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Limb Regeneration

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INTRODUCTION

I will begin with a brief summary of the events that occur in normal limb development, and then progress to a similarly brief outline of the processes involved in normal limb regeneration as it occurs in some vertebrates, with an attempt to demonstrate the similarity between ontogeny and epimorphic regeneration.

After comparing regenerating and non-regenerating forms, I will describe the efforts to alter regeneration by electric and magnetic field stimulation.

NORMAL LIMB DEVELOPMENT

The events leading to the formation of a normal limb during development have been studied and reviewed extensively (1-3). At the outset, the limb in the embryo is induced to begin its development by the somites in the future limb-forming region (4). The appearance of the limb is manifested by the formation of a small bump which contains a core of mesenchyme (loose embryonic connective tissue) covered by epithelium. The epithelium rapidly becomes specialized at the tip of the bump into a thickened ridge, usually called the apical ectodermal ridge (5). An epithelial–mesenchymal interaction ensues between the underlying tis sue and this ridge such that the ridge directs differentiation and outgrowth of the limb, while the underlying tissue produces a substance or substances that preserve the existence of the ridge. Much literature documents the details of this interaction (6-9). In addition, events occur which are not entirely understood, but which result in polarization of the outgrowing limb along the proximodistal, dorsoventral, and craniocaudal axes. The polarization of the craniocaudal axis has been ascribed to the activities of a caudal organizing region near the base of the limb (9). In any event, the limb continues to extend and the tip flattens into a paddle-shaped structure. Internally, some of the mesenchyme condenses into models for the future bones.

Next, the paddle becomes altered by the appearance of bumps at its free margin. These are the finger buds, which gradually elongate to form the fingers. Internally, the mesenchymal bone models become chondrified, and the tissue between the fingers degenerates to separate them into discrete digits. Development is finished when the cartilage–bone models ossify, and the details of the fingers are laid down.

EPIMORPHIC REGENERATION AND ITS CONTROL

In some vertebrates, notably the Salamandridae group of Urodeles, adult animals can grow new limbs if the old ones are cut off. All of these adult regenerators are aquatic forms. Limb regeneration also occurs in many other amphibian larvae, including *Anurans* (frogs and toads). Again, these larvae are aquatic.

Land-dwelling forms generally do not regenerate as adults, though their offspring, if young enough, often do show some evidence of regenerative ability. This is true especially for animals whose young are born in a very immature state, and Mizell has provided a prime example with the opossum (10). Baby rats can exhibit regeneration (11), as can sub-pubertal humans who regenerate fingertips if the wound is not surgically closed (12).

In the case of the salamanders which regenerate complete limbs, the process mimics normal development quite closely. First, the epithelium closes over the wounded surface. Then it develops into a thickened structure similar to the apical ectodermal ridge. Simultaneously, a collection of loose, undifferentiated mesenchyme-like cells appears beneath the epithelium. Taken together, this combined structure is called a blastema. The origin of the mesenchyme-like cells has been a subject of much controversy for years. Some investigators have felt that they come from preexisting stem cells, while others have insisted that the limb tissues dedifferentiate into cells which can then redifferentiate into the new tissues as required. Hay (13) appears to have shown that muscle cells can dedifferentiate, and Oberpriller (14) has demonstrated that cells from a blastema derived from a regenerating intestine can be incorporated into the regrowing limb. The former observation seems to settle the question of whether old cells can dedifferentiate, and the latter lays to rest any requirement for specificity. The regrowing limb simply makes use of whatever cells it has at hand to make whatever tissue needs to be made.

The regenerating limb then follows a course that mimics normal limb development: a paddle-shaped structure forms, which then develops finger buds and elongated fingers. Internally, bone models form, chondrify, and then ossify as in normal development. A good review of the process, including a discussion of the evidence for various control systems and cell origins, is given by Rose (15). Generally speaking, the adherents of various control schemes have strongly propounded their own ideas.

What concerns us is the system responsible for initiating regeneration and polarizing the regenerate. Obviously, the animal must somehow learn that the limb has been cut off, so that it also knows that regeneration is necessary. At the conscious level this seems obvious. However, such awareness at the tissue level is far from obvious, and we are actually unaware of the nature of the signal. It has long been known that an injury produces a wound potential, generated by leakage from damaged cells (16). This potential makes the wounded surface strongly negative with respect to the surrounding tissue. Since the body can respond to electrical signals, the wound potential is a possible signal. Another possibility is suggested by experiments which show that the size of each part of the body appears to be regulated by products that it produces which inhibit the further development of more of the same tissue. This concept of specific inhibition

suggests that when a part of the body is removed, these inhibitory products are also removed, and the remaining part of the body is then free to develop more of the tissue until the original volume is replaced (17). This concept works well for relatively homogeneous tissue such as the liver, but it is less obvious how it would operate in the case of a complex array of tissues like a limb. As we shall see, there is strong evidence that some kind of specific inhibition does exist for the limb.

