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Bioelectricity and Regeneration

Richard B. Borgens, Joseph W. Vanable, Jr., and Lionel F. Jaffe

Ever since the time of Galvani and Volta, about two centuries ago, biologists have been aware of electrical phenomena in living organisms. [See Geddes and Hoff (1971) for an interesting review of these early days.] However, most of the research in this area has been concerned with potentials generated across cell membranes, particularly of nerve and muscle. Less well-appreciated are the stable electrical potentials generated across whole cells or even tissues. These steady, trans-cellular and trans-tissue potentials play an important role both in the day-to-day existence of organisms and in their growth, development, and regeneration. We will focus here on the effects that these potentials have on growth, development, and particularly regeneration.

There is a classical literature on so-called bioelectric potentials, which goes back to at least 1905 when Ida Hyde measured potentials across turtle and fish eggs. Such bioelectric potentials have generally been measured by taking the cell or organism out of its aqueous environment and measuring surface potential differences with the cell or organism in air. For example, the remarkable pioneer, Elmer J. Lund, made some careful measurements on stem sections of the hydroid *Obelia* (1925). He found that the end of the stem that in time would regenerate a head was substantially electropositive with respect to the other end. This would indicate that steady current flows out the regenerating end.[1]

However, there are several serious difficulties in interpreting this measurement and others like it. In order to get a measurable signal, the stem was placed in moist air rather than in its natural seawater environment. The voltages were produced by currents driven by the stem along the thin film of moisture on its surface during the measurements. The stem does not, in fact, regenerate in moist air, and one cannot be certain to what extent the currents that flow in this environment are present during regeneration in seawater. Even if present during regeneration, these currents would not produce the large voltages measured. Indeed, the size of the measured voltage is more or less arbitrary, depending as it does on the thickness of (and hence the resistance along) the film of surface moisture. Nevertheless, the same amount of current that produced large surface voltages during measurement may also traverse the stem during regeneration in seawater. However, even if this were true, we cannot determine the size of this current from the published data, since the resistance along the surface film was not measured. (This discussion is not put forth to dismiss the whole body of older bioelectric potential measurements. In many or most instances, they may well be valid indicators of the direction of developmental currents, but little or no quantitative information can be inferred from them.)

Three more reliable measurements, which form a transition to the modern era, have been carried out on developing plant systems: regenerating *Acetabularia*, corn coleoptiles bending upwards in response to gravity, and fucoid eggs germinating away from unilateral light. The stem segments of *Acetabularia* were placed in seawater and actually regenerated during the measurements. However, the ends of each segment were sufficiently insulated from each other by greased partitions to allow the measurement of voltages by conventional means. Starting 40 hours before cap regeneration began, large voltage pulses appeared, which indicated pulses of current entering the future regenerating end (Novak and Bentrup 1972).

Unlike most systems studied electrically, the corn coleoptile grows naturally in air. Grahm and Hertz (1962) developed an ingenious system using vibrating centimeter-square plates, which did not

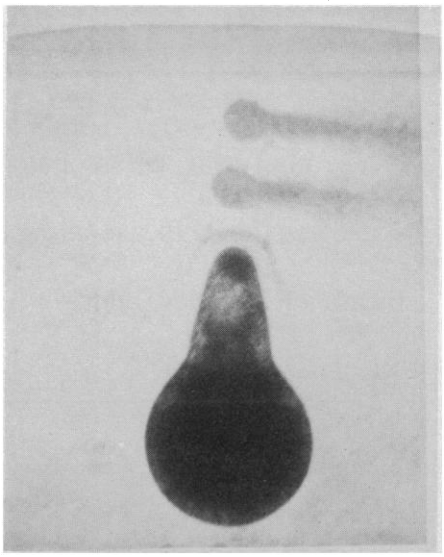

Fig. 1. Photograph of a probe vibrating at 200 cycles per second in front of a day-old fucoid embryo growing in seawater. (Photographed under illumination by a stroboscope flashing at 400 c.p.s. to visualize the probe in its two extreme positions.) The tip of this probe consists of a platinum black ball about 25 μm in diameter. It registers the minute voltage difference between its extreme positions. From this voltage difference, together with the resistivity of the medium, one can calculate the current density driven between these points by the embryo. (From Jaffe and Nuccitelli 1974)

