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# COMPARISON OF WOUND HEALING AND/OR REGENERATION IN MAMMALS AND URODELES

PAR

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CONTENTS



RÉSUMÉ

Un traumatisme tel qu'une amputation est suivi d'une réparation tissulaire qui conduit soit à une cicatrisation simple soit à une régénération, selon que l'on s'adresse à un mammifère ou à un urodèle. Chez ce dernier la régénération est la règle mais la cicatrisation simple se produit aussi parfois, chez le mammifère la cicatrisation est la règle et la régénération ne se produit que dans des conditions expérimentales modifiant le cours habituel de la réparation. Il nous a semblé utile de revoir dans cette étude bibliographique les travaux consacrés aux phénomènes de cicatrisation et de régénération, et de les comparer, pour préciser dans la mesure du possible les similitudes et les différences non seulement en considérant le produit final, mais aussi au cours des processus engagés. Il en ressort que les similitudes sont plus nombreuses que les différences. Celles-ci portent plus sur l'intensité et la rapidité que sur une différence fondamentale. Les trois différences importantes constatées sont les suivantes: il y a au cours de la régénération, un contact intime entre cellules épidermiques et mésenchyme, la présence de nerfs et une fibrillogenèse retardée. On ne retrouve pas ces caractéristiques lors de la cicatrisation simple.

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Les récents travaux de THORNTON (1971) montrent l'importance de l'action du système nerveux au cours du développement, tout se passe comme si les pattes au cours de leur innervation perdaient leurs capacités régénératrices autonomes et ne les regagnaient qu'en présence suffisante de tissu nerveux. Quelles sont les substances apportées par le nerf et assurant cette commande, au niveau moléculaire, est une des questions majeures qu'il reste à résoudre.

## I. INTRODUCTION

All living organisms have the ability to respond in some corrective manner to injury.

One kind of injury that can be corrected is that which occurs when an internal organ (e.g. a kidney, a salivary gland, a gonad, or part of the liver or of the brain) is partially or completely removed from the body. The kind of reaction which occurs is a compensatory reaction. This type of response is restricted to cells homologous to those whose loss is being compensated, or to other kinds that may be physiologically related to them. It is achieved primarily by cellular hypertrophy and hyperplasia, and only rarely does it involve extensive morphogenetic reorganization (Goss, 1965).

Wound healing is probably the most common response on the part of an organism to injury. With the exception of teeth, there are no tissues in an organism which are not capable of *repairing* localized injuries. Wound healing is most commonly thought of in terms of the epidermis or the full thickness of the skin in which a lesion is healed over by migration of tissues, but it also occurs equally well, if not better, in the internal tissues of an organism. Wound healing not only involves the cellular proliferation and differentiation characteristic of compensatory hyperplasia, but also cell migration and morphogenesis. Some cases of wound healing, in fact, involve high degrees of histological morphogenesis (Goss, 1965). If a bone is broken, for example, the two ends are joined again by the production of a fracture callus which differentiates into cartilage and is then replaced by bone.

A third type of response, regeneration, can occur in some organisms when an appendage is amputated. Many invertebrates are capable of regeneration. Among the vertebrates, however, the urodele amphibians have considerably greater capability. When a vertebrate appendage (e.g. a limb, a tail, a fin, or a taste barbel) regenerates there are certain prerequisites that have to be met.

They are as follows: (1) There must be a source of cells of mesodermal origin from which a blastema is produced. The blastema or bud then differentiates into the regenerate. (2) Neuroplasm must be present in sufficient quantity for the establishment of the blastema (Singer, 1965). (3) An epidermis must be present to provide the thickened wound epithelium (apical cap) shown to be essential to regeneration by Thornton (1957). Regeneration will not normally occur if any one of these conditions is not met.

*The purpose of the following discussion is to compare the role of the various tissues involved in the healing of skin wounds in mammals with regeneration of amputated limbs in larval and adult urodeles.*

## II. SKIN

### A. **Epidermis.**

The first response to either a mammalian skin wound or amputation of an amphibian limb is the formation of a blood clot. The clot, which serves to close the wound quickly and prevent further bleeding, consists of tissue debris, fibrin, erythrocytes, leucocytes and fluid.