Since regeneration does occur, we may assume that some sort of initiation signal does exist, whether electrical, chemical, or both. We now must examine the phenomenon of orga1lization of the regenerate. It is intuitively evident that the regenerate must be polarized. Otherwise, the out growth would be a disorganized mass of tissues, bearing no visible relationship to a limb. What is the nature of the polarization? How does the information travel about so that every cell knows where it is in relation to every other cell? I will first discuss the presence of polarization, and then its transmittal to all tissues.

Proximodistal polarization of the regenerating limb has long been known. By cutting through reversed bits of limb grafted onto stumps, Kurz (18) discovered that the regenerates were always replacements for the tissues distal to the level of the cut surface. In other words, the graft. always regenerated a limb, never a new salamander. Myriad experiments since then confirmed the existence of this Rule of Distal Transformation. There must be information present that allows the limb to distinguish distal from proximal (15).

The rules for polarization of the dorsoventral and cranio-caudal axes have been worked out more recently. An elegant exposition has been presented by Vernon French and Peter and Susan Bryant (19). In their model, there exists a system of polar coordinates. If one examines the cut end of a limb, the polarizing information exists at a series of discrete points around the circumference of the limb, and also as a series of points along radii extending outward from the center. Thus, any cell can be located by a distance from the center of the stump and a kind of compass bearing.

The validity of this model has been extensively tested by grafting experiments, and it se ems to explain most of the results seen so far, even the rather bizarre duplication seen when bits of limbs are recombined in various positional combinations (20). The result of this experimentation has been the establishment of rules which show that each cell exists in a defined three-dimensional network which allows it to know exactly where it is and what it is to become. The nature of the information is unknown.

Having established the existence of polarization of the regenerate, we come to the question of how the information is relayed. Some of the answers to questions about control modalities seem to have been answered for some time. Harrison (21,22) established that information travels only along axes of polarity. Turning a piece of tissue so that the axes no longer align releases that bit of tissue from control by the surrounding tissues. In 1946, Monroy (23) demonstrated that lines of control exist in the tissue, and that if grafts are made in such a way that the lines cross at right angles, regeneration is totally blocked. These lines are evidently labile, since they can be destroyed by X-rays (24). Indeed, X-rays can block the transmission of the morphogenetic

information necessary to build regenerates, yet leave the cells alive and able to remain apparently healthy for years. This peculiar phenomenon has been known since 1937 (25). The regenerative ability can be restored by grafting either internal tissues (26) or epidermis (27) back into irradiated limbs, suggesting that the control influences can come from either source, although skin grafts resulted in greater restoration of regeneration. These results, together with some very interesting experiments on coelenterates, led Rose in 1962 to postulate that there is a system of information transfer within the limb stump that he called a "tissue arc" (28). The experiments with *Tubularia* indicated that the morphogenetic influences could migrate for short distances through agar bridges, and that, most importantly, the transmission occurs in only one direction. Material from the distal hydranth will pass down the stem toward the base, but not in the other direction.

The stage is now set for a discussion of the nature of the polarity that causes this unidirectional flow. Matthews (29) discovered in 1903 that coelenterates are electrically polarized. This was reconfirmed for coelenterates and a number of other forms by Lund in the 1920's (30,31). At about the same time, Child discovered that gradients of oxygen consumption existed in the same organisms, as well as others (32). This suggests that the electrical gradients seen in organ isms are the result of redox potential gradients. This polarity, and the demonstration by Lund (33) that growth and differentiation could be controlled by applied direct electrical currents, led Burr and Northrop (34) to postulate in 1935 an "electrodynamic theory of life," modifications and extensions of which have been the foundations of the experiments discussed later in this Chapter, and in other chapters of this volume.

Taking these findings together with the discovery of the phenomenon of electrophoresis, the notion naturally arose that morphogenetically important molecules might be charged, and might be moved about in the organism by its own naturally-occurring fields. It simply remained to be shown that this could occur. I will discuss the use of electric fields to control regeneration later. Here I will only cite some experiments to show that morphogenetically important molecules can be moved in electric fields.

