Chapter 7

INJURY, IONIC CURRENT, AND REGENERATION

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The notion that naturally produced, relatively steady, electrical fields are involved in the development of animals and plants is not a new one historically. Interest and active investigation into these matters began slightly before the turn of the century and reached its zenith in the late 1930s. However, interest began to fade in the late 1940s, and these provocative ideas and measurements of "bioelectricity" have lain fallow until recently (for reviews of this classical literature and the renaissance of new modern approaches, see Jaffe and Nuccitelli, 1977; and Jaffe, 1979).

Even much earlier, physiologists were aware that steady electrical fields were also produced by injury to specific tissues, even whole organisms. The first man to measure the action potential in nerve, E. DuBois-Reymond, also made interesting and insightful measurements of steady injury potentials in severed peripheral nerve (1843) and wound potentials in human skin (1860) (Fig. 7-1). Herlitzka (1910) made further investigations into the existence of wound potentials in human skin and summarized the existence of similar injury-induced electrical activity in a variety of animals. The measurements of "demarcation potentials" in transected nerve were elaborated upon by Lorente de Nó (1947), who felt that they were involved with the capacity of peripheral nerve to regenerate. This classical work influenced the famous Italian embryologist Alberto Monroy. He wondered if steady electrical fields were produced around an injured and regenerating salamander limb. Monroy was the first to actually make measurements of a potential difference along the limb stump of regenerating newts. These measurements were begun in the late 1930s and reported in 1941. Monroy's essential observation was that the distal portion of the stump is electropositive with respect to areas of the limb nearer the body. From this polarity one can infer that electrical current leaves the regenerating tip and enters at points more proximal. Although this observation has been essentially confirmed by others in more modern times (Becker, 1960; Rose and Rose, 1974; and Lassalle, 1974a, b), *all* such measurements of "translimb potential differences" share serious problems in interpretation with the bulk of the classical measurements of bioelectric potentials. In almost all cases, organisms were removed from a primarily aquatic environment and kept in an aerial environment for the duration of