1. *Migration of the epidermis.* After the initial inflammatory response, which will be discussed later, migration of cells from the wound periphery begins. In the amputated limb of the adult newt, *Diemictylus viridescens* " the wound surface is covered by epithelial cells as early as 4-6 hours following amputation " (NORMAN and SCHMIDT, 1967). Though Schotte and Smith (1959) found evidence of epithelial migration as early as 6 hours in amputated mouse digits, in most human and animal wounds the epithelium begins to migrate from the margins of a wound within 24-48 hours (GILLMAN, et al., 1955; VIZIAM, et al., 1964; ODLAND and ROSS, 1968; CROFT and TARIN, 1970; JOYNSON and PIERCE, 1970).

Ultrastructural studies of this process show it to be quite comparable in the amputated adult newt limb, and in mouse and human skin wounds (NORMAN and SCHMIDT, 1967; ODLAND and ROSS, 1968; CROFT and TARIN, 1970). These studies reveal a loss of epithelial contact (hemidesmosomes) with the adepidermal membrane (newts) or basement membrane (mammals) and a reduction in the number of intercellular desmosomes prior to migration. The freed epithelium accumulates at the wound margin adjacent to the early blood clot then moves beneath the clot following the junction between the clot and living tissue beneath.

The exact plane of epithelial migration has been of more interest to those studying mammalian wound healing than to those studying urodele regeneration. From their EM studies of human wound repair Odland and Ross (1968) conclude that " the advancing epidermis moves initially through a serous exudate containing fibrin and some red blood cells. Inflammatory cells do not regularly lie in proximity to the advancing elements of the epidermis. The viscosity of the exudate could not be determined, but the cells appeared to be deformed only by structured elements of the exudate." Croft and Tarin (1970) from EM studies of mouse skin wound healing state that the epithelium " cuts through" connective tissue, extravasated blood and fibrin following the junction between necrotic material which is to form the scab and living connective tissue. According to Odland and Ross (1968) the migrating epidermal cells establish a physical attachment to the fibrin substrate,

whereas, Croft and Tarin (1970) suggest that the epithelial cells appeared to use the fibrin band as a support, but did not report physical attachments. Wessels (1964) has pointed out that basal epidermal cells require attachment to some form of physical substrate in order to undergo normal mitosis. In mammalian wound healing this substrate attachment may be important in view of the work of Viziam, Matoltsy, and Mescon (1964) with rabbit wounds which suggests that mitosis continues at a high rate after wound closure resulting in a thickening of the epidermis. Though Schmidt (1968) says that the migrating epidermis of the adult newt " glides over the wound between the blood clot and the injured tissues beneath ", Norman and Schmidt (1967) intimate that the epidermis moves over the blood clot which later dissolves with the cellular and extracellular debris being removed by macrophages. In either case fibrin could be used as support. No physical attachments have been reported, however. Schmidt (1968, p. 79) states that there are " no hemidesmosomes along the basal interfact between the epithelium and substratum " and that this " reflects the lack of a suitable substratum for cell contacts." Such a substrate attachment would likely not be required in the regenerating urodele appendage whose thickened wound epithelium (apical cap) is formed principally by migration from the proximal epidermis. Hay and Fischman (1961) labeled the cells of the apical epithelial cap of a newt by a preamputation injection of thymidine and noted that apparently only one cell division occurs (3-4 days post-amputation) and that by 20-28 days after the initial injection of thymidine the labeled cells of the apical cap can be seen undergoing desquamation indicating a lack of proliferative activity.

Keratinization and desquamation of the upper layers of the thickening and differentiating epidermis of mammalian wounds results in the dislodging of the scab formed over the wound by the original clot and other necrotic material.

2. *Subepidermal membranes.* The submicroscopic adepidermal membrane and the prominent basement membrane (lamella) of the newt skin and the basement membrane of the mammalian skin are absent under the wound epithelium (TABAN, 1955; SINGER and ANDREWS, 1956; SALPETER and SINGER, 1960; NORMAN and SCHMIDT, 1967; ODLAND and ROSS, 1968; CROFT and TARIN, 1970; and JOHNSON and PIERCE, 1970). The adepidermal membrane in the newts and the basement membrane in the mammals appear to be comparable structures consisting of distinct laminae densa and lucida. The newt basement membrane (lamella) on the other hand consists of several well ordered layers of collagen.