In 1963, I showed that molecules responsible for specific inhibition of anal collars in marine worms could be moved (35). Rose did it for *Tubularia* in 1966 (36). For higher vertebrates, I demonstrated in 1965 that the specific inhibitor of newt lens regeneration could be isolated electrophoretically (37), and Shaw demonstrated mobility of similar information-bearing molecules in frog embryos in 1966 (38). This concept is now well established, and the only remaining question is whether naturally-occurring fields are sufficiently large to do the same within the organism. Rose's experiments certainly seem to suggest that they are (39).

In summary, we can assume that a limb which is removed is recognized as being lost, that in some animals a signal stimulates the replacement of the lost tissues, and that control of the regeneration is carried out along straight lines between cells, using some form of regionally specific information which appears to move in an electric field, and which somehow gives each cell its specific location and task.

DIFFERENCES BETWEEN REGENERATORS AND NON-REGENERATORS

HISTOLOGICAL DIFFERENCES

The earliest event in regeneration, the rapid covering of the wound by epithelium, occurs in both kinds of animals. Thereafter, the responses diverge. In regenerators, the epithelium thickens into a wound cap, and tongues of cells penetrate the underlying tissue. The epithelial cells may either act as phagocytes to remove debris from injured deep tissue cells, or contribute to the blastema (40). In addition, regenerating nerves send many neurites into the interstices between the overlying epithelial cells, thereby establishing a neural–epithelial link. As we shall soon see, this may be vital to regeneration. Dedifferentiation and morphogenesis ensue, ultimately restoring the lost parts.

In non-regenerators, after the wound is covered by its epithelium, connective tissue cells begin to position themselves beneath the epithelium. These cells form a layer several cells thick beneath the epithelium, and begin to lay down large amounts of collagen, thus forming a kind of scar. The connective tissue seems to block the invasion of the underlying tissues by epithelial cells, and also to prevent the contact between epithelial cells and regenerating nerves. If one examines the junction between this kind of healed skin and the underlying tissues, one sees that the regrowing nerve fibers have been diverted, growing parallel to the newly formed collagen fibers rather than into the epidermis.

It is now unquestioned that prolonged contact between the epidermis and deep tissues is an absolute requirement for limb regeneration. Tornier (41) demonstrated it in 1906, as did Schaxel in 1921 (42). If an amputation wound is covered by a seal of whole skin, regeneration is blocked. Godlewski (43) showed that if the wound is even sewn shut, regeneration is blocked in salamanders. More recently, there have been indications (12) that even in humans, the common clinical practice of closing amputation wounds may inhibit a substantial potential for regeneration. When whole skin covers a wound, the dermis seems to prevent the interaction between epidermis and underlying tissues which is a necessary factor for regrowth of a lost part.

The role of nerves is apparently equally important. Singer (44) and Taban (45) first showed the intimate relationships which were established between nerves and epidermis in the early stages of regeneration. Hay (46) subsequently demonstrated that junctions between nerve processes and epidermal cells exhibit many of the characteristics of synapses. The requirement for this contact in regeneration was first shown by Thornton (47). The full role of nerves in regeneration has been studied in detail by Singer and his students (44). The evidence shows that regeneration cannot occur unless there is a sufficient proportion of nervous tissue in relation to the other tissues of the extremity. If nerves are severed or blocked from regenerating, morphogenesis does not ensue. Conversely, regeneration can be stimulated in a variety of forms, including frogs (48), lizards (49,50), chicken embryos (51), and even mammals (10,52) when the nerve supply to the limb is augmented. Regeneration is not always perfect in these instances, but the attempt is at least made if the ratio is raised to a threshold level.

So, the roles of epidermal contact with underlying tissue and of nerve contact with epidermal cells seem clear. What about animals that regenerate as larvae but not as adults? The shift between regenerating and non-regenerating stages occurs at maturation, when the skin becomes toughened and more resistant to drying, and when the response to an injury becomes rapid closure with a connective-tissue scar.

Why the preponderance of aquatic forms or stages among regenerators? The question is easy to answer with respect to physical characteristics, but more difficult in terms of selective advantage. Aquatic forms tend to have thinner, less heavily keratinized skin which does not heal rapidly by formation of a scar. Thus, there is sufficient time for the interactions between epidermis, underlying tissue, and nerve to ensue following amputation.

