal sarcoplasm of the transected muscle fibers by macrophages. Occasional muscle fibers undergo a discoid type of degeneration without the participation of macrophages. Osteoclasts have not yet appeared along the bone. The distal areas of damaged tissues contain a light to moderate number of inflammatory cells, both mononuclear and polymorphonuclear, throughout this period. By three days eosinophils appear in increasing numbers.

Stage of dedifferentiation (DD) —
(6-11 days after amputation)

External appearance (fig. 1b). The rounded edges of the limb stump are due in part to the breakdown of distal tissues and in part to a mild edema which affects some areas of the skin. Neither the extent nor location of the edema is consistent from one limb to another. The wound epidermis is noticeably thicker and less translucent than it was during the previous

stage. The foci of deep hemorrhage and other evidence of acute trauma are no longer evident, but the end of the bone is still white. An apical epidermal thickening (AET) with ill-defined borders appears. Beneath the AET, the apex of the limb appears avascular and white to gray in color. Melanophores are not seen in the apical area. The "basal" or peripheral area of the wound surface, between the apex and stump skin, presents a different appearance. The epidermis is thinner and more translucent than that of the apex; it is underlain by expanded pigment cells; and blood vessels with an active circulation are evident below.

Histology (fig. 3). The wound epidermis, with Leydig cells scattered throughout, begins to develop a distinct AET by days 8-9. The basal epidermis forms a fairly regular layer of cuboidal cells, but within the AET there is virtually no distinguishable layering of cells. Occasional osteoclasts are seen along the end of the humerus as early as seven days, and they are numerous by days 9-10. Around the distal part of the bone is a loose layering of fibroblastic cells. Although this layer eventually turns into a cuff of hyaline cartilage, there is yet no disposition of matrix or even morphological activation of nuclei. There is a roughly inverse relationship between the decline in the early inflammatory reaction and the appearance of the dedifferentiative reaction seen in the soft tissues, but there is no evidence for or against a causal relationship. By the end of this period, there is a scattering of blastemal cells throughout the distal regions of the stump, particularly in areas of loose connective tissue, but as yet little apical accumulation of these cells is evident.

Stage of early bud (EB) — (12-15 days after amputation)

External appearance (fig. 1c). The earliest externally visible blastema has the form of a low cone, usually symmetrical although sometimes oriented slightly posterodorsally with respect to the body.

The apex of the blastema is still whitish, avascular and unpigmented. The AET is shaped more like a poorly defined cap than a ridge. The base of the blastema is highly vascular and well pigmented. Both blood vessels and pigment cells show a definite orientation toward the apex of the blastema. The border between the skin of the stump and the base of the blastema becomes increasingly difficult to discern. During this stage, the blastema elongates rapidly, and by the end its still cone-shaped tip has become clearly oriented toward the posterodorsal quadrant of the body axis.

Histology (fig. 4). A well-defined AET is present at the apex of the limb. A regular basal layer of high cuboidal cells characterizes not only the AET but the entire epidermis covering the blastema. Within the AET the epidermal cells above the basal layer are disorganized and not readily definable into layers. The cells of the AET are mitotically active. Leydig cells are also present.

Osteoclastic erosion of the humerus remains intense, and the loose layer of fibroblastic cells surrounding the distal shaft of the bone has changed little. The

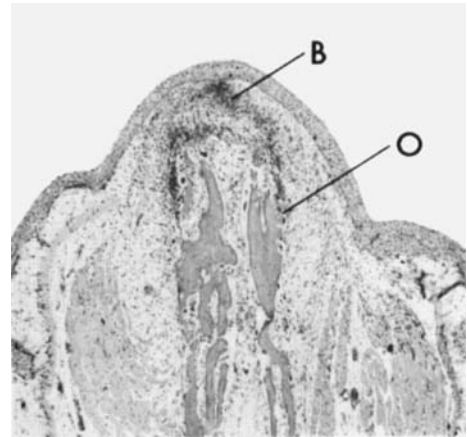


Fig. 4 Fifteen-day regenerate in *early bud* stage. Blastemal cells (B) are beginning to accumulate distal to the end of the bone. Osteoclastic activity (O) around the bone is marked. Cells in the basal layer of the wound epidermis are regular cuboidal in shape. H & E. $\times 15.75$.