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[1]We use the convention, which goes back to Benjamin Franklin, that the direction of current flow is the direction of movement of positive charges. Moreover, all biological currents are carried by ions and not by electrons. For example, in this case, the current along the surface of the *Obelia* stem would be largely composed of the two major ions found in seawater: Na^+ ions moving away from the regererating end and CL^- ions moving toward it.

touch the coleoptiles but sensed the fields in the air around them. This enabled them to measure reliably the voltages generated across these organs in response to unilateral gravity. The coleoptiles develop potential differences of 80 mV or more across themselves, with their lower (and faster growing) side the positive one. Unlike most surface potential measurements, these directly indicate the natural voltages developed across the growing tissue. Furthermore, one can infer that current emerges from the outer plasma membranes of the epidermal cells on the faster growing side and enters those on the opposite side, although the current path in between remains obscure.

Jaffe (1966) measured voltages across fucoid eggs while they developed in seawater by placing several hundred eggs in series within a loose-fitting glass capillary and measuring the total voltage across the capillary. The voltages per egg were very small (about a few hundred nanovolts) but indicated substantial currents with densities of the order of 1 μamp/cm^2 entering the growing tip of the embryo.

A modern era in measuring developmental currents was initiated with the introduction of an ultrasensitive vibrating electrode (Fig. 1). When placed in the aqueous medium outside the cell or tissue, this probe enables one to plot and measure the pattern of current densities around the object and infer the direction and densities of current going through it. Use of the vibrating probe facilitated a detailed study of natural currents through developing fucoid eggs. Nuccitelli (1978) found that these currents entered the presumptive growth tip many hours before it was visible. Furthermore, once growth does begin, the rate of elongation is roughly proportional to the current entering the growing tip (Nuccitelli and Jaffe 1974). Studies using radioactive tracers, as well as the probe, have shown that much of the early current traversing the egg before growth begins, and indeed before the direction of growth is irreversibly established, consists of calcium ions. Thus, very early in development, calcium ions enter the presumptive growth point and leave its antipode (Robinson and Jaffe 1975).

