ELECTRICAL STIMULATION OF PARTIAL LIMB REGENERATION IN MAMMALS

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SELF-REPAIR is a primary property of living systems. It reaches its highest expression in the regenerative growth processes evidenced by some of the amphibians, which are capable of the regrowth of a complete and fully organized vertebrate extremity containing varied tissues. Unfortunately man retains only vestiges of this capability; in man true regenerative healing occurs only in fractures. All other processes of repair in man demonstrate less competent mechanisms, including scarification or the simple enhancement of normal processes of cellular replacement.

It is important to characterize true regenerative growth accurately as the process that begins with the formation of a blastema. This mass of primitive and apparently undifferentiated cells formed by a variety of cellular processes can differentiate into the complete range of cell types necessary to replace the missing part. Following the formation of the blastema, mitotic activity rapidly produces the cellular mass necessary for replacement; a wave of differentiation producing the complex missing structure begins proximally and extends distally from the original site of injury. Obviously this is a highly effective process. If it could be restored to man, it would be of very great value, not only in replacing the complex parts of an extremity, but perhaps more importantly in the

This study was supported in part by grants from the Public Health Service, National Institutes of Health, and the Veterans Administration Research Service.

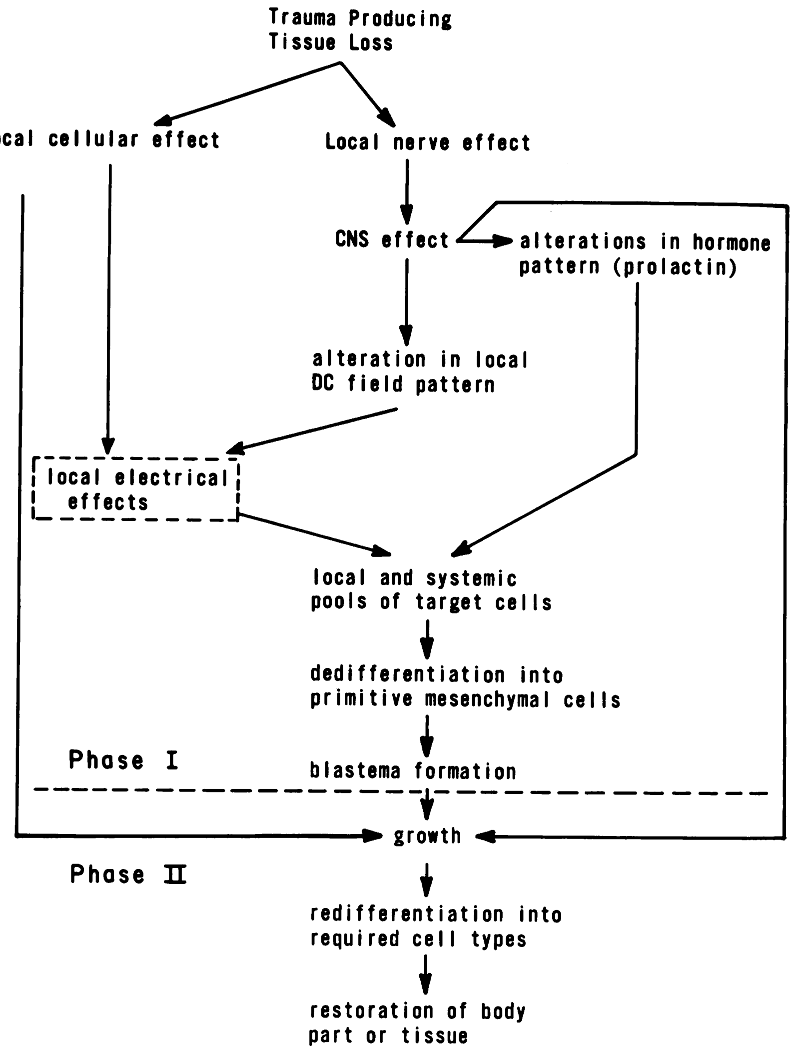


Fig. 1. Theoretical control system governing regeneration. The local electrical effect, measurable as the current of injury, is the resultant of a number of factors and is the primary trigger for initiating the cellular response, Phase I. The two phases are regulated by separate control systems.

efficient replacement of single tissues or organs that have been damaged or lost.

While regeneration has been studied since Spallanzani's first written report in 1768,[1] it is only within the past few decades that a chain of events has taken place that enables us to gain insight into the primary mechanisms involved and permits us to manipulate the process itself. Thus we are no longer limited to simple observations.