There is some evidence that these membranes in both urodeles and mammals are reconstituted by the epithelium. Odland and Ross (1968) find in their EM studies that in human wound healing the basement membrane and hemidesmosomes are formed where the epidermal sheet establishes contact with fibrin. Since there does not appear to be any relationship to collagen or fibroblasts in the formative stages, and since organelles usually associated with synthesis and secretion of extracellular proteins can be seen in the epidermal cells, they conclude that the basement mem-

brane is produced by the regenerating epidermal cells. Croft and Tarin (1970) agree that basement membrane formation is an epithelial function on the basis of their EM work on mouse skin wounds. The electron microscope autoradiographic studies of Hay and Revel (1963) on the regenerating forelimb of a larval urodele *(Ambystoma maculatum)* also give support to these conclusions. They demonstrated that the epidermis secretes a protein to the basement membrane (lamella) forming adjacent to the epidermis. They believe this protein to be collagen.

The time and pattern of the formation of these membranes present the first histological difference in mammalian wound healing and urodele regeneration encountered in this discussion. The basement membrane is apparently reconstituted much more rapidly in mammalian wound healing than are the subepidermal membranes in urodele regeneration. Johnson and Pierce (1970) found an " apparently normal basement membrane was present " in a mouse skin wound 72 hours after wounding. Also using mice Croft and Tarin (1970) noted that basement membrane formation was just beginning at 72 hours and that " at first this only occurred in limited areas." By day 7 the membrane was complete. According to Salpeter and Singer (1960) during the period of formation of the early regenerate (6-8 days) in the adult newt there is no structural barrier between the basal cells of the wound epithelium and the accumulating mesenchymatous cells of regenerating. As regeneration proceeds (8-9 days), the adepidermal membrane forms *de novo* near the base of the regenerate and development proceeds distally. It does not form as a progressive and continuous structure, but appears first as a discontinuous one, and is entirely lacking at the tip of the regenerate even in late stages of regeneration (Salpeter and Singer, 1960). The basement membrane may form after the adepidermal membrane or simultaneously with it. The basement membrane has its beginning at the point of amputation also, and extends distally with the growth of the regenerate. " However, " states Schmidt (1968), " at no time is there a fibrillar intervention between apical epithelium and underlying cells during the course of regeneration. Intimate contact is evident even between the apical digital epithelium and the subjacent prochondral blastema cells of the differentiating cartilaginous digit."

3. *Role of the Epidermis.* A significant role of the wound epidermis is the removal of wound debris. This may be accomplished by phagocytosis, by tongues of wound epithelium moving down into underlying tissues and engulfing scattered debris or by a movement of cells or dissolved materials into the wound epithelium where some of it may be digested and utilized and the remainder gradually moved to the surface and discharged (SINGER and SALPETER, 1961, review).

Taban (1955) observed striking irregularities in the basal layer of the epidermis when he deviated the brachial artery and associated sympathetic nerves of the newt to the body wall. The regenerating epidermis intruded deeply and encompassed the deviated structures as well as a blood clot that formed at the site of operation. He observed debris of various sorts within the wound epithelium. Others who have

observed tongues of wound epithelium extending into underlying tissues and/or evidence of phagocytosis in the newt are Rose (1948), Scheuing and Singer (1957), Bodemer (1958), Schmidt (1958), and Norman and Schmidt (1967). Goss (1971, lecture) reported a similar phenomenon in the wound epithelium formed in full thickness wounds in the web membrane of bats. Both Odland and Ross (1968) and Croft and Tarin (1970) demonstrated phagocytic activity in the migrating epithelium of human and mouse skin wounds, respectively, and the latter investigators suggest it is part of the " mechanism of epithelial invasion." This invasion is also described by Taban and Connelly in newts allografts and axolotl to newts xenografts (unpublished).

Evidence of the movement of cellular and particulate matter through the wound epidermis has been reported by many workers. Singer and Ray (1957), for example, infused Nile-blue sulphate from below into the early regenerate. After first being concentrated within the mesenchymatous cells of the regenerate, the Nile-blue sulphate appeared a few days later within the epithelium, the moved through the epidermis to the outside. Bodemer (1958) placed charcoal on an open wound in the newt and found that it was culled by the epidermis as it closed over the wound and then was expelled. Riddiford (1960) covered a tritium labeled newt blastema with an unlabeled epidermis. The epidermis became progressively labeled presumably by movement of cells and cellular debris from the blastema and with the continuous displacement and desquamation of epidermal cells the labeling was gradually lost. Singer and Salpeter (1961) suggest that, though cells and larger particles may be moved to the outside due to the movement of the epidermal cells, smaller particles and dissolved substances may be carried outward by a steady stream of fluid. The fluid pressure within the edematous regenerate may be great enough to promote such a movement (SINGER and SALPETER, 1961). Indeed, it is entirely possible that the epidermal cells are engaged in active excretion as well.