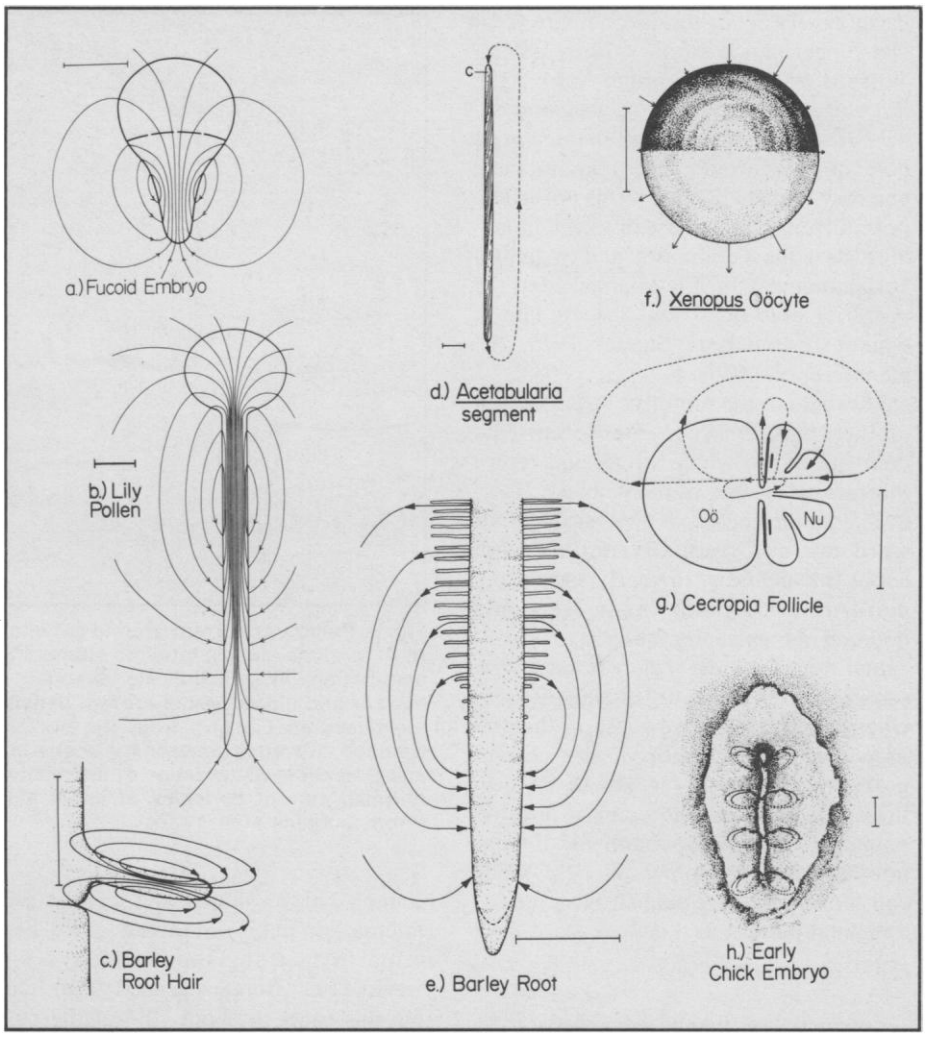

Fig. 2. Some examples of developmental currents. The left-hand column (a-c) shows three tip-growing plant cells. Current enters the growing end in all cases, with peak entry densities of the order of 1 μamp/cm^2. (Scale bar is 0.05 mm.) The middle column shows two larger and/or more complex plant systems. The arrows through the regenerating, anucleate stalk segments of *Acetabularia* (d) indicate the direction of periodic, 100-second-long current pulses (rather than the steady currents indicated elsewhere). They enter the end that will regenerate a cap and seem to have peak current densities of the order of 10 to 1000 μamp/cm^2. (e) Current enters the elongating zone of the barley root, with peak entry densities of the order of 1 μamp/cm^2. (Scale bar is 1 mm.) The right-hand column shows three animal systems. (f) Current enters the animal pole of the fully grown *Xenopus* oöcyte with a density of about 1 μamp/cm^2, and leaves its antipode with about this same density. (g) Current is relatively concentrated, with densities of up to 20 μamp/cm^2 as it enters the nurse cell end (Nu) of the *Cecropia* follicle. (The nurse cell pole of the insect oöcyte roughly corresponds to the animal pole of the other animal oöcytes.) (h) Current leaves the primitive streak of the early chick embryo with densities of the order of 30 μamp/cm^2. (Scale bar is 0.5 mm.) [For the fucoid egg current, see Nuccitelli & Jaffe (1975); lily pollen, see Weisenseel et al. (1975); barley root, see Weisenseel et al. (1979); *Acetabularia*, Novak & Bentrup (1972); *Xenopus* oöcytes, Robinson & Jaffe (unpublished); *Cecropia* follicles, Jaffe and Woodruff (1979); chick embryos, Stern & Jaffe (see text footnote 2).]

COMPLEX ORGANISMS

Transcellular currents appear to play a key role in the development of more complex organisms, also. Exploratory studies with the vibrating probe have already revealed large developmental currents through a wide variety of forms (Fig. 2 and Jaffe 1979). Good examples are the ovarioles of the American silk moth, *Cecropia* (Jaffe and Woodruff, 1979) and the chick embryo in the primitive streak stage (Stern and Jaffe[2]).

During most of the growth of the cecropia oöcytes, large currents (with densities of up to 20 μamp/cm^2) flow into the nurse cell end of each follicle (or oöcyte-nurse cell complex) and leave the oöcyte end. Since the nurse cell end of each oöcyte will become the anterior end of the egg, one may reasonably ask if the anterior-pole currents somehow help establish the head-favoring factors, which are laid down in the front end of various

[2]C. D. Stern and L. F. Jaffe, "Large Electrical Currents Leave the Primitive Streak of Early Chick Embryos," unpublished paper.

insects early in oögenesis (compare Kandler-Singer and Kalthoff 1976, Nüssslein-Volhard 1977). Furthermore, during the last stages of development, a second entry current is established at the posterior pole of the forming cecropia oöcytes; one may reasonably ask if this posterior-pole current is somehow involved in laying down the germplasm and/or tail-favoring factor, which is established at the posterior pole of various insects late in oögenesis (compare Sander 1976, Illmensee et al. 1976).