The first events in the recent series were the report by Rose in 1945[2] and the subsequent work of Polezhayev the following year,[3] which indicated that the initial injury was related causally to the regenerative process and did not merely provide the need for the process to occur. Implicit in the work of both these authors was the germ of the idea that the extent of the injury was related quantitatively to the process, perhaps in threshold fashion. Sometime later Singer[4] established an almost mathematical relation between the total mass of nerve in an extremity and the total mass of the extremity that demonstrated a true threshold for regenerative ability. Augmentation of either injury or nerve supply in experiments on animals not normally capable of regeneration resulted in some measure of regeneration. The nerve augmentation procedure has been reported by Mizell[5] to have produced some measure of regeneration of the hind limb in the newborn oppossum. The oppossum, however, is born in a relatively "fetal" stage with the hind limb particularly undeveloped and capable of some measure of regeneration after simple amputation. While it has been known for some time that hormonal factors were essential to regeneration, it remained for Thornton and his group[6] to identify the responsible hormone as prolactin. Throughout this period, however, attempts to restore regeneration to postfetal mammals and to identify some specific chemical entity as the initiator of the process in nature were unsuccessful.

The approach adopted in our laboratory in 1957 was unique primarily in its basic concepts. We looked upon regeneration as a cellular process regulated by a precise control system which should be amenable to attack by application of engineering control-system theory. Further, we postulated that cells and tissues possessed solid-state or electrochemical properties that permitted small electrical currents and potentials to act as important control signals in the control system. This approach has proved to be a powerful tool, with potential applications beyond the present study.

We have pursued these concepts in experiments on the measurement of low-amplitude electrical phenomena in a variety of tissues and in an analysis of their solid-state characteristics. It has been possible to describe what appear to be functionally significant electrical steady-state phenomena related to growth processes[7-13] and to demonstrate properties

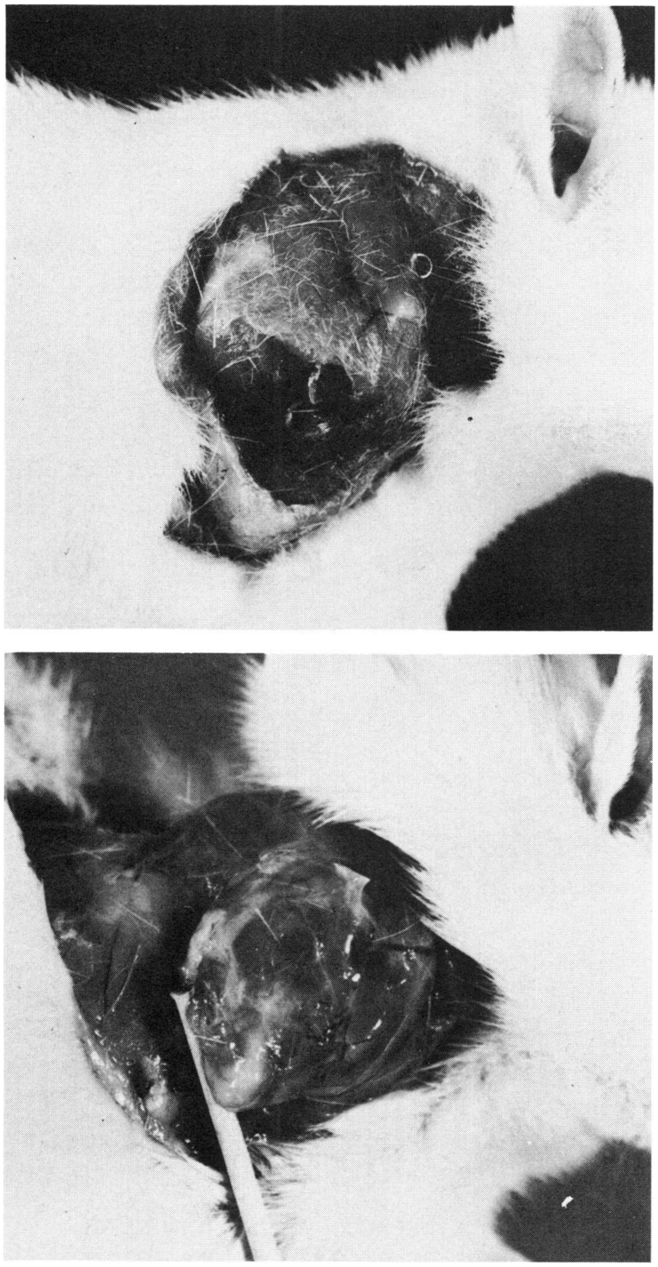


Fig. 2. Gross photographs of amputation site: *Top:* immediately after insertion of bimetallic device. The guillotine-type amputation is evident and the proximal loop in the inserted device is visible emerging from the musculature lateral to the shoulder joint. The skin is subsequently closed over the wound and the animal given one injection of 0.2 cc. of procaine penicillin (Pfizer). *Bottom:* A typical cone-shaped regenerate seven days after implantation of an effective device.