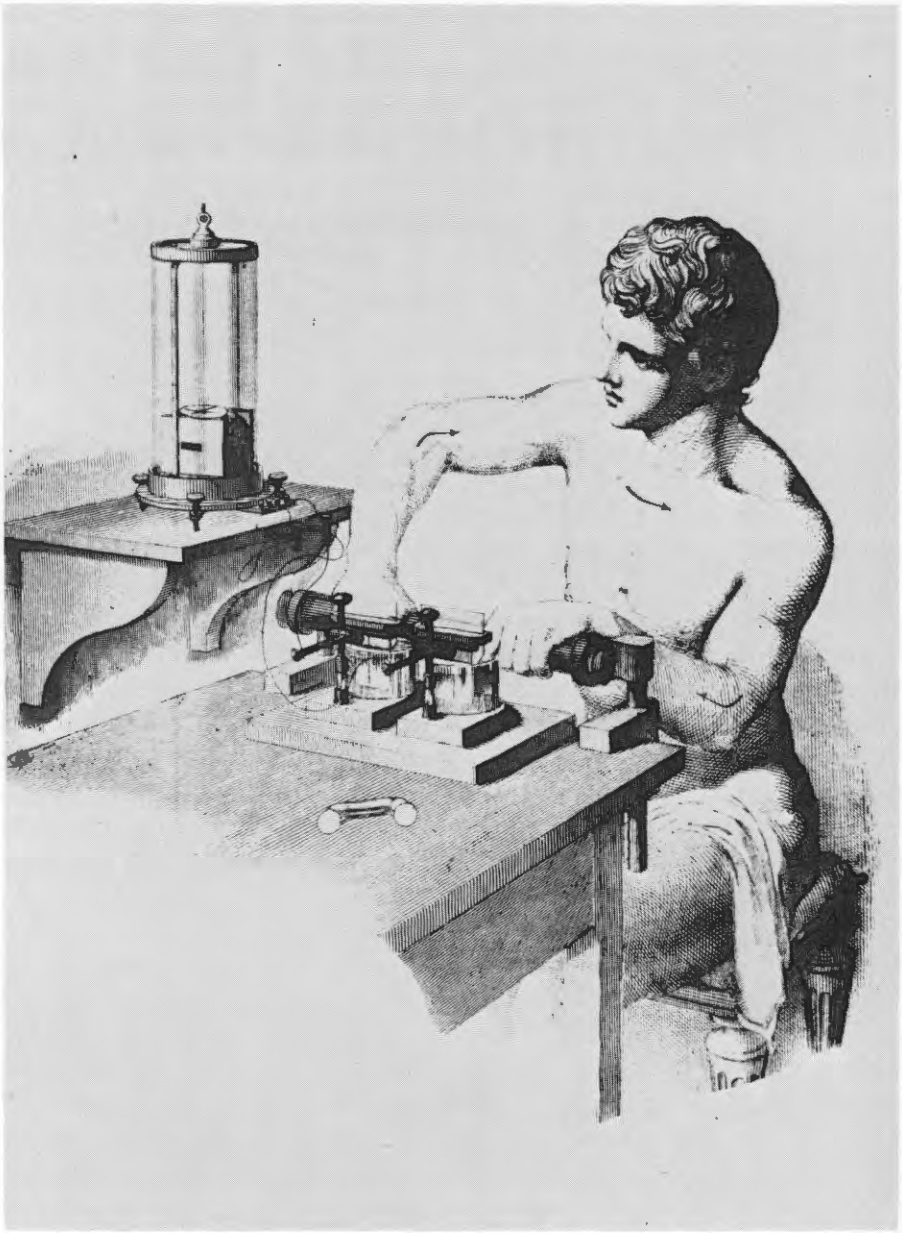


Figure 7-1. An early investigator measuring a wound potential produced by a small wound to his own finger. A small lesion was made in one finger and the other undamaged finger was used as a reference. Both fingers were held in saline baths in series with a galvanometer. (From E. DuBois-Reymond, 1860.)

the measurement period. For this reason about the only aspect of these measurements that one can rely on would be the *polarity* of the external electrical field.

For example, in all of the voltage measurements made on salamanders, the animals were taken from an aquarium and a potential difference was measured between two electrodes contacting the surface of the limb stump. This voltage drop is, of course, associated with a natural current flowing along the film of moisture covering the stump. Moreover, the absolute magnitude of the voltage measured is *dependent* on the thickness, hence the resistance, along this thin film of moisture. It is certain that these potential differences would be much lower (to almost immeasurable values) when the animal was returned to the extended aqueous environment of the aquarium or the pond. Additionally, one cannot even be certain that the current flow that produced these voltages would be the same in both environments. This is because one cannot determine the size of this current without knowing what these pertinent resistances were, and no such data was provided in these experiments.

It was in part due to these uncertainties that Joseph Vanable, Jr., Lionel Jaffe, and I began studies on the possible role of ionic currents* in the regeneration of salamander limbs. Our electrical measurements were made using a unique device, developed by Lionel Jaffe and Richard Nuccitelli (1974), called the "ultrasensitive vibrating probe." When this vibrating electrode is moved to a position near a cell or tissue immersed in a natural medium, it enables one to measure the pattern of current density entering or leaving this biological source with great precision.

The measuring electrode in this system is a single platinum black ball about 25 μm in diameter. This electrode is vibrated about 400 CPS between two positions, typically 30 μm apart. When this vibrating electrode is placed in an electrical field, the electrode detects the minute *difference in potential* between the extremes of its excursion by means of a lock-in-amplifier tuned to the frequency of vibration. Since one can easily measure the resistivity of the medium in which the measurements are being made, the current density entering or leaving this source (be it a biological source or a calibration electrode) can be easily calculated using an analog of Ohms Law for extended media. Using such a vibrating probe system one can measure densities of current 100 to 1000 times smaller than can be detected by conventional means. In part this is due to the fact that the single vibrating electrode is self-referencing and practically eliminates the effect of electrode drift on the measurements.

* Note that electricity in living systems is carried by *ions* not electrons, and that the direction of current flow is taken by convention to be the direction in which positive charges move.

Large Electrical Currents Leave Regenerating Amphibian Limb Stumps

Exploratory measurements of *intact* newt limbs made with the vibrating probe showed that at almost all of the measuring positions around the limb, small currents (0.01-1.0 $\mu A/cm^2$) enter the surface (Fig. 7-2a). Immediately after amputation, the pattern and intensity of this current is radically altered. Intense currents (with surface densities of 10.0-100.0 $\mu A/cm^2$) now *leave* the end of the stump and are balanced by currents with densities of 1.0-3.0 $\mu A/cm^2$ that enter the more proximal surface (Fig. 7-2b). With respect to the time course of this outcurrent (as well as the absolute magnitude of the peak current) there was variation between animals. In general, the large outcurrents finally fell to quite low values (even reversing their polarity for short periods) about the time of visible blastema formation (5 to 10 days post amputation). However, these outcurrents did not necessarily decline in magnitude *immediately* after amputa-

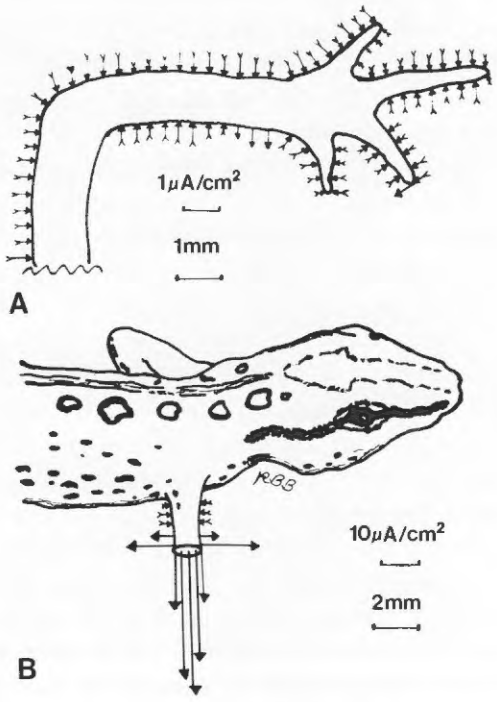


Figure 7-2. The pattern of current around an intact newt forelimb (A) and the forelimb stump twenty hours after amputation (B). Arrows indicate the direction and magnitude of current density, measured about 0.3 mm from the surface. Note that in A, the current density scale is tenfold smaller than in B to permit visualization of the relatively small current densities of intact skin. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *Proc. Natl. Acad. Sci. USA, 74*:4528, 1977.)