Throughout the primitive streak stages of the chick embryo, intense currents pour out of the whole streak and return throughout the rest of the epiblast. However, the epicenter of this strong outward current gradually moves from about the middle of the early streak to a more anterior point, near Hensen's node. This epicenter seems to correspond roughly to the region of tissue that shows the greatest organizing activity when transplanted elsewhere in the embryo. Within the embryo, the current probably returns to the streak through the space between the epiblast and hypoblast. One may reasonably ask if these internal currents somehow help guide cell movement or establish basic developmental pattern in the chick.

VERTEBRATE REGENERATION

Trans-cellular currents appear to play a key role in vertebrate regeneration as well. For some time, it has been known that there are surface potential differences between various parts of the adult salamander. Monroy (1941) discovered that the distal part of a limb is positive with respect to the proximal part. After amputation, this potential difference becomes far larger. Monroy's discovery has been essentially confirmed by Becker (1960) and Lassalle (1974a), as well as by Rose and Rose (1974).

These surface potential differences along early regenerating urodele limbs suggest that current should be leaving the cut surface of the regenerating limb stump and reentering at points proximal to this. Measurements with the vibrating probe show that this indeed is the case. Before amputation, current enters the skin at nearly all points of the limb surface (Fig. 3A); after amputation, there is an increase in incurrent density and a dramatic efflux of current from the cut surface of the stump (Fig. 3B). These large outcurrents have densities of the order of 10 to 100 μamps/cm^2 (usually 50 μamps/cm^2 or more) and persist with fluctuation until just about early blastema formation, about 10 days to 2 weeks later (Borgens et al. 1977b). During this time, in about 70% of the measurements made, the peak currents occurred in the postaxial region. As regeneration continued, the outcurrents dropped to less than 5 μamps/cm^2 and from time to time reversed to equally small incurrents. The reversal was never steady, and the incurrents were usually small; only occasionally did they even reach a value of 10 to 20 μamps/cm^2.

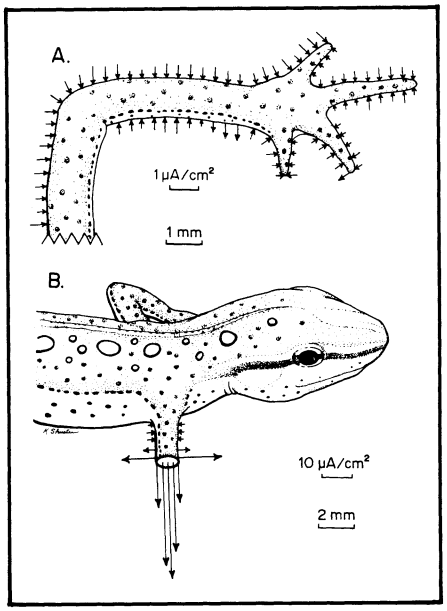

Fig. 3. Pattern of current around (A) intact newt forelimb and (B) forelimb stump after amputation. Arrows indicate direction of current and magnitude of current density, as measured 0.3 mm from the surface. Note the difference between the scales in A and B to allow visualization of the relatively small current densities of intact skin. (From Borgens et al. 1977b)

SOURCE OF URODELE LIMB CURRENTS

What might be the source of such currents? Becker has claimed that nerves are the source of the potential differences measured on the surface of regenerating newt limbs (Becker 1960). However, several facts argue convincingly against this claim. Both Monroy (1941) and Lassalle (1974b) showed that the surface potentials are practically unaffected by denervation. Furthermore, Lassalle found that the potentials disappear when the skin is removed.

Our own work has confirmed and extended these conclusions. Amphibian skin can pump sodium ions inwards (see Ussing 1964). These Na^+-dependent incurrents are in the same direction as the incurrents we have measured entering intact newt limb skin, and therefore could theoretically be the source of the outcurrents we observed after amputation (Fig. 4A). The fact that these currents are Na^+-dependent permitted us to test this hypothesis. When we remove Na^+ from the medium in which the newt is immersed, within 15 minutes there is a dramatic reduction in outcurrent from the cut surface of the limb (Borgens et al. 1977b). In most cases, current densities in sodium-depleted media are less than 1 μamp/cm^2, approximately a 50-fold reduction from control values. Furthermore, if the Na^+ concentration is raised fivefold over normal levels, there is an average of a fivefold increase in current density leaving the end of the stump.