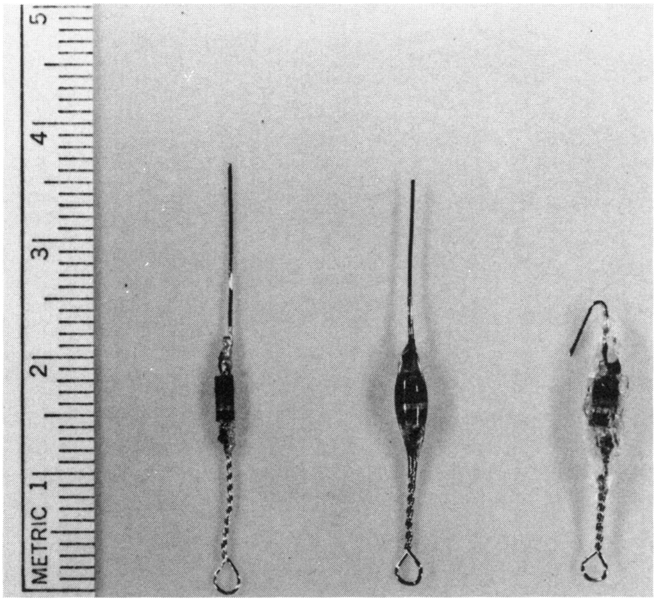


Fig. 3. Stages in construction of bimetallic device. *Left:* the platinum wire *(upper)*, resistor and silver wire *(lower)* are soldered together and the proximal loop formed in the silver wire. *Center:* the resistor and both solder joints are encapsulated in epoxy cement and permitted to cure. *Right:* the epoxy-encapsulated portion and both wires are coated with medical grade silicone (Dow Corning), leaving exposed the proximal loop and the bent distal portion which is inserted into the open medullary cavity of the humerus.

very suggestive of such solid-state properties as electron transfer and semiconduction.[14-18] Several of these studies have provided a sequential series of experiments that led us to our present state of understanding of regenerative systems of growth.

In 1961, in our initial publication[19] in this series, we described differences in the current of injury versus time at limb amputation sites in vertebrates capable of regeneration, such as the salamander, *Triturus viridescens*, and in closely related animals not capable of this activity, such as the adult frog, *Rana pipiens*. Six years later Smith[20] reported the restoration of some measure of limb regeneration to the frog by implantation of a bimetallic electrogenic coupling which crudely simulated the current of injury observed in the salamander. In the interim a long series of investigations on the self-organizing aspects of bone[21-23] led us to identify the control system that regulates the healing of fractures in amphibia as one that possesses electrical phenomena as significant control