tion. In some cases the peak current densities leaving the stump were reached several days after amputation. These stump currents also showed a strong tendency to leave the postaxial region of the stump's cut surface; in about 73 percent of the cases the peak outward currents measured were postaxial; in about 20 percent they were central; while in only 7 percent they were preaxial in origin (Borgens et al., 1977b). Noting the remarkable character of these stump currents, we next asked what their source might be.

It is certain that the flow of this current exiting the cut end of the stump and returning to the undamaged portions of the regenerating forelimb through the pondwater on the outside of the animal produced the trans-limb potential differences that Monroy and others have measured. At least one of these latter investigators has claimed that nervous tissue within the animal was the source of these voltages and the electrical current that could be inferred from them (Becker, 1960, 1961). We did test this notion experimentally. Newts were denervated by severing all three roots of the hrachial plexus servicing one forelimb. After three days, we made sure the effected limb was indeed functionally denervated, and then we amputated *both* forelimbs of each surgically treated animal. When stump currents were measured with the vibrating probe, we found that the denervated limb usually generated somewhat *more* current than the contralateral control stump, certainly not less.* This demonstrated that nerves within the limb stump are not the source of the large outcurrents that leave its cut face.

Amphibians possess another electrically active tissue that could be a candidate for the source of the stump's outcurrent: their skin. Alberto Monroy found that denervation, decerebration, removing the heart, and other procedures had little effect on the voltages he measured along regenerating limbs. Thus, he suggested skin as the source of these voltages, more or less by this process of elimination (1941). Lassalle (1974b) found that by removing the skin from the forearm stump of salamanders he could eliminate the measured potentials, and likewise suggested skin as the source of these voltages. However, a consideration of certain aspects of the physiology of amphibian skin allowed as a much more direct test of its possible role in these matters. Amphibian skin is well known to produce substantial voltages (60 to 100 mv) across itself by taking up Na^+ from the outside medium and pumping it inward (Ussing, 1964; Kirschner, 1973). Isolated skin preparations can drive substantial current as well, on the

* As Lorente de Nó correctly inferred, injury current actually *enters* the ends of transected nerves (see below). This current entering the ends of severed nerves in the stump would actually *subtract* from the net current leaving that stump. Therefore, by removing the nerves by denervation, one would expect the denervated stump to produce slightly *more* current than the stump containing nerves.

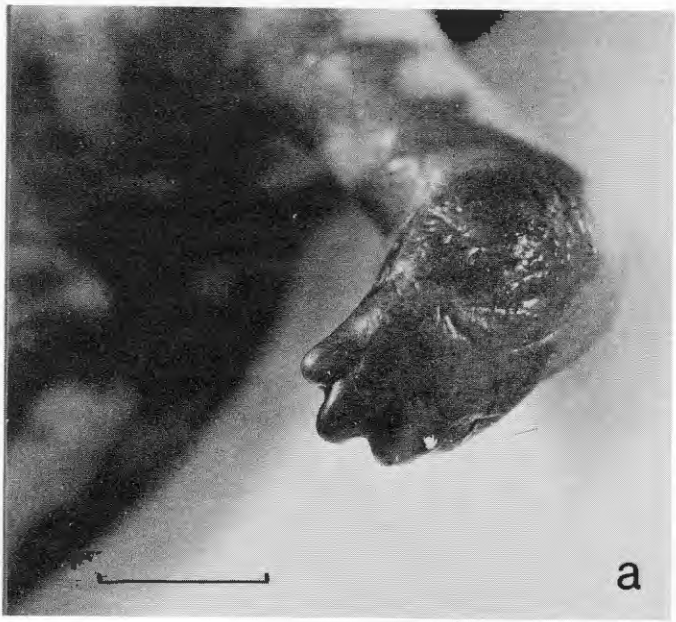
order of 50 $\mu A/cm^2$ (Ussing, 1964). Since this transcutaneous Na^+ "battery" is dependent on the concentration of the Na^+ in the medium bathing the skin, this provided us an opportunity to test whether the skin's sodium battery might be the source of the stump currents. When we raised the concentration of Na^+ in the artificial pondwater in which we made electrical measurements about fivefold (from 1.7 mM to 8 mM), we observed the current density leaving the stump to increase an average of fivefold (Borgens et al., 1977b). When we lowered Na^+ to near zero, the density of the outcurrent was remarkably reduced by about 90 percent within minutes. In another set of experiments, we used compounds (amiloride and methyl ester of lysine) known to specifically block the uptake of Na^+ by skin when in dilute concentration in the medium bathing the outside of the skin. We found that the application of pondwater containing either compound reduced the intensity of stump currents by about 90 percent.