It would seem just as important for a land-dweller to have fingers as for an aquatic salamander—perhaps even more so. The answer may lie not in the advantage of regeneration, but in the disadvantage of slow wound healing. A small animal whose metabolism is relatively slow in cool water, and whose skin allows for a considerable degree of direct oxygen exchange, may be relatively insensitive to blood loss. Also, for an aquatic form, dehydration is a negligible problem. If an animal can get along without much blood, and is in no danger of drying out, it can afford to repair a loss at a leisurely pace. A land-dweller has no such luxury. Skin thin enough to exchange gas would also lose water vapor. Keeping exchange through the skin is necessarily rather limited. If metabolism is very rapid, or the animal is very large, oxygen exchange potential via the blood becomes critical, and neither blood nor fluid loss can be borne with impunity. For both reasons, rapid wound healing is a decided advantage in a land-dweller. Land forms have apparently given up the luxury of slow healing and regeneration in favor of fast fluid-tight healing.

ELECTRICAL DIFFERENCES

The first systematic study of electrical events during regeneration (at least in amphibians) was undertaken by Alberto Monroy in 1941. He examined surface potentials in salamanders (*Triton*) during tail regeneration (16). Similar studies have been done on limb regeneration at intervals ever since. The seminal work was done by Becker in 1961 (53). For regenerators, the initial measurements of the tip of the limb stump revealed that the wound surface became strongly negatively charged with respect to the center of the back. This initial charge is attributable to the injury potential and the products of damaged cells. As soon as the epithelium covered the wound, the potential reversed, and became strongly positive. In regenerators, Becker found that the potential then reversed again after about 4 or 5 days, becoming negative at about 10 to 25 mV. During the course of morphogenesis, the potential gradually rose toward a very slightly positive baseline value. The picture was different for non-regenerators. They underwent the initial negative spike, and the reversal to positive, but never reversed again to the negative potential of the regenerators. These findings have been the source of much controversy. Becker concluded that potentials arose in nerves, and demonstrated that they were abolished by

anesthesia and denervation. Later investigators like Rose (54), Borgens, et al. (55), and Lassalle (56) did not find the denervation effect. Borgens and Lassalle attribute the electrical events to transepithelial potentials, produced by pumped ionic flow across the skin, and modifiable by changing the external ionic composition or by altering ionic permeability by various means. Lassalle has shown the same basic pattern of potential differences between regenerators and non-regenerators as reported by Becker. Lassalle attributes all measured potentials to epithelial status, but it is possible that the mechanisms postulated by both him and Becker are operative. It is clearly not reasonable to attribute all limb potentials in amphibians to transepithelial ionic passage. On the other hand, perhaps it is reasonable to conclude from Lassalle's data that surface potentials do not normally control regeneration in limbs. The story becomes more confusing when the data of Borgens et al. (57) are considered, since they found that artificial enhancement of the surface potentials can enhance limb regeneration in and that lowering it can inhibit regeneration in newts (58).

It is certainly possible to measure significant potential differences between the center of the back and the tips of the limbs in humans (unpublished data). Since humans are not usually wet, it would seem difficult to attribute these differences to ion-pumped transepithelial potentials. There may be many sources for such potentials, including potentials along nerves, streaming potentials derived from blood flow in arteries, and muscular activity.

The development by Jaffe and Nucitelli (59) of a miniature vibrating-probe electrometer has materially aided the study of electrical events associated with regeneration. Using this device, Borgens et al. (54,60) found that the charge distribution differed be tween adult newts, which regenerate, and adult frogs, which do not regenerate. They found that currents at the center of the limb in frogs were much lower than in newts, and attributed the difference to the large lymphatic spaces beneath frog skin which can shunt currents (61). These findings reflect the histological observations that only regenerators retain tight contact between epidermis and underlying tissues. They found no relationship be tween the stump currents and innervation.

Perhaps, as Lassalle suggests, the positive potentials observed in the limbs of amphibians are principally the result of transepithelial ionic passage, and can be altered slightly without affecting limb regeneration. The later-appearing strong negative potentials seen in regenerators may reflect nerve penetration of the epidermis. The final potential occurring at the surface of a limb during regeneration is perhaps a sum of the transepithelial potential which can vary considerably, and the internally derived potential, which may not have the same phase relationship as the transepithelial surface potential. Such a synthesis might explain some of the seemingly contradictory results obtained by the various laboratories investigating the phenomena.

In any case, it seems clear that the electrical behavior of regenerating and non-regenerating forms is quite different, whatever the source of the potentials. It now remains to be seen whether those differences can be exploited to advantage.