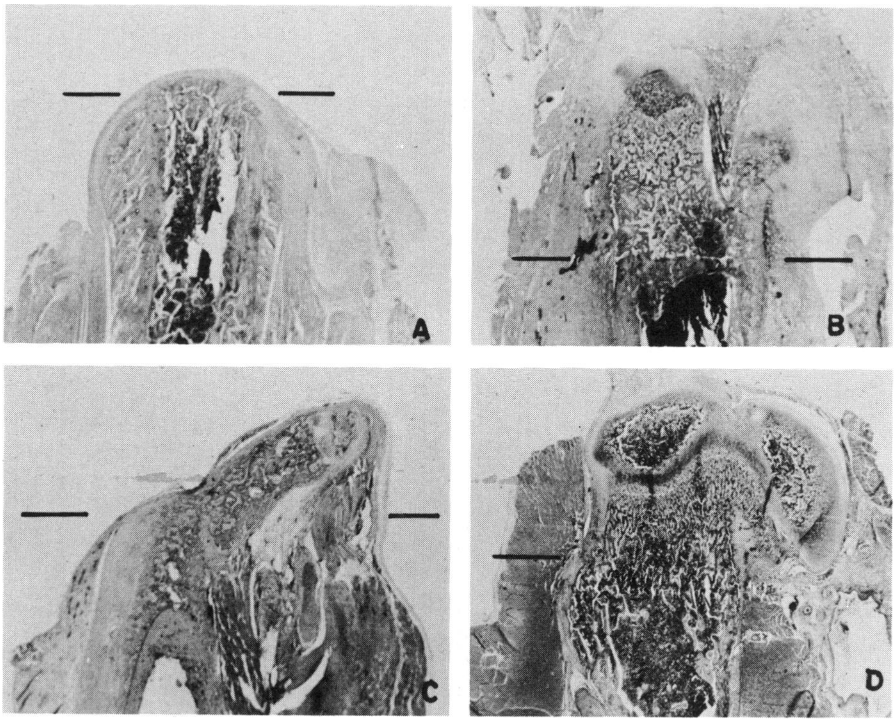


Fig. 4. Photomicrographs of longitudinal sections of the distal humerus on the seventh postoperative day (except third postoperative day for D). All sections were stained by standard H. and E. technique and are enlarged 10 times. The line in each instance indicates the original level of amputation.

A. Control, no device inserted. There is subperiosteal osteogenesis and a tendency to bridge across the cut surface with new bone. A small fibrous cap forms across the end of the bone and the muscles retract proximal to the original section.

B. Ten megohm silver-platinum bimetallic device. Longitudinal growth of the humerus has occurred with the formation of two distal "condylar" structures consisting of cartilage cells. New muscle fibers are visible surrounding the shaft as well as in the cleft between the two cartilagenous masses. The original humeral shaft has completely closed off with a bridge of bone.

C. Ten megohm silver-platinum bimetallic device, section taken obliquely. Original humeral shaft is visible as an elliptical structure on the lower portion of the print. The new bone between it and the line of amputation is not longitudinal growth but an oblique section through the subperiosteal new bone. Longitudinal growth of the humerus has occurred past the amputation site containing a well formed distal epiphyseal plate and an epiphysis containing cells resembling hematopoietic marrow. Again skeletal muscle regeneration is visible around the new bone growth. The curvature of the regenerate is rather typical and appears to follow the electrical field.

D. Ten megohm silver-platinum bimetallic device. The animal in this case was sacrificed on the third postoperative day and an almost complete restoration of the normal distal humerus is evident. The insertion point of the electrode is visible as a band of fibrous tissue entering just proximal to the new growth on the right. Note that the regenerate is displaced laterally toward the side of insertion of the device. The low level of amputation in this instance indicates that a small portion of the original epiphyseal plate may have been retained.

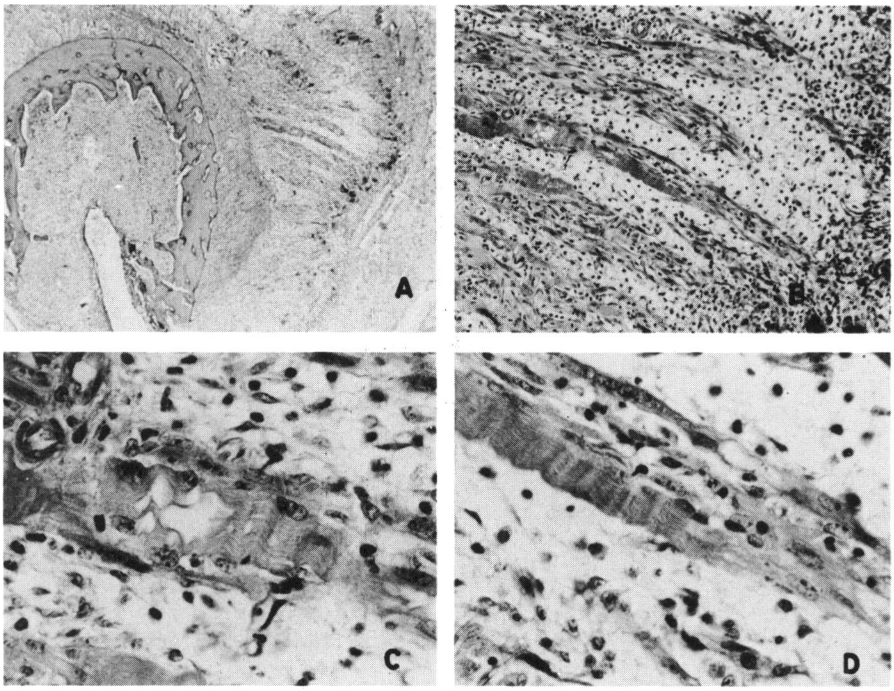


Fig. 5. Muscle regeneration as evidenced by a transverse section through level of insertion of device.

A. Section viewed at 10× magnification. The original position of the distal electrode is evidenced as a cap in the bony shaft at the bottom left. There is early subperiosteal osteogenesis laterally and a radial arrangement of separated muscle fibers in a loose cellular stroma.