Another role of the wound epidermis is that of secretion. The ultrastructural studies of Salpeter and Singer (1960) indicate that many cells of the newt wound epidermis possess a cisternal type of endoplasmic reticulum which has been associated with cells engaged in the secretion of a protein-rich product. These cells frequently have a rather broad cortical region devoid of mitochondria or endoplasmic reticulum. Singer and Salpeter (1961) believe that secretory products accumulate in this region which are discharged gradually through the cytoplasmic membrane. Odland and Ross (1968) describe similar cortical zones among the cells in human wound epidermis.

Taban (1955) observed the liquefaction of a blood clot by the wound epithelium. Thornton (1968, review, p. 213) points out that " since wound epithelium intensely incorporates methionine-35S (BODEMER and EVERETT, 1959; ANTON, 1965) but not thymidine-3H (HAY and FISCHMAN, 1961), this protein synthesis is apparently not involved with mitosis but may be associated with proteolytic enzyme production ".

It may be that proteolytic enzymes are discharged from the epidermal cells of the regenerate into the intercellular spaces and subepidermal regions. The secretion may contribute to the widespread histolysis of the distal stump tissues resulting in their dissociation and modulation, as well as inhibiting the development of subepidermal membranes and the dermis for a time. Eisen and Gross (1965) found that epithelial cells from tadpole tail fin formed a collagenolytic enzyme in culture. Collagenolytic activity has not been reported in the wound epithelium of regenerating urodele limbs though it has been found in the combined tissues of the newt regenerate (GRILLO, 1964 *b*). It has been reported in mammalian wound epithelium, however. Grillo and Gross (1967) found collagenolytic activity in tissue cultures of wound edge epithelium from guinea pigs and suggested that this activity might facilitate the release of the cells from their attachments to basement membrane. This would allow the epithelium to migrate. Johnson and Pierce (1970), using immunofluorescent techniques were able to correlate a loss of antigenicity in the epithelial basement membrane with a change in the ultrastructural appearance of the membrane immediately prior to the migration and proliferation of epidermal cells in the closure of mouse skin wounds. They felt that the changes in the membrane might be due to the action of a " collagenase " synthesized by the injured epithelial cells and postulated a similar role to that suggested by Grillo and Gross (1967).

An additional role of the epidermis of considerable importance is that of influencing the aggregation of blastema cells. The work of Thornton (1954, 1956, 1957, 1958, 1960, 1965), Thornton and Steen (1962), Steen and Thornton (1963), and Thornton and Thornton (1965), provides considerable support for this function. Thornton (1954) induced excessive regression in unamputated larval *Ambystoma* limbs by denervation and injury. When the limb was reduced to a small moud of dissociated cells and nerves were permitted to regrow. The nerves penetrated the apical epidermis and it transformed into an apical cap. The dissociated cells then accumulated beneath the newly formed apical cap and developed into a typical regeneration blastema ". Removal of the apical wound epithelium in *Ambystoma tigrinum* larvae prevented blastema formation and regeneration (THORNTON, 1957). Treatment of the apical cap with ultraviolet light (25374 Å) also prevented apical cap formation and accumulation of blastemal cells (THORNTON, 1958). When the apical cap was moved to an asymmetrical position in innervated (THORNTON, 1960) or aneurogenic (THORNTON and STEEN, 1962) limb stumps the blastemal cells aggregated beneath it in corresponding asymmetry, and asymmetrical regenerates developed from the acentric blastemata. Thornton and Thornton (1965) found that a secondary outgrowth of blastemal cells was induced when an apical cap was transplanted to the base of a limb blastema. The nature of the activity of the apical cap in influencing the aggregation of blastema cells is not known.

Apical cap formation and blastemal cell aggregation do not occur in amputated limbs of adult frogs and higher vertebrates. With a few exceptions, *mammals do not*

*exhibit wound epithelial thickening and mesenchymatous cell aggregation in wound healing* to the extent noted in a regenerating system. It may be that the inability or prevention of the wound epidermis in exerting this influence is a factor in the loss of regenerative ability in mammals.