Altogether, the results of these tests lent strong support to the notion that the skin of the forearm stump (and perhaps that of the trunk too) is the battery that generates the large currents flowing out of the stump immediately after amputation, until the formation of a regenerative blastema.

Inhibiting Stump Currents Interferes with Regeneration

A fair question one might ask is, "Do these stump currents even influence the process of limb regeneration in any way?" Indeed, they could be an epiphenomenon. During our experiments to determine what was the *source* of the stump current, we had developed techniques for modulating the flow of this current. We decided to use these same techniques over a long period of time to see what their consequence might be on the process of limb regeneration. For example, in one set of experiments, we topically applied a solution of 0.5 mM amiloride dissolved in well water to the cuff of skin surrounding the stump of adult tiger salamanders *(Ambystoma tigrinum)*. We began these treatments immediately after amputation through the mid forearm, and continued them every other day for the next seven weeks in one experiment; nine weeks and eleven weeks in two more similar experiments. Control groups were treated identically except their stumps were swabbed with *only* the water in which the amiloride was dissolved. All of the animals were kept in moist plastic containers, as free as possible from pools of water that might wash off the amiloride. All of the thirty-four control animals in these three studies were regenerating digits at the time their experiment was concluded (Fig. 7-3). From a total of thirty-three experimentals, only 48.5 percent regenerated as normally as did the controls. The rest were either grossly malformed (15.1%) or *completely inhibited* from regenerating (36.4%) (Fig. 7-4) (Borgens et al., 1979a).

We have observed a comparable inbibition in newts and tiger salamand-



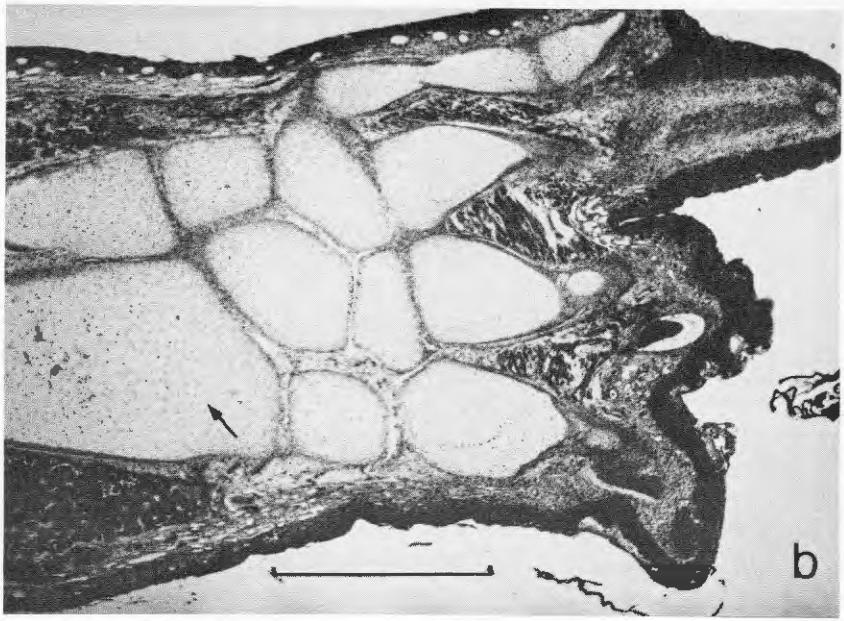
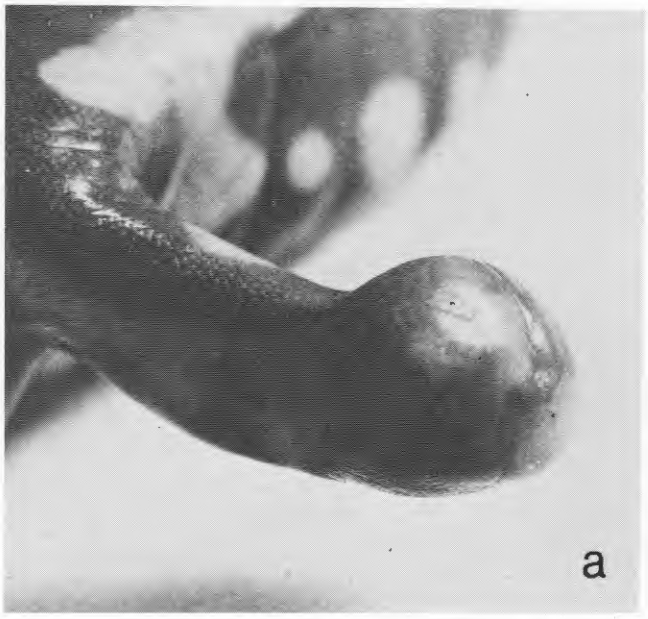


Figure 7-3. External view *A* and photomicrograph *B* of a control regenerate *(A. tigrinum)* eleven weeks after amputation. Note the well-defined radius, ulna (→), carpals, and metacarpals. The rest of the digits were in other sections. Calibration line in *A* equals 5 mm; in *B*, 2 mm. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool.*, 1979, in press.)