B. View of the muscle area at 40×.

C. and *D*. Magnification at 100× of the central portion of *B*. Linear syncytial arrangements of cells are visible with some cross-striated fibers evident.

signals for cellular activity.[24, 25] The importance of this work lay in the fact that we were able finally to quantitate the effective parameters of electrical activity and to establish a coupling mechanism that had cellular activities related to the process of healing.

By late 1970 we were able to state that injury to living organisms results in a series of complex electrical events at the site of injury and that these are directly responsible for such cellular events as dedifferentiation and mitotic activity. We were then able to correlate many of our studies with work done by others and to propose a theoretical scheme for the control system that regulates regenerative healing (Figure 1).[26] The key element in the scheme was the concept of regeneration as a

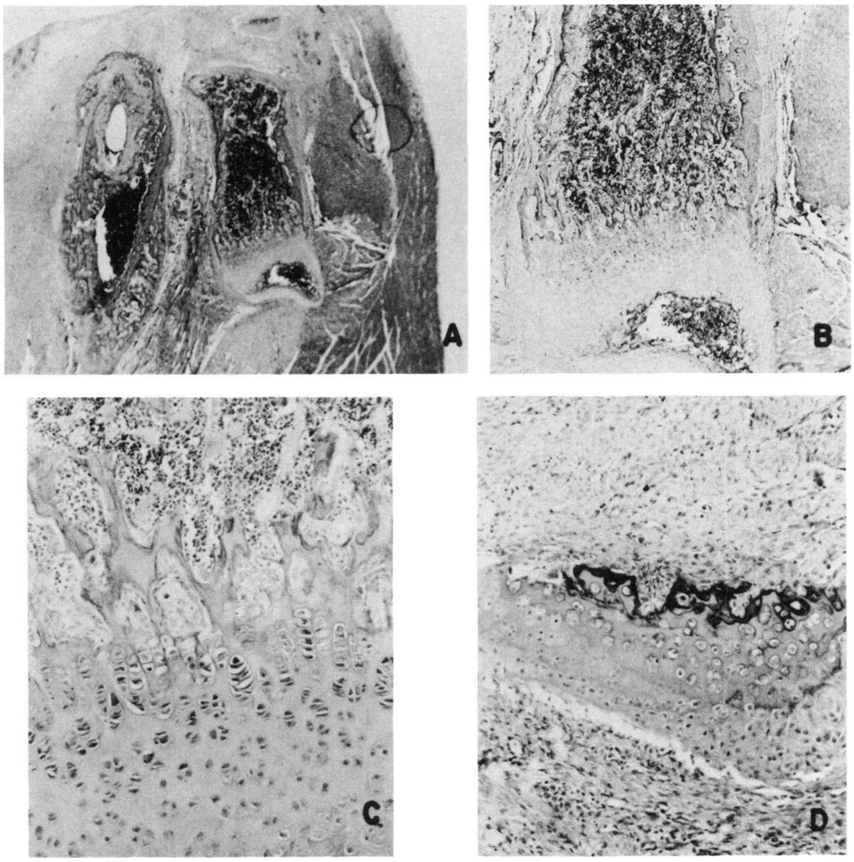


Fig. 6. Formation of a supernumerary limb adjacent to residual shaft of humerus. A 10 megohm silver-platinum device was inserted and the animal sacrificed on the seventh postoperative day.

A. Longitudinal section 10 times enlarged. The residual humeral shaft is sectioned obliquely and is visible on the left of the section. A large mass of undifferentiated tissue is present adjacent to this, containing a well-formed humerus with single proximal epiphysis, epiphyseal plate, bony shaft containing marrow cells, and a distal cartilaginous surface. Proximally there is a tissue condensation suggestive of the scapula and shoulder joint and, distally, two small osteocartilaginous anlagen, one visible, presumably radius and ulna.

B. Detail of the proximal end of the supernumerary humerus at 40× magnification. The organization with a single proximal epiphysis, epiphyseal plate and bony shaft is evident.

C. Enlargement at 100× of the proximal epiphyseal plate of the supernumerary humerus showing normal histological and cytological detail.

D. Enlargement at 100× of the distal radial or ulnar anlage, with a zone resembling cartilage that is presumably calcifying on its distal aspect and with an adjoining mass of active cells.

two-step process that functions under different sets of controls. The first process was conceived of as a simple electrical trigger mechanism, with threshold values, stimulating the cellular activities that result in the appearance of the blastema. The second step was viewed as an initial complex transmission of data to the blastema, with its subsequent establishment as a self-organizing system capable of growth and redifferentiation as an appropriately organized and located extremity.