A slightly different approach has also provided data consistent with the hypothesis that the Na^+-dependent skin pump is the battery that drives the current leaving the regenerating surface of the stump. Amiloride and methyl ester of lysine (MEL) each prevent the generation of these skin currents by blocking the channels through which Na^+ gains access to the Na^+ pumps (Benos et al. 1976, Kirschner 1973). When amputated newts are immersed in 0.5 mM amiloride or in 3 mM MEL, the stump current density is sharply reduced: In the amiloride, currents leaving the limb stump fall from their initial level by 68% within 15 minutes, and at 50 minutes, by 90%; in MEL, in most cases, the values fall by 90% within 15 minutes. Finally, the reduction in current produced by each of these methods is reversible by placing the animals back in their normal water (Borgens et al. 1977b).

In view of Becker's claim about the role of nerve in generating the surface potentials that he and others have measured, we have also investigated the effect of denervation on the currents we measure with the probe. We find that currents leaving arms that were denervated three to four days prior to amputation are, if anything, larger than those leaving the contralateral innervated stump[3] (Borgens et al. 1977b). Therefore, we agree with Monroy and Lassalle about the source of the current that we measure: All of our evidence points to the skin as the battery that drives the current leaving the surface of regenerating limb stumps prior to the early bud stage of blastema formation.

[3]Note, however, that we do *not* claim that the denervated limbs would regenerate without their nerves.

RELEVANCE OF CURRENTS THAT LEAVE LIMB STUMPS

Are these currents a critical part of the process of limb regeneration or just an epiphenomenon? There is good evidence that currents help polarize the developing fucoid egg and determine the point of germination. Since comparable evidence had been lacking for vertebrate limb regeneration, we set about testing whether the stump currents we measure are relevant to amphibian limb regeneration. We decided to reduce the natural current and see what consequence this has on regeneration, using two methods: long-term blocking of the Na^+ channels by amiloride and long-term immersion in low Na^+ water.

If the same molarity of amiloride that is effective in drastically reducing newt stump currents is applied topically to only the stump skin of *Ambystoma tigrinum* adults every other day, there is a marked interference with normal regeneration of the stump. All of the control tiger salamanders (treated just with pond water) regenerated typically, in every case forming digits. In contrast, 12 of 33 amiloride-treated animals did not regenerate at all, and 5 regenerated with gross abnormalities; only 16 regenerated as normally as the controls (Borgens et al. 1979a).

The second approach involved immersing newts in artificial pond water (APW) from which Na^+ was omitted, rather than blocking its entry into the skin. For five to six weeks, the animals immersed in this low-Na^+ medium did not, on the average, even reach the early blastema stage (regenerate length averages 0.4 ± 0.2 mm), whereas on the average the controls produced paddle-stage regenerates (length 1.5 ± 0.2 mm). However, in the subsequent four weeks, the low-Na^+ animals escaped this inhibition and caught up in both length and developmental stage with the controls (Borgens et al. 1979a).

These results clearly show that reducing Na^+-dependent stump currents drastically interferes with normal regeneration, providing evidence that they are a critical component of urodele limb regeneration. However, there remains the question of why this inhibition is incomplete. In the case of the low-Na^+ animals, we discovered that after several days in such water newts began producing stump currents that do *not* depend on Na^+. It is possible that amiloride-treated animals can also adapt in such a manner. Such currents could easily account for the escapes that we have seen, and deserve more careful study.

THE QUESTION OF ANURANS

Why don't adult frogs regenerate limbs, since the classical work on Na^+-dependent skin current has been done with these creatures? Of course, while stump currents might be necessary for limb regeneration, they are not sufficient. Frogs, then, might be missing other essential components. However, one can initiate a degree of regeneration in these animals by drawing currents through adult frog-stump tissues that are in the same direction and of similar density, duration, and distribution as natural newt currents (Borgens et al. 1977a, Smith 1974, and see Fig. 4B and 5).