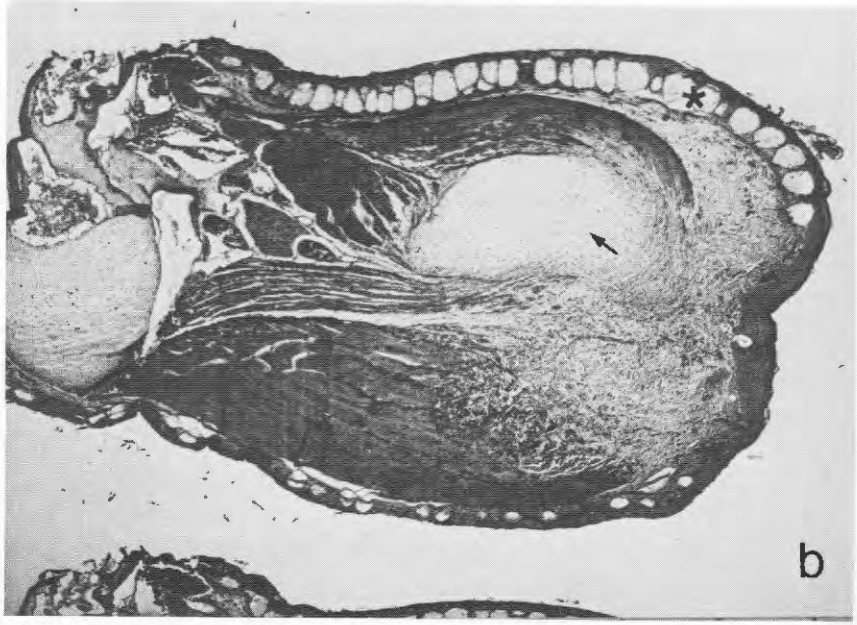


Figure 7-4. External view *(A)* and photomicrograph *(B)* of a nonregenerating amiloride-treated limb *(A. tigrinum)* eleven weeks after amputation. Note the extensive callus formation (→), mature skin glands (*), and the absence of wrist and hand elements. Calibration as in Figure 7-3. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool.*, 1979, in press.

ers maintained in an artificially prepared pondwater that was kept very low in sodium (Borgens et al., 1979a). Animals kept in such Na^+ dilute media scarcely regenerate for about a month; however, they "escape" this inhibition, ultimately catching up with the controls in both regenerate length and developmental stage. There are two aspects of this "escape from inhibition" that are especially curious. One is that you would not expect to see *any* such escape if you considered the histology of apparently inhibited animals sacrificed before they showed signs of regeneration. In many, the limb stumps were covered by a full thickness of skin and the terminal portions of the stump contained a large callus and cicatrix formation, indicative of a nonregenerating amphibian. It is extraordinary that such animals would ever regenerate at all. Second, the rate of regeneration after the animals excape the inhibitory effects of the low Na^+ media is remarkable. It appears as if some component of regeneration is *accumulating* during the period of inhibition. But what?

One might reasonably ask why such an escape of inhibition occurs at all in low Na^+ media. Additionally, why were the amiloride treatments only about 50 percent effective in inhibiting normal regeneration? With regard to the low Na^+ experiment, for the first day or two after amputation, it is very difficult to keep the animal's media very low in Na^+. There is a continual loss of body fluids, high in Na^+ content, from the wound itself. Therefore, the low Na^+ media would not be expected to be as effective in inhibiting stump currents for the first 30 hours or so as the amiloride treatments. Additionally, using the vibrating probe system, we have confirmed the existence of Na^+ *independent* stump currents, which are generated by animals who have incubated in the Na^+ deficient media for several days. It is possible that the skin adapts to this environment in some way, begins to produce stump currents insensitive to the concentration of external Na^+, and this ultimately induces tbe current-dependent component of the regenerative process. In the amiloride experiments, it was curious that the treatments either completely inhibited limb regeneration or had no effect at all. Intermediate responses were very infrequent. This suggested to us that the frequency of molting by the experimental animals may have affected these results. It is possible that there is a critical period for the current to help initiate limb regeneration, and that some animals molted during this period. Besides sloughing off the amiloride treated skin, the current-generating properties of the newly exposed skin are known to be peculiarly resistant to amiloride (Nielsen and Tomilson, 1970). Also, it is possible that some of the animals washed off the amiloride by contacting the beads of condensed moisture in their plastic containers. From our own unpublished measurements, and from the literature (Benos and Mandel, 1978), we know that amiloride is washed off easily, and that its inhibitory effect on the sodium "battery" of the skin is completely reversible.