distal stump musculature has been removed in the dedifferentiative reaction, but deeper within the stump the histological picture of dedifferentiation and early tissue regeneration of isolated muscle fibers continues. Occasionally, basophilic cuffs, representing early post-fusion myotubes, are seen around the sarcoplasm of old muscle fibers.

The distal part of the limb stump appears relatively empty, with blastemal cells scattered throughout the areas of loose connective tissue. Although increasing numbers of blastemal cells are accumulating distal to the end of the humerus, significant numbers are still found at levels proximal to the end of the bone.

Stage of medium bud (MB) — (16-20 days after amputation)

External appearance (fig. 1d). Early in this stage, the white tip of the cone normally has a pronounced posterodorsal to dorsal orientation (fig. 10A). At this point occasional superficial pigment cells are seen on the dorsal surface of the blastema. By the end of this stage, the black pigment cells are arranged in a discrete band. At the base of the blastema, the melanophores are more deeply situated, and the texture of the basal skin progressively approaches that of normal stump skin.

Within a couple of days, the ventral face of the blastema becomes strongly convex, and occasionally has an almost keel-like configuration. In contrast, the dorsal face is concave and is sometimes distinctly flattened. The apex of the blastema remains white and pointed, but by the end of this stage it begins to flatten. The AET begins to become more prominent. Sometimes it is so thick that it can be more properly called an apical epidermal lobe (Faber, '59).

Histology (fig. 5). A well-established distal blastema capped with a prominent AET dominates the histological picture during this stage. Cells of the basal layer of the epidermis covering the blastema retain their regular orientation, particularly along the lateral surfaces of the blastema. The

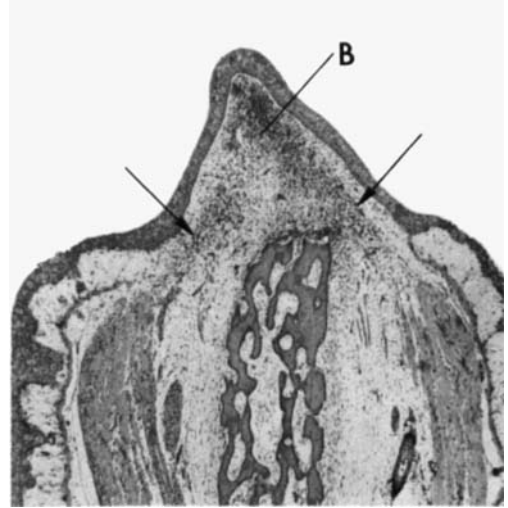


Fig. 5 Seventeen-day regenerate in the *medium bud* stage. The blastema (B) is now a prominent cone-shaped outgrowth. Areas of cellular density (arrows) are seen distal to the transected ends of the muscles. H & E. $\times 16.50$.

blastema itself is morphologically undifferentiated, but within it the density of the cells is not homogeneous. The cellular density is often greatest directly distal to the cut ends of the muscles. Nerve fibers are present throughout the blastema, but the vasculature appears sparse. No special injections of the blood vessels, however, were made. Active sarcolysis in the stump muscles continues, and in some degenerating muscle fibers, macrophages are present. During this period, the numbers of osteoclasts undergo a definite decline, and concomitant with that, the nuclei in the connective tissue surrounding the shaft of the bone begin to swell. There is a definite temporal relationship between the decline in numbers of osteoclasts and the initiation of deposition of periosteal cartilage. By the end of this stage, occasional small areas of cells alongside the bone have begun to produce a distinct cartilage matrix.