DIRECT CURRENT STIMULATION

The initial experiments aimed at electrical stimulation of limb regeneration were carried out by Bodemer (62) in 1964, He used an indirect method, that of stimulating the brachial plexus of frogs after forelimb amputations, and evoked a regrowth of tissue and elongation of the stump. I think that perhaps my efforts came next (63). Becker had suggested in 1961 that imitation of the regenerating electrical pattern in a non-regenerator might produce regeneration. The problem lay in devising a convenient means to do so. At the time, implants seemed too large for the small animals being used, and electronic miniaturization had not progressed to its present remarkable state. My thought was rather simple: why not let the frog be its own battery? Accordingly, I decided to implant a simple bimetallic couple consisting of silver and platinum wires, insulated except at their tips. I chose the metals based on their relative biocompatibility and electrochemical activities, hoping for some current flow in the salty extracellular fluids without major toxic effects of the metals. As it happened the choice was serendipitous, and the pair of metals generated the proper current levels to induce regeneration, I reported the results in 1967, demonstrating a rather remarkable degree of regeneration, including all of the tis sues normally present in a new limb, plus some attempts at organization into wrist-like structures. Becker tried the same method in postnatal rats (64), adding a resistor between the silver and platinum to control the amount of current generated, and reported remarkable results. His paper generated a good deal of controversy, based on the brief period required to produce the results. To my knowledge, however, nobody ever has exactly repeated his experiments, so the criticisms were and are entirely speculative, and can be ignored until an accurate reproduction of the experiments is undertaken.

In these early experiments, the electrodes were simply inserted into the ends of the bones (into the marrow cavity) distally, as a means of fixing them in place. In my experiments maximal regeneration ensued if the silver end was placed distally. The results of these experiments were disappointing in that the organization of the regenerates did not closely resemble that of a normal limb. As information developed regarding control of polarity in the normally forming limb, I decided that the problem might lie in how the implants were being made. I reasoned that better results might be had if the stimulating electrode were placed at the wound surface in the spot where the apical ectodermal ridge normally forms. To do this, I had to design a new type of implant, using a battery in the center of the frog's back, with a long cathodal lead wire coming down the limb which could then be fixed in place at the end. This work was reported in 1974 (65). It confirmed that placement of the electrode in any position other than the dorsal postaxial quadrant of the wound surface resulted in poor regeneration. If, however, the placement were appropriate, regeneration of remarkable completeness could be obtained. In one instance, a perfect hand formed. Movable digits regenerated in 23% of the cases.

Borgens et al. (66) were concerned that electrode products might be causing the results rather than the electric fields, so they modified the experiment by using conducting wicks instead of wires to deliver the current to the end of the limb. They also succeeded in inducing limb

regeneration, but did not obtain particularly good organization of the regenerated tissues. They used a current of 0.2 μA compared to 0.103 μA used in my study. Whether this difference or whether the electrode products their method was designed to eliminate may have contributed in my experiments are unresolved issues.

Sisken et al. (52,67) reported the results of a long series of experiments in rats in 1979 and 1984, showing tissue regeneration and outgrowth. The response was augmented by the simultaneous injection of nerve growth factor. Libbin et al. (11) demonstrated electrical stimulation of rat limb regeneration. In 1981, I reported that implantation of electrodes into the dorsal postaxial position in subadult rats initiated regeneration at a current of about 0.1 $\mu A/mm^2$ (68). The regenerates exhibited joints, muscles, cartilage, bone, and some suggestion of organization into wrist-like elements, but no complete regenerates were obtained. Sisken et al. (52) reported that implanted bimetallic strips enhanced the response of regenerating rat limbs to implanted fetal nerve tissue.

Thus, the evidence shows that regeneration can be initiated in normally non-regenerating forms. The regeneration generally proceeds only partially, and includes the formation of all of the new tissues required to form a limb. The final organization of the new tissues is incomplete, except in extremely rare instances, and then only in *Anurans*.

Why do DC fields stimulate regeneration? I think the answer may lie in the considerations raised in the previous Section. Which of the requirements for regeneration are satisfied by DC stimulation? Firstly, there seems to be no doubt that a DC field of appropriate strength provides an adequate signal to initiate regeneration. Experience in my laboratory suggests that the thresholds for such stimulation are narrow. Currents of less than 10 nA/mm^2 are ineffective, as are currents of 1 $\mu A/mm^2$. I obtain the best results at a current of about 100 nA/mm^2. The experience of Borgens et al. (66) suggests that even 200 nA may be too much in *Rana pipiens*, the same test subject that I used. If the current is too high, the regenerate begins to consist more and more of just disorganized connective tissue. Above 1 $\mu A/mm^2$, only scar is produced.