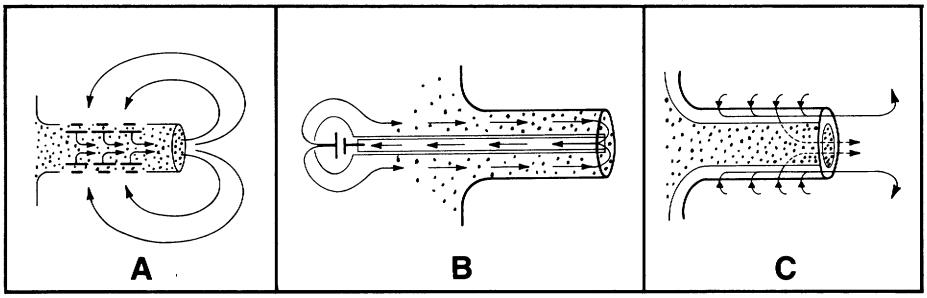

Fig. 4. Current paths through limb stumps. A. Diagram of current driven by skin battery through newt stumps. The skin's Na^+ pump drives Na^+ ions from outside to inside. The resulting voltage drives positive charges toward and out of the leak at the end of the stump. The circuit is completed by a return of charge to the outside of the skin via the medium. (From Borgens et al. 1977b). B. Path of current driven through frog stumps by implanted battery assembly. The insulated wick cathode pulls current out through the tissues of the limb stump; the same direction as the endogenous currents in the newt stump (compare with Fig. 4A). C. Path of current driven by skin battery through frog stumps. Current is driven through the skin, as in the newt (Fig. 4A), but much of it leaks through the subdermal lymph space rather than being forced through the core tissues. Therefore, the amount of current being forced through the core tissues (stippled) is greatly reduced. (From Borgens et al. 1979b).

Using the probe, we have recently measured natural Na^+-dependent skin currents leaving limb stumps of adult frogs. The current densities measured are comparable in magnitude to the ones in urodeles, and they diminish after two or three weeks to the level of 5 to 10 μamps/cm^2. In the cases in which current is drawn through frog limb tissues by a battery, the calculated densities immediately below the stump electrode are comparable to those leaving newt stumps, but these high densities are found in only a small fraction of the stump area (Borgens et al. 1977a). Therefore, as a first approximation, it appears as though a battery's supplement to the frog's natural stump current would be almost negligible. However, this would be the case only if current densities leaving frog-limb stumps are uniform. We have found that they are not: There is a large current leak between the skin and the tissues below, so that little current traverses the muscle and other subdermal tissues (Fig. 4C).

This leak is absent in the urodeles we have measured. Current densities are highest in the central and postaxial regions, rather than between the skin and the tissues below the skin (Fig. 3B and 4). A reasonable explanation for this difference in current profile might lie in a difference in anatomy: Frogs have a loose skin, with ample lymph space beneath; urodele skin usually adheres tightly to the underlying musculature. In the frog, the imposed current drawn by the battery has to pass through the core tissues of the limb stump; therefore, this supplement assumes considerable importance, in view of the fact that much of the natural frog current leaks through the lymph space and so is ineffective (Borgens et al. 1979b).

MODE OF ACTION

If natural skin-driven currents are a critical component of regeneration, what is their mode of action? Rather than the flow of current *per se*, it probably is the field associated with the current that is perceived by the responding cells or tissues. The heat produced by the minute currents is exceedingly small (of the order of microcalories/second) and would be negligible in view of the capacity of tissues to dissipate heat.