Stage of late bud (LB) — (21-23 or 24 days after amputation)

External appearance (fig. 1e). All blastemas are now distinctly flattened so that

an axis can be defined running dorsoventrally or from the anteroventral to posterodorsal direction with respect to the body of the animal. The ventral edge of the blastema is always convex whereas the dorsal edge is flat or even concave. The orientation of the blastema as a whole is asymmetrical, and when the regenerating limb is viewed directly end-on, the tip of the blastema is directed toward the dorsal or posterodorsal region of the limb (fig. 10A). Apical lobes persist on the dorsalmost tip of many blastemas. Blood vessels are present throughout the blastema, and in some cases, marginal veins have begun to take shape.

Histology (fig. 6). Overt cytodifferentiation within the blastema has not yet begun, but segregation of the cells is occurring so that one can often distinguish pre-cartilage from pre-muscle masses. The cellular density is considerably reduced in the region between the future muscle masses and the skin. The AET is still readily visible, but the prominence of the apical lobe varies considerably. Figure 6 shows a blastema with a prominent apical epidermal lobe. The basal layer of epidermis, so regular

along the sides of the blastema, loses its regularity at the tip of the regenerate. Very few osteoclasts remain along the humerus. During this period differentiation of the cuff of periosteal cartilage occurs rapidly. This takes place before differentiation of cartilage within the blastema. Early regeneration continues at the distal edge of the stump musculature.

Stage of palette (Pal) — (24-28 to 30 days after amputation)

External appearance (fig. 1f). During this stage the blastema is strongly flattened, with the axis of flattening extending from an anteroventral to a posterodorsal direction. The first digital primordium, followed shortly by that of the second, appears on the anteroventral (preaxial) side of the blastema. The intervals between the appearance of the third and fourth digital primordia are longer than that between the first and second. There is considerable variability, however, in the time of appearance of any of the digital primordia. The anterodorsal (extensor) face of the blastema is flat to concave in outline whereas the posteroventral (flexor) face remains convex.

The posterodorsal (postaxial) edge of the blastema is rimmed by a strong epidermal ridge, and a weaker ridge is seen on the preaxial edge. The posterodorsal ridge often extends proximal to the old skin of the stump. The apical epidermal lobe persists over the area of the second and third digital primordia, but it is losing its prominence. The postaxial edge of the regenerate is characterized by a darkly pigmented streak underlain by a marginal vein.

This stage of regeneration can easily be subdivided into substages by taking note of the number of digital primordia.

Histology (fig. 7). At the start of this stage, the differentiation of cartilage is occurring in the bones of the zeugopodial skeleton. Blastemas of the zeugopodial flexor and extensor muscles have formed and the cells in them are at the myoblastic stage of development. More distally, the

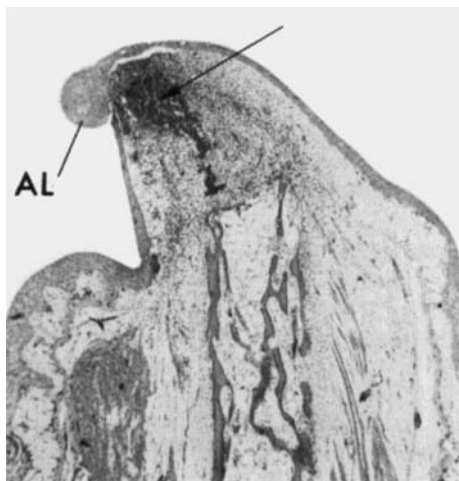


Fig. 6 Twenty-day regenerate in the *late bud* stage. The same regenerate as that illustrated in figure 1e. The apical epidermal lobe (AL) is prominent. Within the blastema is an area of pooled blood (arrow). H & E. $\times 15.75$.