Secondly, a DC field seems able to act as a polarity- inducing stimulus, or at least one which can act in concert with naturally occurring polarity. Our experiences with varying electrode placement supports this idea, although more work is needed.

Thirdly, some sort of in formation transfer surely does occur in these regenerates. The degree of organization, though generally imperfect, suggests that the regenerates are not simply bits of disorganized flesh. In *Anurans*, the degree of perfection of the regenerates varies widely, but can approach perfection quite closely on rare occasions. Something must be organizing the tissues. One possibility is that the field acts directly to organize information transfer, which in turn organizes the epithelial–mesenchymal interactions necessary for the initiation and completion of regeneration. This option implies that all of the information-bearing molecules are present normally, and that regeneration in non-regenerators only requires the right stimulus. A second possibility is that the DC field simply induces the rapid regeneration of neurites, which then penetrate the wound epithelium before scar formation can prevent it. It is the completion of

a neural-epithelial circuit which produces the regeneration. It is possible to encourage nerve growth with fields and direct neurites toward a cathode. Thus, it is conceivable that the electrodes simply speed up neurite formation and direct their growth into a particular point in the epithelium which then establishes the necessary polarity and interactions to engender regeneration. So far as I can determine, there has not been a systematic study

of this possibility. I have made preliminary observations that indicate that neurites do indeed penetrate the epidermis. in frogs, but not in the large numbers characteristic of salamanders. Treatments that delay scar formation (69-71) also induce partial regeneration, so this option may indeed be correct. The nerve augmentation studies also support this hypothesis. A third possibility is that the fields somehow stimulate the cells to begin formation of the information necessary to regeneration, and also to proliferate to provide the tissue raw materials. Our own experience (72) suggests that pulsed fields can enhance thymidine uptake by cells in the skin's basal layers, and other chapters in this volume fully explore other stimulative cellular effects. There is a wealth of evidence to suggest that electric fields can engender transcription and cell proliferation, so this option is also a viable one. Perhaps tissue organization occurs as a result of a combination of these mechanisms.

It is necessary to consider why regeneration is so seldom carried to completion, or is so often poorly organized, especially in mammals. Given the obvious fact that regeneration can be stimulated to begin, and that even mammals have the obvious capacity to at least partially organize a regenerate, why don't we obtain perfect limbs in non-regenerators? There are two possibilities to consider. Regeneration may stop because scar formation cannot be delayed indefinitely. Scar prevents information transfer, thereby halting regeneration. There have apparently been no experiments combining suppression of scar formation (with cortisone, collagenase, or some such agent) with electrical stimulation. Another possibility arises from the work of Stocum with salamanders (73). In some combinations derived by grafting bits of limbs together, the regenerates appear to progressively lose more of their organizational maps as extension of the regenerate occurs. This finally results in a spike-like outgrowth, rather than a normal limb. Under most grafting conditions, gaps of missing positional information are intercalated to restore a perfect map. However, if like areas are approximated, the limb may not recognize that something is missing, and fail to regrow a perfect regenerate. One of the characteristic forms of regenerates seen in *Anuran* and mammalian experiments is a spike-shaped outgrowth. This suggests that *Anurans* and mammals do not have complete positional information maps, and that regeneration therefore cannot be expected to be complete. This is a discouraging possibility because it suggests that there is no hope of inducing perfect limb regenerates in non-regenerators. But even if the pattern is incomplete, hope looms on the horizon in the form of retinoids. These analogues of vitamin A are apparently capable of inducing pattern duplications in the transverse axes of limbs (74). Their action is apparently stage-specific (75), but it may be possible to use retinoic acid in con junction with DC fields to produce complete regenerates.

The final chapters of DC field stimulation of limb regeneration have by no means been written. The area remains largely unexplored, and should provide the basis for much experimentation.

PULSED MAGNETIC FIELD STIMULATION

One of the objections often raised in experiments with DC fields is the matter of implantation of electrodes. Aside from the irritation and potential for infections introduced by implantation procedures, one must consider the myriad electrochemical reactions that occur at an electrode surface. Study of the metabolic consequences of these reactions is in its infancy. It would therefore be highly advantageous to be able to produce the same effects as DC fields using a non-invasive method. The simplest way to achieve this effect is to subject the animal to pulsed magnetic fields (PMF) which induce currents in the tissue that are directly proportional to the conductivity of the medium, the strength and shape of the pulses, and the geometry of the system. Use of pulsed magnetic fields introduces a whole new set of complexities, but they are at least a little better understood than the complexities at an electrode.