On the other hand, the field strengths associated with these currents would not be negligible; they could easily be of the order of millivolts/mm or more (Jaffe and

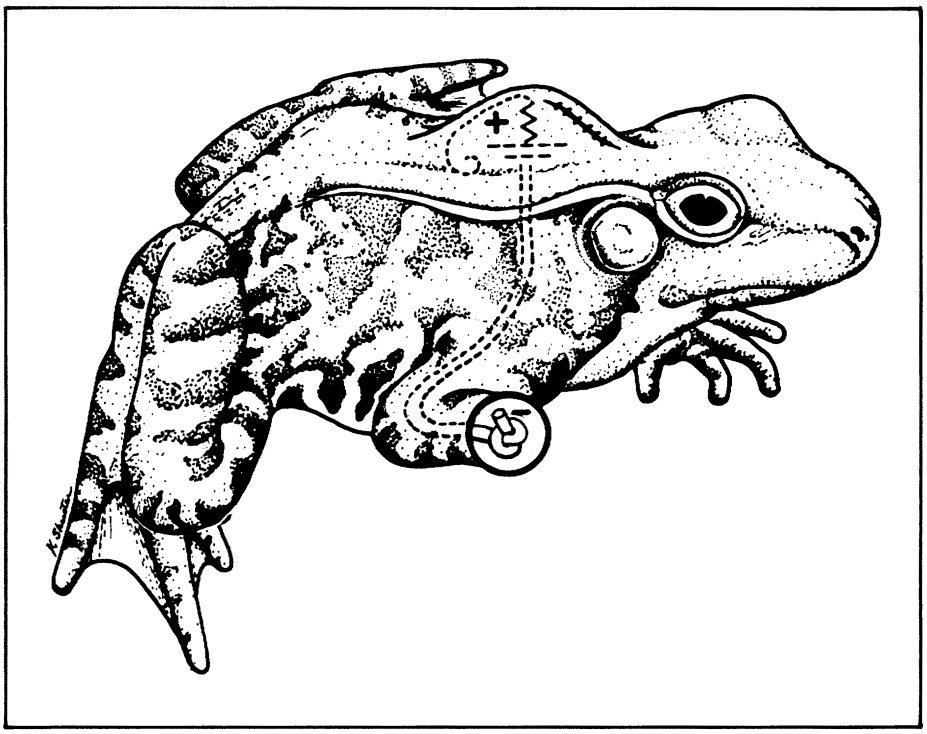

Fig. 5. *Rana pipiens* implanted with a small insulated hearing aid battery assembly. The positive lead is in contact with the tissues beneath the battery, and the uninsulated portion of the wick cathode is in contact with the tissues at the surface of the stump. These batteries were implanted beneath the skin at the time of amputation and removed about four weeks later. (From Borgens et al. 1977a)

Nuccitelli 1977). When considering candidates for a target of such fields, it is natural to focus on cells that are unusually long in the direction parallel to the field and/or cells or tissues that have an unusually high resistivity. Muscle, and especially nerve tissue, is made up of cells that are elongate and therefore might be able to perceive quite shallow fields. Epidermis, on the other hand, with its closely adhering cells, has a higher resistance than most tissues and therefore would have a relatively steep potential gradient across it.

The epithelium covering the regenerating blastema is a critical component of regeneration (Thornton 1968) and so might well be under some influence of the steady fields associated with regeneration. However, we have not yet tested this experimentally. Instead, we have begun to direct our attention to nerves: It is well-known that normally nerves are a *sine qua non* of limb regeneration (see Singer 1974). Furthermore, a remarkable quantity of nerve grew into the cathodally stimulated regenerates that we produced in *Rana pipiens* (Borgens et al. 1977a), suggesting that perhaps the imposed field somehow stimulated and/or directed this growth. In similar experiments with *Xenopus laevis*, we also have an indication that nerve outgrowth is stimulated by a distally negative imposed field (Borgens et al. 1979c).

There is in vitro evidence that nerve growth can be so affected: Marsh and Beams (1946) found more neurite growth from explanted ganglia occurring toward the cathode than toward the anode, a polarity of effect that would account for increased growth in both the normally regenerating newt limb and in the cathodally stimulated *Rana* and *Xenopus* limbs. Marsh and Beams' findings have been confirmed in our laboratory and are now being extended. We hope, also, to check soon the hypothesis that nerve growth is affected by the fields associated with stump currents, by comparing nerve growth in newt amputees that are regenerating normally with that in amputees whose capacity to drive skin currents (and to regenerate normally) has been reduced by amiloride or MEL.