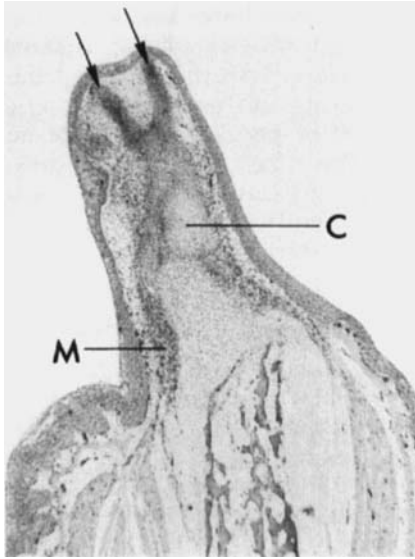


Fig. 7 Twenty-day regenerate in the *palette stage*. Differentiation of cartilage (C) and muscle (M) is occurring in the blastema. Digital primordia are prominent (arrows). H & E. $\times 15.00$.

level of differentiation decreases as has been previously described (Grim and Carlson, '74). The epidermis along the sides of the regenerate possess an extremely regular layer of cells which are now columnar in shape. Toward the apex (particularly over the tips of the distal primordia) this layer decreases in both height and regularity. The apical epidermis is still thickened although it is continuously losing its former prominence. No epidermal indentations are present between the digital primordia. More proximally in the stump, the cuff of periosteal cartilage is well differentiated and a prominent landmark. By this time osteoclasts are rarely seen.

Stage of digital outgrowth (DO) — (30+ days after amputation)

External appearance (figs. 1g,h). By the time the fourth digital primordium has formed, the first digit has already entered a phase of active outgrowth. During subsequent days, digits 2-4 also begin a period of rapid elongation in a pre-to-postaxial sequence. As the digits grow out, prominent epidermal wedges appear between them.

These wedges are most prominent between digits 1-2 and 2-3. Their overall form and time of appearance in the axolotl are similar to Iten and Bryant's ('73) description of them in the newt.

The beginning of digital outgrowth is also coincidental with the last stages of disappearance of the epidermal specializations that are prominent during the period of blastemal outgrowth. The apical epidermal lobe has usually completely regressed by the time when digits 2 and 3 begin their outgrowth. At about the same time, the posterodorsal ridge loses its prominence. The smaller anteroventral ridge has normally already disappeared.

Grossly the regenerate soon looks much like a miniature forelimb. A well defined elbow appears, and the handplate can be distinguished from the distal forearm. The flexor aspect of the regenerate has lost its convexity, and the extensor aspect is no longer concave. By 40 days it is often difficult to distinguish between the skin of the stump and the proximal skin of the regenerate. At 50 days, the digits are still more stubby than those of the normal limb, and the regenerated limb has not yet attained normal dimensions. The dissociation between morphogenesis and growth is readily apparent in regenerating limbs of large axolotls for after the period of gross morphogenesis has been completed, the fully formed but miniature limbs then undergo a prolonged phase of growth, and most regenerates eventually become indistinguishable from normal limbs.

Histology (fig. 8). At the start of this period, all of the elements of the skeleton are recognizable as cartilaginous rudiments although a proximodistal gradient of differentiation is still apparent, especially within the digits. At the base of the regenerate individual muscles can be identified, and the muscle cells are in the myotube stage. A basement membrane is reforming beneath the lateral epidermis. It is more prominent on the preaxial side than on the postaxial side of the limb. At the level of the light microscope, it is not yet possible to recognize a basement membrane beneath the di-

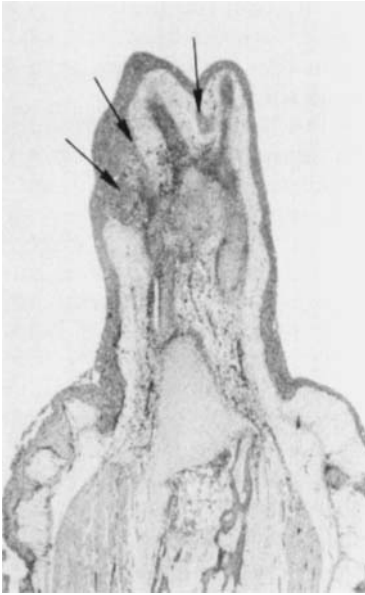


Fig. 8 Thirty-day regenerate in the *digital outgrowth* stage. Epidermal wedges (arrows) are found between the digital primordia. H & E. $\times 12.00$.

gital epithelium. Reformation of the basement membrane follows along a roughly proximodistal sequence.