The role of the epidermis in the reconstitution of the subepidermal membranes has already been discussed.

B. **Dermis.**

In urodeles and larval anurans wound healing is accomplished by movement of epidermal layers alone and the dermis does not intervene between the wound epithelium and subjacent tissues until regeneration is nearly completed, several weeks after amputation. In adult frogs, which lack the ability to regenerate, the healing of an amputated limb is accomplised by the movement of whole skin (dermis and epidermis) over the wound surface such that direct contact between epidermis and underlying tissues is precluded. This direct contact is also absent in the healing of " full thickness " mammalian wounds, i.e. wounds in which both epidermis and the full thickness of the dermis are removed (JAMES, 1965). In this type of wound fibroblasts enter the wound area very quickly and within 48 hours begin to deposit collagen and mucopolysaccharides. By 4 or 5 days the original deficit is filled with a highly vascular loose connective tissue (granulation tissue). Between the 5th and 10th-12th day the granulation tissue contracts drawing the surrounding skin inwards over the wound bed. The granulation tissue then matures into a collagenous scar.

*The absence of the dermis in a regenerating system and its presence in a non-regenerating one is the second histological difference encountered in this discussion. Both of the differences noted involve a barrier or lack of it between the wound epithelium and underlying mesodermal tissues.*

Undoubtedly, the absence of a dermal barrier and direct contact between the apical wound epithelium and underlying tissues is essential to regeneration in urodeles. Simply transplanting intact normal skin (which includes both epidermis and dermis) will inhibit regeneration. On the basis of reports by Gross and Lapiere (1962) and Grillo (1964 *b*), who found collagenase-like activity in amphibian tissues and newt limb regenerates, respectively, Schmidt (1968) suggests a mechanism for retarding the development of the dermis and subepidermal membranes in the regenerating system. He infers that early deposition of stable collagen fibers within the blastema as well as premature formation of dermis and subepidermal membranes during the course of regeneration could interfere with necessary remodeling and regenerating processes. An enzyme system to catalyze the digestion of collagen could retard the development of dermis and subepidermal membranes by removing extracellular collagen fibrils as fast as they are produced. This activity would, of course, have to gradually become localized in the apical region, where it might continue until regeneration is almost complete.

Rose (1944) and Gidge and Rose (1944) stimulated regeneration in normally nonregenerating frog limbs by treating the amputation surface with a hypertonic salt solution that disrupted the apical wound epithelium. They concluded that regenerative capacity in frogs is inhibited by rapid dermal growth intervening between wound epithelium and mesodermal tissues.

Amputated rat digits were partially regenerated following an alternating application of trypsin and $CaCl_2$ solutions which removed the scab material and kept the wound " open " (SCHARF, 1961, 1963).

Joseph and Dyson (1966) found " that full-thickness defects of the rabbit's ear of 2 to 3 sq. cm. in area were filled by a growth of new tissue and to some extent by contraction." Some marked similarities to regeneration can be noted in the histological studies of this tissue replacement. Some of these are as follows: (1) Seven days after injury the collagenous dermis was not found over the injured surface; the epithelium having migrated independently of it. (2) Fourteen days after injury a regenerative blastema had formed beneath an apical cap some 15 cells thick. (3) Forty-two days after injury the blastema was still growing, but the production of dermal collagen had increased and the dermis now extended to the growing tip of the blastema.

Goss (1971, lecture) repeated the above experiment and achieved the same results. However, when he tried the same experiment in rat, mouse, deer, sheep, and guinea pig ears the wound margins healed, but tissue was not replaced.

Full thickness defects through the web membrane of the fruit bat healed in a similar manner to the rabbit ear (CHURCH and WARREN, 1968). Again characteristic features of a regenerate are found in the description of histological changes which occurred during tissue replacement. For example: (1) The epidermis over the wound surface increased to a thickness eight times that of the normal epidermis near the wound margin. (2) The dermis thinned out to form only a thin layer of loose connective tissue between the thickened wound epidermis and the underlying granulation tissue. The " granulation tissue ", in this case, resembles a blastema and contains only a small amount of collagen.

It appears that the usual barrier found between the wound epidermis and underlying tissues in mammalian wound healing is lacking or reduced in these two cases.