Since the appearance of a blastema at the site of injury in a mammal has never been observed except in the case of the fracture "callus," the question of whether the mammal had sufficient data-transmitting ability to accomplish the second step of the process has remained unanswered. What was certain, however, was that in all tissues except bone the mammal lacked the ability to produce a blastema. The schematic control system predicted that this deficiency was due to one of three factors: insufficient current of injury at the site, insufficient or inappropriate hormone responses to the injury, or inability of mammalian cells to respond to appropriate electrical and hormonal factors by either dedifferentiation or mitosis.

We determined to attempt the simple experiment of simulating, in crude fashion, an appropriate current of injury at a site of amputation of a mammalian limb. The test animal was the 21-day-old male Sprague-Dawley rat with complete surgical amputation of the forelimb at the level of the mid-humerus (Figure 2). The electrical environment was simulated by implantation of bimetallic electrogenic devices (Figure 3) modified from Smith's type by the insertion of a current-limiting resistor. This latter was necessary to bring the current level of the simple bimetallic coupling into the maximally effective range previously established in our study of fracture healing.

The results of these initial experiments have far exceeded expectations[27] and we have continued to evaluate the electrical parameters only, as yet without recourse to hormonal supplements. In those instances in which optimally effective types of devices were implanted, we have succeeded in producing a process of growth which approximated the late cone stage in salamander regeneration of the limb of the salamander and involved complete restoration of the missing portions of the humeral shaft, with reasonably appropriate organizational pattern (Figure 4). Regeneration of skeletal muscle accompained this osseous growth by a variety of pathways (Figure 5). In one instance a blastema was formed

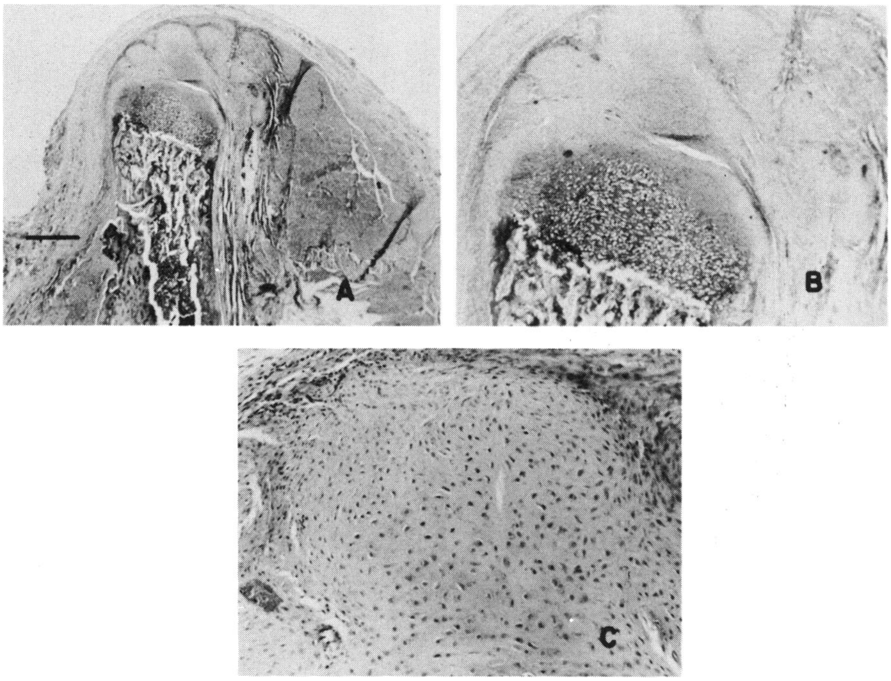


Fig. 7. Longitudinal section through limb sacrificied at 21 days after implantation of 1,000 megohm device.