Interdigital epidermal wedges are prominent as this stage begins, but with continuing digital outgrowth, they disappear. Ultimately the tissues within the regenerate assume a mature appearance, but the skeleton remains cartilaginous for an extended period of time.

Time course of regeneration

The progression of forelimb regeneration according to stages is illustrated in figure 9. The regenerates used in compiling this curve are those which were observed daily during the formulation of the staging system.

After the initial wound healing period the relationship between time and mean stage of regeneration followed a remarkably linear course up to the final stage of digital outgrowth. The magnitude of the

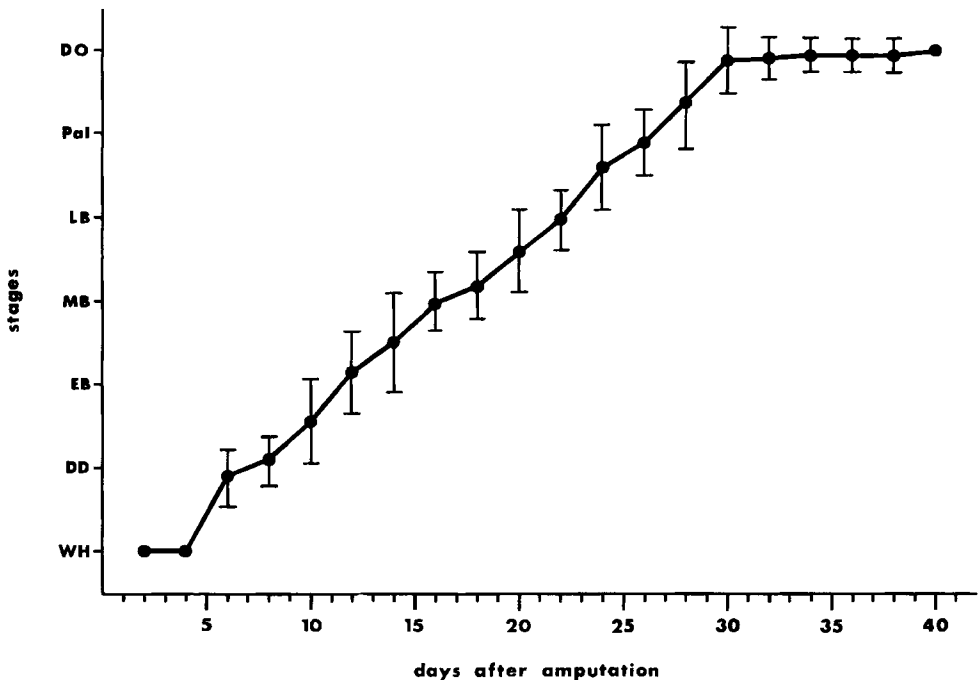


Fig. 9 Graph illustrating the mean progress of regeneration according to stages and the variation at various times. Each bar represents \pm one standard deviation. To obtain these, the stages of regeneration were assigned numerical values from 1 (WH) to 7 (DO).

standard deviations shows that at any given time after regenerative outgrowth can be detected, variation in development amounting to about one defined stage above and below the mean for that time can be expected. By the time the regenerates have all reached the digital outgrowth stage, morphogenesis is virtually complete. The regenerates must then undergo a prolonged period of growth (several months) during which time some internal changes (such as ossification of the cartilaginous skeleton) occur.

DISCUSSION

In any experimentation involving the manipulation of amphibian limbs during the process of regeneration, a set of defined developmental stages based upon external morphology must be used as a reference point in order to obtain comparable results. Both previous experience and the results of this study (fig. 9) have demonstrated marked variability in the progress of regeneration as a function of time after amputation. Even among members of an extremely homogeneous group of animals, this variability is too great to allow time after amputation to be used as the basis for critical experimental manipulation or sampling.