Last, we should consider if these results can be explained by any other means than by the reduction of the skin generated stump current. Since we reduced the stump current by two different and modest treatments, it makes it rather unlikely that either one of the treatments *both* inhibited current production *and*, independent of this effect, somehow otherwise interrupted the regenerative process. However, one might suggest that the net effect of these treatments would be to deprive the animals of Na^+ and that such Na^+ depleted animals are poor regenerators. First, it is well known that amphibians easily survive prolonged immersion in Na^+ depleted environments *without* a loss of total body sodium (McAfee, 1972). Moreover, we made measurements of blood Na^+ in tiger salamanders chronically immersed in the Na^+ depleted pondwater; there was no significant difference between the controls and experimentals (Borgens et al., 1979a). In the amiloride-treated animals there is not even any reason to expect such a depletion, since less than 1 percent of the total animal's skin surface was treated with amiloride, and the animals were all well fed. Probably the most convincing argument against a possible toxic effect is the fact that salamanders will regenerate nicely during similar application of amiloride *if* the treatments are *not* begun immediately after amputation (Vanable and Datena, Personal Communication). (This also suggests that there might be an early period after amputation where the presence of stump currents are most critical for regeneration.)

If the presence of the electrical currents leaving the limb stump were not a necessary component in the control of limb regeneration in salamanders, then we should not have expected any of these treatments aimed at reducing the stump currents to have any effect on regeneration. However, such does not appear to be the case. In fact, our experiments suggest the opposite; if you interfere with the flow of ionic currents that leave the limb stump as a consequence of amputation, then you also interfere with the normal regeneration of that limb.

Do Adult Rana Produce Stump Currents?

To this point, I have discussed experiments designed to demonstrate the necessity of skin-generated stump currents to the process of limb regeneration in salamanders. The knowledgeable reader might suggest that such stump currents should exist in adult frogs as well; this person would be correct. In fact, the majority of experiments undertaken to gain an understanding of the nature of the Na^+ dependent skin pump in amphibians were performed on frog skin. Moreover, adult ranids were most commonly used, these individuals being noteworthy in their *lack* of regenerative ability. However, there is a substantial difference in anatomy between the limbs of Urodeles and Anurans, which might help explain this apparent paradox. Adult frogs possess large subdermal lymph sinuses that

develop late in metamorphosis. In Rana, these sinuses are particularly well developed; the skin is very loose and separated from the underlying limb musculature by these lymph spaces. On the other hand, salamanders *do not* possess these lymph sinuses. Their skin tightly adheres to the underlying musculature. (Urodeles possess numerous lymph hearts for the circulation of lymph throughout the body). Interestingly, Oscar Shotté and Margaret Harland (1943) noticed that the appearance of the lymph sinuses during metamorphosis occurred at the same level of the limb that was undergoing a loss of regenerative ability. This observation is one of the few close correlations between a change in developmental morphology and the loss of regenerative ability during the metamorphosis of tadpoles to frogs.

We suspected that lymph spaces in frogs might be effective current "shunts," deviating the flow of skin generated current *around* but not through the underlying tissues where tbe blastema arises. Could this shunting of current be responsible for the loss of regenerative ability in tadpoles? There have been no careful studies of stump currents in tadpoles; however, one might expect such a current to exist since tadpoles probably maintain an inwardly positive potential difference across their skin. This potential difference has been detected by passing a microelectrode through the skin in living Xenopus larvae. Moreover, this "battery" appears very early in development; it is certainly present by the time the neural tube has formed (McCaig and Robinson, Personal Communication). An earlier report (Taylor and Barker, 1965) that the skin battery appears very late in bullfrog metamorphosis is frought with difficulty. These investigators removed patches of belly skin, which was tested for a transepithelial potential difference. It is not clear whether the absence of potential they reported was due to damage to the younger and more delicate skins, or to a leakage of current back around the edges of the skin after it was placed in what were apparently "Ussing" chambers.

We have, however, obtained evidence that such a shunting of current through lymph sinuses does occur in *adult* frogs. Large skin-driven currents (20-50 $\mu A/cm^2$) do leave the limb stumps of adult frogs for about ten days post amputation. The general features of this stump current are in many respects similar to newts and salamanders. However, a close inspection of the *pattern* of this current flow leaving the limb measured before a wound epithelium (a limiting resistance) covers the stump surface suggests that lymph spaces do indeed behave as current shunts. Right after amputation, the density of current leaving the adult frog's stump is greatly depressed in the central areas, averaging only 7 $\mu A/cm^2$. This value is remarkably small, not only in comparison to the density of current leaving the periphery of the same stump but also in comparison to the density of current leaving the central areas of a comparable newt stump (which is on the order of four times greater) (Fig. 7-5). In general, we found that the