## III. SUBCUTANEOUS TISSUES

### A. Nerve Tissue.

It has been known since 1823 (TODD) that nerves must be present for an amputated urodele limb to regenerate normally. The type of nerve is not important since either sensory or motor nerves will support regeneration, but the number of

nerve fibers can be correlated with regenerative ability (SINGER, 1952, review). In the adult newt the mid-upper arm has approximately 24.5 fibers/100 microns $^2$ of amputation surface. When this number is decreased by selectively destroying the 3rd, 4th or 5th spinal nerves or combinations of them or the sensory or motor component, regenerative ability remains provided the number of intact fibers exceeds a range of approximately 9.0-13.0 fibers per unit wound area (SINGER, 1952). When this theory was extended to the primitive frog, *Xenopus*, which can also regenerate, it was found that its limb is very sparsely innervated in comparison to the adult newt. It has only 2.6 fibers/100 microns $^2$ of amputation surface. This is very few more than the nonregenerating *Rana* which has 1.8 fibers per unit wound area (RZEHAK and SINGER, 1966). Realizing that nerve activity may be a factor not only of number of fibers, but also of their caliber, Singer, Rzehak and Maier (1967) measured the axon diameters in the nerves of *Triturus*, *Xenopus* and *Rana* and calculated the mean cross-sectional area. In comparing the estimated amount of neuroplasm per unit area of amputation wound it was found that *Xenopus* and *Triturus* have comparable neuroplasmic volumes but that nonregenerating *Rana* has substantially less. They concluded that the volume of cytoplasm in the axon, as measured by its cross-sectional area, is directly related to the effectiveness of that axon in its action or regeneration.

The quantitative nerve requirement does not appear to be absolute, however. Since an adequate response of the tissues is involved in addition to quantity of nerve fibers, if the response of the tissues could be heightened then the nerve requirement may be reduced. Singer and Mutterperl (1963) observed such a circumstance when a limb was transplanted to the back of a newt. Regeneration occurred in some of these grafts even though the number of nerve fibers was well below the normal threshold number. The results were interpreted to mean that the trauma of transplantation had altered the threshold.

Not only can dependence on the nerve be reduced but in some cases it can be dispensed with altogether. Yntema (1959 *a*, *b*) showed that when the limb of the salamander develops in the embryo in the absence of nerves, it does regenerate upon amputation. These results were confirmed by Thornton and Steen (1962) and Steen and Thornton (1963).

Nerves have two functions: conduction and maintenance and growth of body parts. The action of nerves in regeneration is independent of conduction and direction of conduction; and it is not dependent upon reflex circuitry (Sidman and Singer, 1951; Ashbaugh and Singer, 1957). The latter or trophic function is important, however. If nerves leading into the adult newt limb are cut during early regenerative growth, growth ceases and the regenerate wastes and shrivels. The nerves must, therefore, supply something to enhance growth. If the nerves are cut in an amputated larval salamander limb, the limb is not only incapable of regeneration, but the stump is completely resorbed in a distal proximal direction unless nerves are permitted to

regrow (Butler and Schotté, 1941; Thornton, 1953). The nerves, in this case, are necessary to maintain morphological integrity as well as for growth.

The trophic agent of the neuron is not known, but it is believed to be chemical in nature. Singer (1964, 1965) proposed that the trophic agent is not unique to the neuron, but because of its tremendous volume of peripheral cytoplasm it has specialized quantitatively in its production. He also suggested that the reason peripheral tissues come to depend on the nerve is that the excess neuronal contribution " quenches the cellular metabolic machinery."

This idea made it possible to explain regeneration in the transplanted limb, even though the number of nerve fibers was well below the threshold number, on the basis that the production of the trophic agent by the non-nervous tissues had been enhanced by the trauma of transplantation (Singer and Mutterperl, 1963). Aneurogenic limb regeneration (Yntema, 1959 *a*, *b*) could also be accounted for. In the absence of nerves the production of the trophic agent continued to be produced by non-nervous tissues in sufficient quantities to support regeneration. There was still the question, however, as to why tissues in a limb maintained in a denervated state for a period of time did not regain their ability to produce the trophic agent. Thornton and Thornton (1970, p. 294) suggest that " perhaps the long-continued functioning of limb nerves during ontogeny and larval development produces such a strong inhibition that interventions in addition to simple nerve withdrawal are needed to reactivate the non-neural tissues... If this is the case, then perhaps a shorter term of innervation will allow limbs, subsequently denervated, to recuperate the ability to regenerate after simple amputation." These investigators found that aneurogenic limbs of *Ambystoma maculatum* larvae transplanted orthotopically and homoplastically to innervated larvae became dependent on the nerves that grew into them by 13 days post-transplantation so that they would not regenerate upon denervation, but regressed excessively. They also determined that if these aneurogenics were allowed to become innervated for periods of 2-3 weeks then denervated and maintained in that state for at least 30 days, they would regenerate upon amputation and continued denervation. Thus, a recuperation of regenerative ability in larval *Ambystoma* limbs after a prolonged absence of nerves was demonstrated.