There is apparently only one published study of the effects of PMF on limb regeneration (76). After mid-forearm amputations, newts were placed in small individual aquaria and subjected to three types of PMF. One was a single pulse repeating at 72 Hz, (waveform 1), and the other two were complex pulse trains operating at 15 Hz. The individual pulses in the pulse-trains had widths of 22 and 6 μsec (Waveform 3 and 4 respectively) (76). The trio of pulses produced different energy inputs to the regenerating system (Figure 1). Figure 2 illustrates a distillation of the results. The single pulse treatment induced a premature differentiation of fingers in the regenerate. The mass of tissue was about the same as that of the control blastema, which had only reached the late cone or early paddle stage. Generally, these animals regenerated only three fingers, instead of four. Waveform 4 stopped regeneration completely. The wound was covered by a thin layer of epithelial cells, indicating that cell migration could proceed, but none of the usual events in regeneration occurred. Even the protruding bone left behind as the stump tissues contracted was not eliminated, as is usually the case within a few days. Regeneration was markedly enhanced by waveform 3. The regenerates at 21 days looked like controls at about 45–50 days. The regenerates were large, with four long finger outgrowths. In effect, the whole process had simply been accelerated, with everything in proper phase, so that a normal regenerate appeared in a very short time.

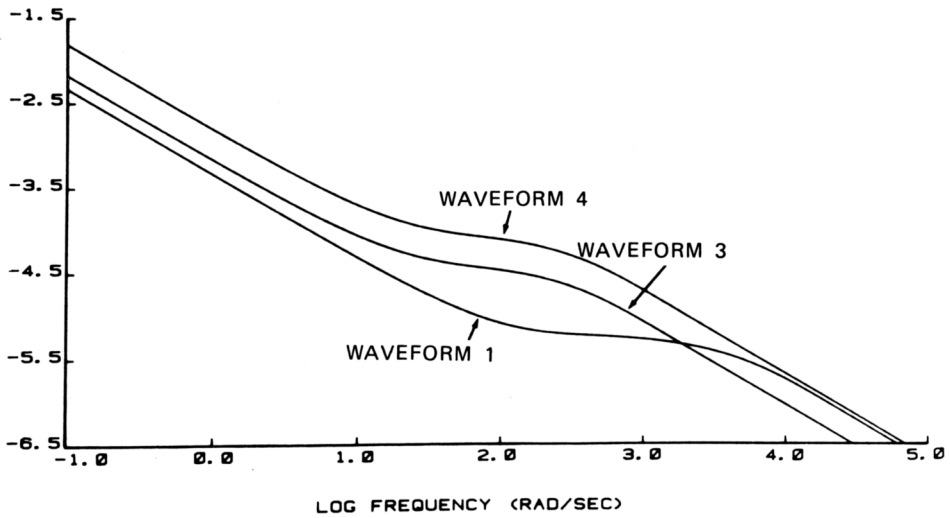

Figure 1. Real axis (LaPlace) frequency spectra for Waveforms 1, 3, and 4. Waveform 1 is a simple asymmetrical pulse repeating at 72 Hz. Waveform 3 is a complex asymmetrical pulse burst with a 22 microsecond component which repeated at 15 Hz. Waveform 4 is similar to waveform 3, except that the 22 microsecond component is replaced by a 6 microsecond component. Note that waveforms 1 and 4 match at high frequency, 1 and 3 are close, but not matching at low frequency, and 3 and 4 are parallel at all frequencies, but not matching in amplitude. The Y axis is a dimensionless logarithmic relative amplitude scale, and is thus not labeled with specific units. (Reproduced from (76), with permission.)

In sum, it appears that PMF can have a variety of effects including altered phase relationships that disturb the normal course of events, inhibition, and acceleration. The inhibition and acceleration may be due to simple modifications in the overall rate-controlling processes of regeneration. The premature differentiation is more difficult to explain. Since cell proliferation was stopped, and differentiation started w ell before their normally appointed times, something selective must have occurred. Since the field was active from the time of amputation, it suggests that there are multiple processes going on simultaneously in normal regeneration, and that the timing of the steps must be precise to obtain a normal regenerate. If one of the steps is accelerated, while others are not, the phase relationships become disturbed, and so does regeneration.