The stages defined here for forelimb regeneration in the axolotl were designed to correspond as closely as possible to those recently published for limb regeneration in the newt (Iten and Bryant, '73). The major difference between these two staging systems is that in the axolotl fewer stages can be clearly defined upon the basis of external morphology alone, particularly during the dedifferentiative and early blastemal periods. In addition, the later periods (palette and digital outgrowth) were kept broad because distinct subdivisions based upon numbers of visible digits (Pal stage) or relative lengths of digits (DO stage) can be easily made to suit the convenience of the investigator. In addition to covering a broader spectrum of the regenerative process than the stages devised by Faber ('59), the present system was

designed to avoid the necessity of relative measurements of the blastema, whose proportions in the axolotl vary with the age and size of the animal.

There are a few consistent differences between the morphology of regenerates in the axolotls used in this study and that of newt and younger axolotl regenerates. The medium and late bud blastemas of the large axolotl are characterized by a pronounced dorsal to posterodorsal curvature, whereas similar blastemas of both newts and younger axolotls are more symmetrical. The epidermal lobe, so prominent in both mature (figs. 1, 3, 6) and younger axolotls (fig. 2 of Faber, '59) is lacking in the newt. This appears to be a reflection of other differences in shape of the apical epidermal thickenings between axolotls and newts.

A consistent finding in mature axolotls is that the basal cross-sectional area of the regeneration blastema is considerably smaller than the cross-sectional area of the limb stump from which it arose (e.g., figs. 1e-g, 7, 8). Such a large degree of disparity in cross-sectional area between blastema and stump is seen in neither larval axolotls nor in newts. Conversely, the difference is accentuated in regenerating limbs of very large axolotls. It is not due to encroachment of stump skin upon the amputation surface because similar differences in proportion are seen in regenerates arising from a limb stump covered only by epidermis instead of full thickness skin (Carlson, unpublished). The disparity in area between limb stump and blastema in larger animals may well be a reflection of an upper limit of absolute size that a blastema can attain and yet carry on normal morphogenetic processes.

Axial nomenclature

For more than a half century there has been a lack of uniformity in designations of the transverse axes of urodelean limbs. The lack of uniformity in nomenclature stems from some differences in interpretation of the mode of embryonic limb outgrowth. These resulted in the use of two methods