It appears that nerves are not essential for wound repair in mammals. When Mank, Marcy, and Valette (1968) made two similar circular cutaneous wounds, symmetrically placed on the two flanks of a guinea pig after a unilateral section of the intercostal nerves, they found a significant delay on the denervated side during the first seven days following wounding, but wound repair was not halted. And, when lesions were made in both webs of fruit bats and the left web of each was denervated, Church and Warren (1968) found no significant difference in any respect in the healing of the innervated and denervated web wounds.

Attempts have been made to induce limb regeneration in nonregenerating forms by augmenting the nerve supply. Singer (1954) was successful in inducing regeneration

in *Rana* by deviating the sciatic nerve from the hindlimb to the forelimb. Deviating nerves in attempts to induce regeneration in the limbs of young rats have been mostly unsuccessful (BAR-MOAR and GITLIN, 1961; KUDOKOTSEV and DANCHENKO, 1966). Mizell (1968) evoked a positive response, however, in 8 of 30 cases when young opossum cerebrum was implanted in the hindlimb of a 2 day old opossum and the limb amputated 4 days later. A recognizable foot-like structure with indications of the fourth and fifth toes could be seen after 18 days of regeneration. Taban (1971) found a trophic effect of grafted nerve in amputated embryo rabbits, but no blastema formation.

### B. Hemopoetic Tissue

The inflammatory response in mammalian wounds has been studied extensively, but very little has been done with this aspect in amphibian regeneration. In a recent study of human wound repair (ROSS and ODLAND, 1968) observations during the first 24 hours revealed that the neutrophilic leucocytes were the predominant cells, although a few mononuclear cells were also seen. By 72 hours the neutrophils had decreased. The mononuclear cells gradually increased in number and became the dominant cellular constituent of the wound by the 5th day. Ross and Odland (1968) suggest that the primary role of neutrophilic leucocytes is cell lysis with the release of their granules, but that " a few neutrophils demonstrate phagocytic action largely involving the uptake of fibrin and serum protein." Schilling (1968) states that the neutrophil also engulfs bacteria and thus is essential in the control of infection at the wound site. Ross and Odland (1968) believe that their work demonstrates that the macrophages active at the wound site are activated monocytes from the blood and that they play a significant role in the ingestion and digestion of fibrin and serum protein. Schilling (1968) points out that monocytes may not only transform into macrophages but also into epithelioid cells and multinucleate giant cells. He also states that liver, spleen, and connective tissue are rich sources of macrophages. Tarin and Croft (1970) reported that 8 hours after wounding mouse skin developed an area of intense eosinophilia in the superficial dermis on either side of the incision. By 16 hours this eosinophilic region was necrotic. Otherwise, the inflammatory response in the mouse skin closely parallels that in human skin. Possible roles of eosinophilic leucocytes, lymphocytes and mast cells in connection with the inflammatory response are discussed by Schilling (1968). Eosinophils " may be serotonin, bradykinin and histamine antagonists. They may transport or modify antigen or protect the body by engulfing or destroying antigen-antibody complexes. It is of interest that antigen-antibody complexes attract eosinophils. Presumably they must play a role in body homeostasis." Lymphocytes may transform into plasma cells, which are mainly responsible for antibody formation. They may also supply nutrients and genetic memory to promote growth in other cells. Mast cells, which are found

mainly in loose connective tissue, degenerate and liberate heparin, histamine and hydrolytic enzymes into the ground substance of connective tissue.

The inflammatory response in amphibians appears to parallel that in mammals in some ways. Following amputation of the adult newt limb " there is local edema, and invasion by granulocytes and some lymphocytes takes place " (SCHMIDT, 1968). Eosinophilic granulocytes were noted in " relatively unusual " numbers in the newt limb stump by Schmidt, 1962. Macrophages are also prominent in the injured tissues, distal to them, and in the apical wound epithelium. " The possibility of encountering any unusual numbers of others cell types such as mast cells, basophils, monocytes, etc., in the subapical wound area is not obvious from available studies " (SCHMIDT, 1968).