PAST LITERATURE: GOODNESS OF FIT

Our observations suggest ways to unify a good deal of the past literature on regeneration. In addition to imposing fields along limb stumps, there have been several other major ways to stimulate a degree of limb regeneration in adult frogs. Rose (1944) produced a considerable degree of regeneration by treating the limb stumps of amputees with solutions of NaCl. Although the general impression in the literature is that very concentrated solutions were essential for this effect, in fact, Rose's best results were achieved with a modest 0.3% NaCl solution, which is more dilute than Ringer's solution. Rose speculated that this effect might be attributed to a prevention of early wound healing and extended trauma; we suggest the alternate possibility that the skin's Na^+ battery was stimulated by his salt treatments to produce greater than normal currents. Although 0.3% (52 mM) Na^+ can hardly be considered irritating, it is 25 to 50 times more concentrated than Na^+ in pond water and should have greatly increased the skin's output and, therefore, the amount of current traversing the stump's core.

Polezhaev interpreted Rose's Na^+ effect as being due to prolonging the period of tissue destruction that normally precedes blastema formation. He repeatedly treated the stumps of adult frogs with 0.8% $NaHCO_3$, which is 95 mM in Na^+ (see Polezhaev 1972). Since this is very close to the molarity of Na^+ in amphibian body fluids, it is hard to envision how this would prolong tissue destruction. However, this Na^+ level is 50 to 100 times higher than the Na^+ of pond water and would, like Rose's 0.3% NaCl, boost the skin's current output.

Even Schotté's remarkable success at stimulating limb regeneration in adult frogs by implanting supernumerary adrenal glands (Schotté and Wilber 1958) can, in theory, be reconciled with this point of view. The adrenals that were implanted certainly produced glucocorticoids, as was emphasized by the authors. Furthermore, it would be reasonable to suppose (as they did) that these glucocorticoids would retard cicatrization and thereby enhance regeneration. However, both the glucocorticoids and the mineralcorticoids produced by the implanted adrenals would also increase the output of the skin's Na^+ pump (Bishop et al. 1961, Mayers et al. 1961) and perhaps encourage regeneration in this way, also.

Finally, if it should turn out that the fields associated with regeneration currents encourage nerve growth into the blastema or even enhance the production or transport of neurotrophic substance, this would provide a satisfying merging of our ideas with the well-established role of nerves in regeneration so elegant-

ly investigated by Marcus Singer (Singer 1965, 1974). The degree of regeneration seen in cathodally stimulated *Rana* (Borgens et al. 1977a) is comparable to that produced in the seminal experiments of Singer (1954), in which he surgically hyperinnervated the forelimb stump of *Rana pipiens*. Since one of the prominent features of these cathodally stimulated regenerates was that they contained an unusually large amount of nerve, it is tempting to speculate here that the imposed field acted by mimicking Singer's neurosurgery. Our work with *Xenopus* regeneration points in the same direction (Borgens et al. 1979c).

HUMAN REGENERATION

Exploring the role of bioelectricity and regeneration has been an intellectually rewarding enterprise. Are these studies of any practical worth? The regeneration routinely achieved by cathodal stimulation of adult frogs has been modest indeed; is there any point in embarking on a search for conditions that would result in reliably producing more complete structures? So far, we have felt that achieving a better understanding of the role of natural regeneration currents in urodeles should precede such a search. We are guardedly optimistic, however, that such a search might ultimately be fruitful.

Already procedures are being developed for the electrical stimulation of healing of skin ulcers in humans (Wheeler et al. 1971), healing of chronic bone fracture nonunions in humans (Friedenberg and Brighton 1974), and the regeneration of articular cartilage in rabbits (Baker et al. 1974). Even without experimental intervention, young humans are able to regenerate lost fingertips with remarkable completeness, at least from external appearance (Douglas 1972, Illingworth 1974). This capacity is retained through the first 11 years.

It would be exciting to investigate the extent to which bioelectricity might be critical for this regeneration. Currents do leave such wounds: Since around the time of the Civil War, currents leaving human skin wounds have been reliably measured (DuBois-Reymond 1860). Such wound currents are widespread in mammals and birds, and are similar in current density and direction to currents leaving the amputated stumps of amphibian limbs (Herlitzka 1910, see also Borgens et al. 1977b). Therefore, there is some promise of practical benefits from studies of bioelectricity and regeneration.

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