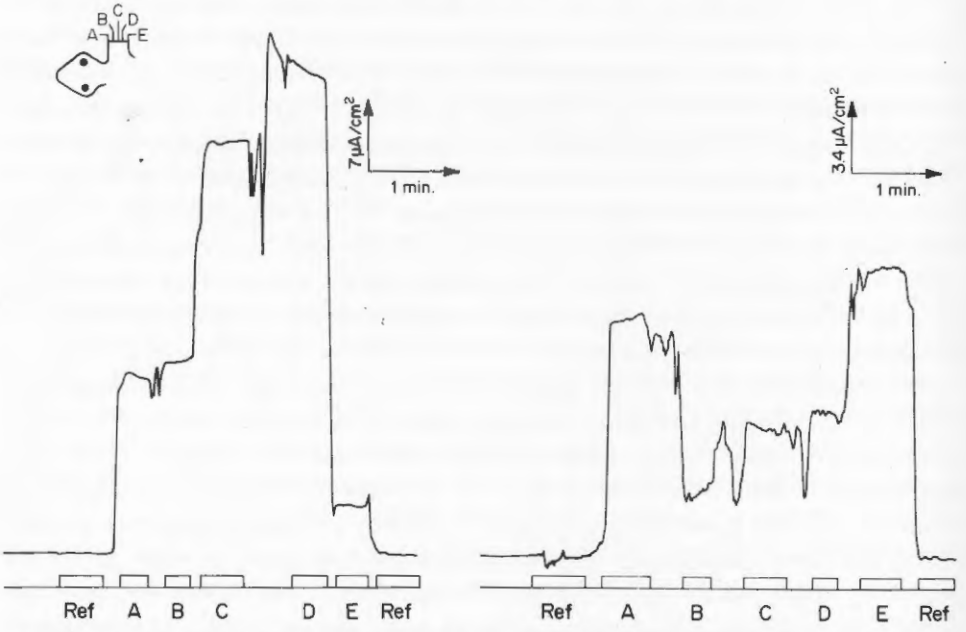


Figure 7-5. Portions of a vibrating probe chart recording showing representative measurements of current density profile across a freshly amputated newt limb stump (left) and a frog stump (right). Measuring positions A through E are diagrammed. Ref indicates a measurement made in the reference position out of the electric field produced by the stump. Oscillations between the fixed measurement positions are artifacts caused by the movement of the probe from one measurement position to the next. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool.*, 1979, in press.)

peak current densities are always found at the periphery of the limb stump in adult frogs, adjacent to the lymph sinus (Borgens et al., 1979b). This is in sharp contrast to the pattern found in newts, where the peak densities are also found leaving the central areas of the stump (usually the post axial regions) (Fig. 7-6A, B). In this report, we discussed other experiments, which suggested that such current shunting by the subdermal lymph spaces continues even after the wound epithelium covers the limb stump of adult frogs. Altogether, our experiments indicate that field strengths sufficient to initiate regeneration in adult frogs do not occur within the core tissues of the stump due to this shunting of skin generated currents.

Enhancing the Deficient Stump Currents in Frogs Initiates Limb Regeneration

One might ask if you could artificially enhance the deficient fields within the core tissues of an adult frog stump, and in doing so, persuade it to begin to regenerate. This provocative experiment was first performed by Stephen Smith (1974). He implanted small batteries into the backs of large

adult frogs and, by carefully placing the stainless steel cathode at the stump's surface, was able to pull small steady currents through the forearm stump. This technique produced a measure of limb regeneration in these animals. (Interestingly, Smith found that the most effective place to locate the stump electrode was in the post axial region of the stump's surface, that area where we have measured the *peak* densities of current *naturally* leaving the limb stumps of newts.) However, Smith's experiment was ambiguous for two reasons: one, he used no sham-treated group to control for the physical presence of the electrode, and the irritation that it would have produced, and two, be used metallic electrodes in contact with body tis-