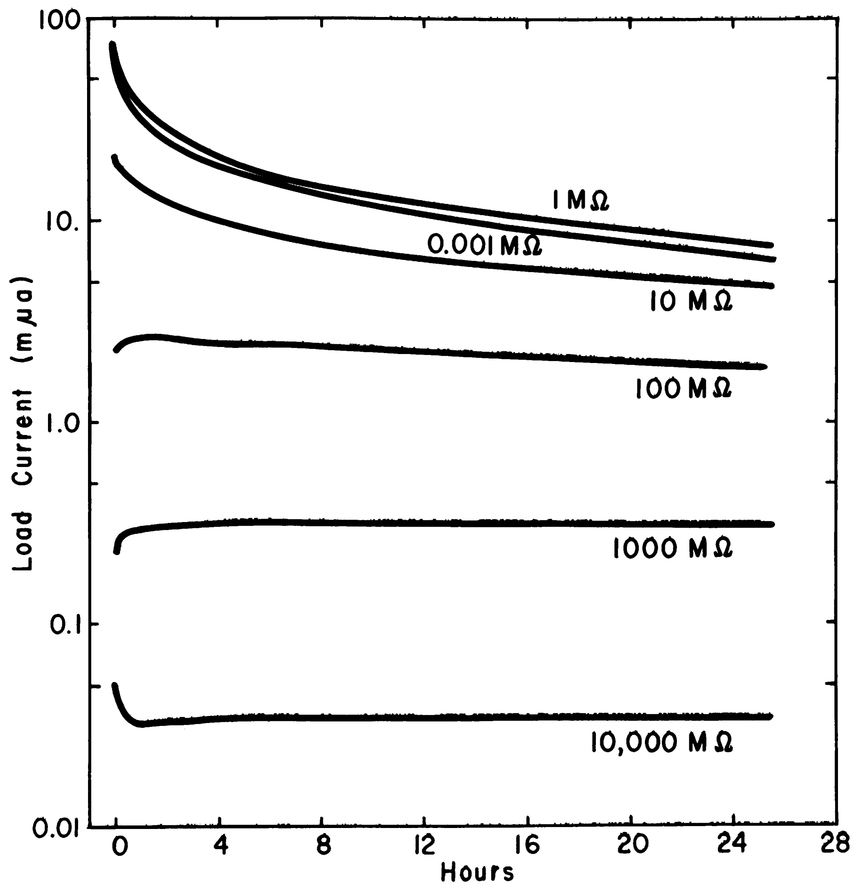
A. Magnification at 10× demonstrating over-all organizational pattern of longitudinal bone growth, with a distal cartilaginous cap and an arrangement of young precartilage cells distally suggestive of digitation. Original line of humeral amputation is indicated.

B. View of the distal cartilaginous cap and "digitation" structure, 40×.

C. Central mass within digitated structure at 100×. The cellular arrangement is typical of that observed in the embryonal digitation of the mammal with a central mass of precartilage cells with peripheral condensation.

which was larger than normal; in addition to longitudinal growth of organized bone with epiphyseal center, a complete supernumerary humerus appeared, with small anlage for radius and ulna and scapula (Figure 6A). This structure was surprisingly well organized and demonstrated all the necessary cell types as well as all the complexities of tissue structure, including a well-formed epiphyseal plate (Figure 6B).

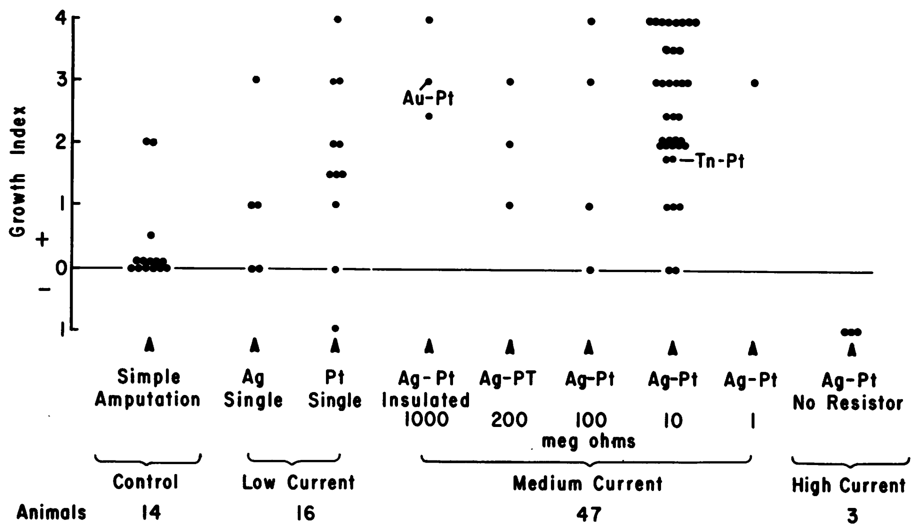
All these results occurred within the surprisingly short period of seven days or less following amputation. Sacrifice of the animals at periods longer than this was disappointing in that growths of lesser organization were seen, except in one case in which, attached to a rudimentary growth of longitudinal bone and cartilage, were five appropri-



Graph 1. Current levels obtained from silver-platinum couplings with various levels of interposed resistors. Measurements were made *in vitro* with a Hewlett-Packard 425A microvolt ammeter with electrodes immersed in normal mammalian saline. Not only is the steady state current altered by the resistor, but the initial current drop is markedly changed as well.

ately sited active centers of growth which approximated the normal digitation (Figure 7). It thus appears that the duration of time that the electrical device is left in situ may have a bearing on the result. It is possible that a deleterious effect is exerted by persistence of the electrical trigger beyond a specific time.