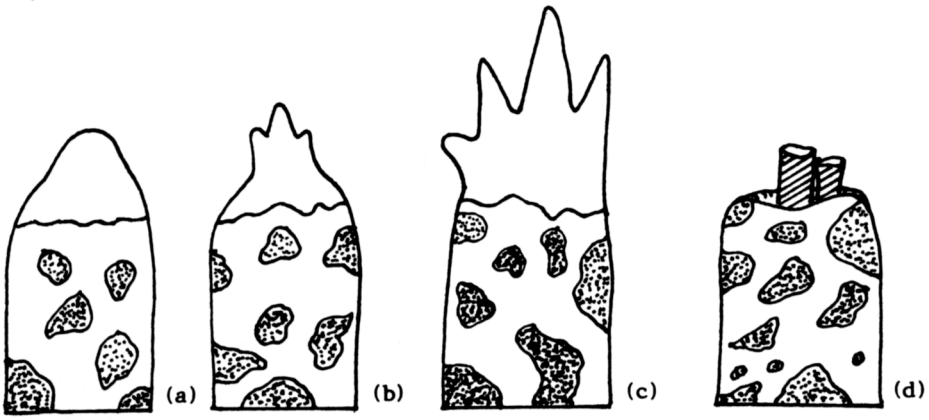

Figure 2. Drawings of typical results of PMF-treated regenerating newt limbs: (a) Control at 21 days. The regenerate is a late-cone early-paddle stage blastema; (b) This is a typical limb treated with waveform 1. The amount of tissue present is not much greater than controls, but differentiation is far advanced, indicating separation of the processes of tissue accumulation and differentiation; (c) A limb treated with waveform 3. Regeneration is far advanced, and apparently normal in morphology; (d) Waveform 4 has stopped regeneration completely. Ordinarily, the tips of the radius and ulna left exposed as the tissue retracts would have been eliminated by osteoclasts in the deep tissues. Here even that process has been interrupted.

The fields induced by PMF are inherently less specifically polarized than DC fields. The induced currents are circular, and lie in a plane perpendicular to the magnetic field lines. However, an animal placed between two coils is not subjected to a field defined only at one particular point. Currents are simultaneously generated everywhere within the animal's body. It would seem, therefore, that unless one could find a pulse that produced a differential effect upon the processes essential to regeneration, PMF would he of use only as a general technique to enhance and speed up the process. The results with the newt limbs suggestthat both may be possible. Some selectivity is obviously possible, as well as generalized effects.

PMF have been used experimentally for at least ten years to stimulate bone healing in non-unions (77) and clinically-approved devices are now available. Dal Monte's chapter in this book reviews the evidence. I think it is safe to state that they are principally of use in stimulating generalized tissue regeneration such as bone healing. I cannot see how PMF can be used to satisfy the polarity requirements of limb regeneration. PMF apparently act primarily through alteration of small ion phenomena at the cell membrane, and unless the cells are regionally programmed to respond selectively, a territorially precise response to PMF seems unlikely. I am not suggesting that PMF are of no use in stimulating limb regeneration. They clearly are. If one of the problems in obtaining limb regeneration is providing enough tissue to make a regenerate, and doing so quickly, or perhaps in inhibiting scar tissue formation, PMFs may play an important role. One could certainly envision a regenerative scheme in which DC fields played a polarizing/ initiating role, while simultaneously applied PMF stimulated blastema formation by cell proliferation, scar suppression, and ultimately a trigger to differentiation.

Such a scheme implies a great de al more information about the controls of regeneration, and about the details of action of PMFs than we now possess. We need accurate models which will predict the effects of an applied field, plus a detailed encyclopedia of the influences controlling normally-occurring regeneration. For example, to say that there is a map of positional information which controls regeneration is a step forward, but it does not allow us to recreate the map. We must know what the map is made of, and what determines how it is constructed. Without a knowledge of what the map constituents are, it is hard to devise precise means of stimulating whatever "map synthetases" there may be in an animal.

None of this is to suggest that experimentation with PMF and OC fields should stop until all of the details of regeneration are worked out in full. Nothing could be less useful. The history of science, biology in particular, is replete with case histories of the cart going before the horse. Most often, something is first found empirically, to be followed by experimentation and elucidation of the mechanisms. Limb regeneration need be no different. I only suggest that a confident prediction of success would be greatly enhanced by more perfect knowledge. We certainly know enough now about effective thresholds and useful waveforms to be actively engaged in experimentation. Sooner or later, I am quite certain that someone will, by rational progression of thought or by serendipity, discover the key to stimulated limb regeneration which progresses to complete perfection in most cases. I have attempted to suggest some avenues of approach which seem reasonable. I hope that someone will explore some of them the potential rewards both for science and the amputee are very considerable.

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