### C. **Connective Tissue.**

The tissue referred to in the following discussion will include the sheaths associated with skeletal muscle, nerves, bone, blood vessels, the dermis of the skin, and the skeleton. Of the normal cell population of connective tissues mast cells, macrophages and fibroblasts (chondroblasts, osteoblasts) are the ones more likely to be involved in wound healing and/or regeneration. Since mast cells and macrophages have been mentioned previously, fibroblasts will be discussed here.

Though the fibroblast is now recognized as the principal cell forming the granulation tissue of repairing cutaneous wounds in mammals and other vertebrates, the derivation of these cells, however, is still unresolved. There is evidence to support a contribution of fibroblasts from the connective tissues near the wound—the dermis, superficial fascia, or perivascular tissues (EDWARDS and DUNPHY, 1958; MACDONALD, 1959; GRILLO, 1963, 1964 *a*, *b*; ROSS and ODLAND, 1968). Though it has not been demonstrated unequivocally that monocytes are converted into definitive fibroblasts, Gillman (1968) questions that " the dividing cells in healing wounds are derived *only* from locally resident cells, be they perithelial or fibrocytic. " He has little doubt that some bloodborne monocytes play a critical role in fibrogenesis and do not function solely as debris removing macrophages.

Regardless of the source of these cells, collagen fibrils are soon being produced in mammalian wounds. Collagen may be identified as early as the 2nd and 3rd day after wounding and significant amounts begin to appear by the 5th day, and by day 7 production is well along (ABERCROMBIE, FLINT and JAMES, 1954; GRILLO, WATTS and GROSS, 1958; TARIN and CROFT, 1970). A scar is formed which slowly matures and binds together the sides of an incision or, by contraction, draws together the edges of an excised wound (JAMES, 1965).

Schmidt (1968) presents extensive arguments for his theory that blastema cells of the amphibian regenerate arise exclusively from connective tissues. Most investigators, however, include all or nearly all of the stump tissues as sources of

blastema cells. Whether connective tissue fibroblasts make up all or part of the blastema, fibrillogenesis occurs in the blastema, though it is considerably delayed (NORMAN and SCHMIDT, 1967). Norman and Schmidt (1967) report finding small intercellular collagen fibers and intracellular collagen-like banded fibrils located deep within the regeneration blastema of 17-day regenerates. Collagen production, however, either proceeds very slowly or collagen fibers are removed as rapidly as they are produced.

Fibrillogenesis apparently begins at the base of the blastema adjacent to the stump tissues. Norman and Schmidt (1967) postulate that the collagen-rich environment thus created serves to condition the extracellular space for the regeneration of striated muscle. Studies by Konigsberg and Hauschka (1965) and Hauschka and Konigsberg (1966), which show that the differentiation of cultured myoblasts was a response to collagenous " conditioned medium," give some support to the idea.

## IV. CONCLUSION

In summary three important differences in urodele regeneration and mammalian wound healing have been noted. *Extended intimate contact between epidermis and mesenchymal cells, presence of nerves, and delayed fibrillogenesis* are characteristic of regeneration, but not of mammalian wound healing. Some speculations on the import of these characteristics and some references in support are as follows:

The intimate contact between epidermal and mesenchymal cells may suggest a controlling or inductive role for either the mesenchyme or the epidermis (GROBSTEIN, 1964; THORNTON, 1965, 1968; BILLINGHAM and SILVERS, 1968; TARIN and CROFT, 1970), or a co-operative action of the two tissues (EISEN and GROSS, 1965).

The need for neural tissue may suggest the release of quantities of nutrients for growth (SINGER, 1965), or the release of some genetic memory (RNA or DNA fragments) (SMITH and CRAWFORD, 1969), or the release of a substance that destroys or inactivates some gene inhibitor (SCHILLING, 1968).

Delayed fibrillogenesis, considered by Schmidt (1968, p. 93) to be the " real distinction between wound repair processes in mammals and limb regeneration in amphibians," may suggest the presence of an enzyme system to maintain the balance of protein turnover (GROSS and LAPIERE, 1962; GRILLO, 1964 *b*), or a physiological environment that interferes with collagen synthesis (SCHMIDT and WIEDMAN, 1964).

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