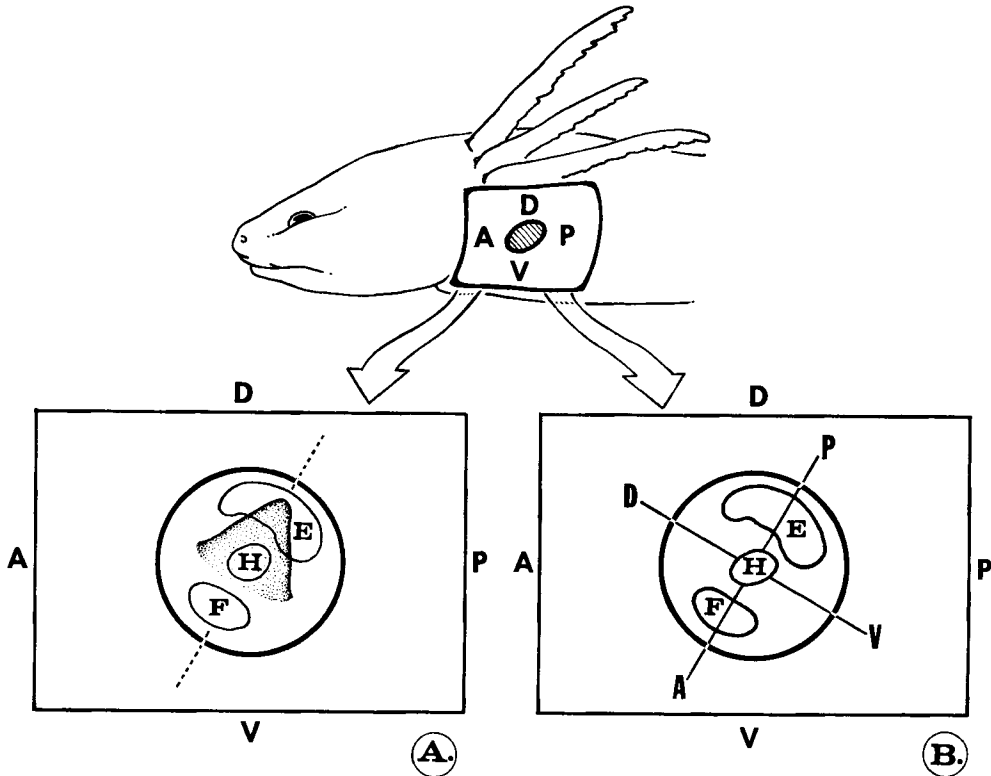


Fig. 10 Comparison between two systems of nomenclature used to designate transverse axes of the axolotl upper arm. The circles in A and B represent cross-sections through the upper arm. H, humerus; E, extensor muscle mass; F, flexor muscle mass. A, D, P, V refer to anterior, dorsal, posterior and ventral. (A) Nomenclature used in this staging paper. All axial designations relate to those of the body of the animal. The dashed line, which marks the direction of asymmetry and later flattening of the blastema, is said to run from an anteroventral to posterodorsal direction. (B) Nomenclature used in morphogenesis experiments in which the basic points of reference relate to the hand [preaxial (digit 1) — anterior; palmar — ventral]. This demonstrates that the A-P axis of the upper arm, as defined in experimental work (Carlson, '74, '75), is equivalent to the axis of asymmetry and flattening of the blastema.

for designating the transverse axes of the limb. The tenor of the older discussions on this point is contained in papers by Przi-
bram ('24) and Harrison ('25), which pre-
sent the evidence for and against the
differing viewpoints.

The result of this lack of nomenclatorial
unanimity is that one must use caution in
comparing the results of studies in which
properties of developing systems are re-
lated to transverse limb axes. In the
Ambystoma forearm, Harrison ('25) desig-
nated the radial (pre-axial) border as an-
terior and the ulnar (postaxial) border as
posterior. Accordingly, the extensor sur-

face of the forearm is dorsal and the flexor
surface is ventral. These axial designations
have been followed by the investigators
who have been recently examining the
effects of stump skin rotation upon the
morphogenesis of urodelean forelimb re-
generates (Settles, '67; Lheureux, '72, '75;
Carlson, '74).

If in the axolotl a mid-dorsal skin incision
is extended proximad from the forearm to
the upper arm, it passes between the flexor
and extensor muscles almost directly over
the cephalic vein. When the body of the
axolotl is used as a reference point, the up-
per arm skin incision is not purely dorsal,

but rather is in an anterodorsal position. Nevertheless, the dorsal designation has been kept for the sake of consistency in reporting experimental results (Carlson, '74, '75a). Thus, in the upper arm of the axolotl the anteroposterior axis has been defined as the plane passing through the humerus and the midpoint of both the flexor and extensor muscles (fig. 10B). This largely intuitive approach is substantiated by experimental results in both Harrison's ('21) experiments on limb buds in the *Ambystoma* embryo and Carlson's ('74) experiments on limb regeneration in adult axolotls, in which multiple limb outgrowth was produced after reversals of tissues along the anteroposterior axis but not along the dorsoventral axis.

In the present report all descriptions of blastemal orientation were based upon the anteroposterior and dorsoventral axes of the body of the animal. They do not correspond exactly with those designations used in the experimental work mentioned above. Thus, when the flattened axis of the palette-stage blastema is described in this paper as extending in an anteroventral to posterodorsal direction with respect to the body axes (fig. 10A) it would correspond to the anteroposterior axis as defined in the experimental studies (Carlson, '74, '75b).

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