In an attempt to learn more about the optimum values of voltage and current we have tested a variety of bimetallic couplings; we have used the same metals—silver proximally and platinum distally—but varied the interspersed resistor (Graph 1). The results are plotted on Graph 2,



Graph 2. Growth resulting from experimental amputations. Growth was assessed on an arbitrary scale of —1 to +4, —1 representing bone and tissue destruction with fibrotic reaction; 0, growth exhibited by control amputation; +1, any more growth than control; +2, formation of an active cellular mass resembling a blastema; +3, the appearance of organized tissue in the regenerate; and +4, the formation of an organized multitissue structure.

where growth is expressed on a scale of –1 to 4+ versus the value of the resistor.

Since it is possible that metallic ions might diffuse from the electrodes, we have begun to use several other nonreactive metals, and we have varied the interposed resistor in order to produce current values close to those in the optimum case of the Ag-Pt coupling. Thus far it is possible to state only that stainless steel produces a destructive response when it is used as the distal electrode despite current levels that should have been within useful ranges. In a few instances gold has been employed to replace platinum as the distal electrode, with good results. More recently we have turned to an *in vitro* method to evaluate possible effects of metallic ions; for this purpose we have used the amphibian erythrocyte as the test cell, and methods previously described.[24] The results to date indicate that, provided the metals are intrinsically nonreactive, the cellular responses appear to be dependent more upon the levels of current and voltage and are generally independent of the type of metal.

It would be well to point out that the physics and electrochemistry of "simple" metallic electrodes in biological solutions are exceedingly complex. Many poorly understood phenomena occur, especially at the liquid-metal interface. It may well be that certain metals are more efficient in the transfer of electrical energy at low currents and voltages in a biological system. This type of information will have to be developed further before the design of effective implantable battery-operated devices will become possible. Such a step is highly desirable, since the complexities of the present bimetallic device are even greater than those associated with simple metallic electrodes of the same metal driven by appropriate types of battery-operated devices.

The results thus far permit us to draw only a few conclusions, which are mostly tentative. The growth response appears to be related more to the electrical factors than to any electrochemical factors associated with the metallic electrodes. While in these experiments we cannot rule out the possible existence of a second-order phenomenon such as the accumulation of specific chemical species in the vicinity of the electrode or micro *p*H changes, previous experiments on *in vitro* systems would seem to militate against such a simple explanation of the effects.[24] Since the growth that results from the implanted devices consists not only of organized bony structures which contain cells of several types but is accompanied also by evidence of the formation of new muscle as well as by ingrowth of nerve fibers, we conclude at this time that to some extent we have stimulated true regeneration of multiple tissues and not merely simple osteogenesis.

The experiments have raised the important questions of the cell or cells from which observed new growths originate. From our preceding experiments on the healing of fractures and on cardiac regeneration in nonmammalian vertebrates, we now lean toward the hematopoietic marrow as the source of the blastema. This concept would appear to be supported somewhat by the observation that monocytes can take part in regenerative processes in limbs,[28, 29] that lymphocytes are capable of dedifferentiation under certain circumstances,[30, 31] and that marrow elements can be induced into osteogenesis.[32] Perhaps the most important conclusion—one that seems inescapable—is that mammals are capable of partial regenerative growth in response to a simple electrical stimulus. The organizational pattern demonstrated by such growth would indicate that whatever the data-transmitting system is that brings about appropriate differentiation of the blastema, it is present in sufficient degree in the mammalia. It would appear desirable to determine in detail the optimal parameters of the stimulus and to ascertain which cells are most susceptible to its effects. The restoration of even a small measure of true regenerative growth to the human would seem to provide a viable alternative to transplantation and prosthetic replacement as well as a much more effective means of dealing with loss or interruption of continuity of single tissues.

A cautionary note is in order. Our results thus far seem to indicate that electrical parameters of low amplitude or second-order associated electrochemical factors are potent stimulatory factors for mammalian cells. It may well be that these parameters are the functionally significant ones that occur naturally in living systems; as such the relation between perturbations in the internal electrical environment and the occurrence of malignant cell changes is not at all clear at this time. While it was demonstrated in 1964 that differential bone growth could easily be produced by appropriate electrical parameters in mammals,[22] the use of this modality for therapeutic purposes in man is in our opinion unwarranted at this time. Such applications should await further knowledge of the cellular response mechanisms involved and a thorough search for the occurrence of undesirable effects including, but not limited to, the stimulation of malignant transformation.

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