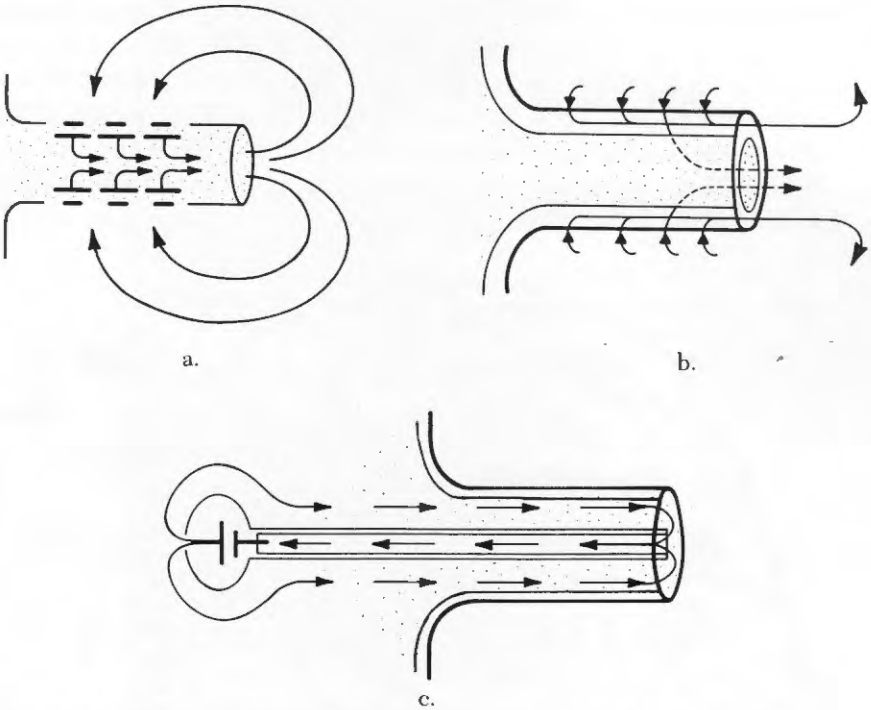


Figure 7-6. Current paths through amphibian limb stumps. *A.* In salamanders and newts the Na^+ battery of the skin drives Na^+ from outside to inside. The resulting voltage drives positive charge towards and out of the leak at the cut end of the stump. The circuit is completed by a return of charge to the outside of the skin via the medium (or the film of surface moisture in semiterrestrial salamanders). *B.* In adnlt frog stumps, current is driven by the skin as in newts and salamanders. However, much of this current is shunted through the subdermal lymph space rather than being forced through the core tissues of the stump (stippled). (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool.*, 1979, in press.) *C.* The path of current pulled through frog stumps by an implanted battery assemhly. The insulated wick cathode draws current out through the core tissues of the limb stump. (From R. R. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *Proc. Natl. Acad. Sci. USA, 74*:4528, 1977.)

sues. This alone made it unclear whether the limb regeneration he initiated was mediated by the presence of electric current or by the presence of the inevitable electrode product contaminants that form where current is passed between a metallic conductor and a complex ionic medium such as body fluids and tissues.

We essentially repeated Smith's effort except that we used Ag-AgCl electrodes and a long, but small bore, salt bridge (or Ringers-filled wick electrode) in contact with the stump tissue (Fig. 7-6C, 7-7). In this way we avoided such electrode product contamination. We were able to report that only 200 nanoamperes of distally negative current was able to initiate varying degrees of limb regeneration in all of our experimental frogs (Fig. 7-8) (Borgens et al., 1977a). Histological examination confirmed that true multitissue regeneration had been initiated, including large amounts of new muscle, organized extension of the radius ulna, large amounts of newly regenerated nerve, and separate cartilagenous structures resembling analagen of hand or wrist bones (Fig. 7-9). Rarely, we even found connective tissue structures resembling ligaments or tendons. All of the sham-treated controls healed their stumps in a manner typical of non-regenerating adult Rana. The tip of the stump was covered by skin, and

Figure 7-7. *Rana pipiens* implanted with a small insulated battery assembly. The anode is in contact with the tissues beneath the battery; the uninsulated portion of the wick cathode is in contact with the core tissues at the stump's surface. These assemhlies were implanted beneath the skin at the time of amputation and removed between three and four weeks later. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool., 200:*403, 1977.)

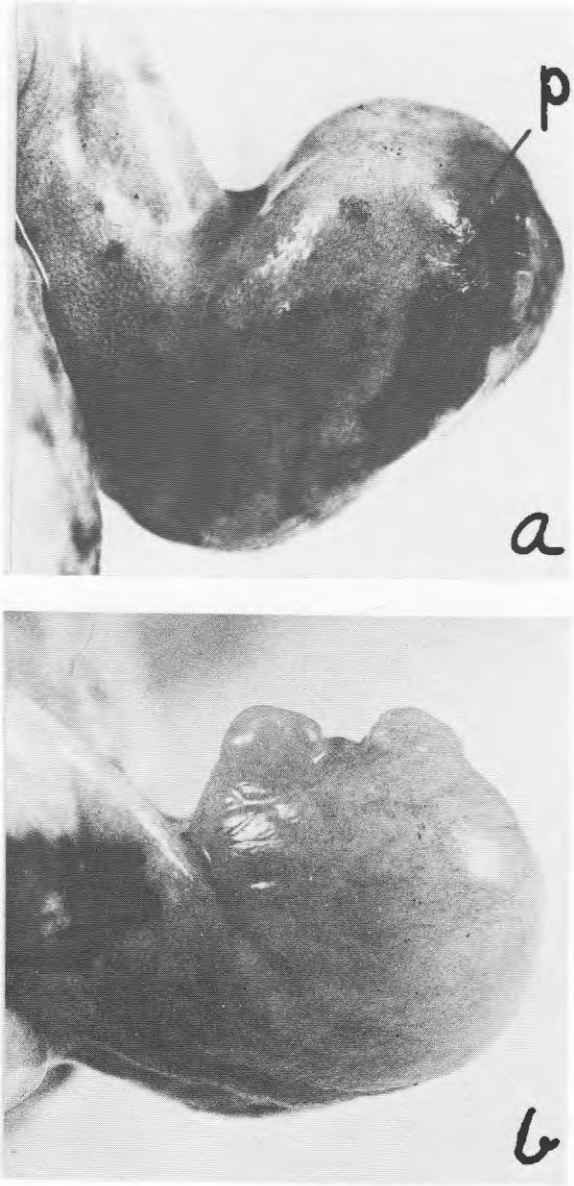


Figure 7-8. External views of a sham treated *(A)* and cathodally stimulated *(B)* frog limb stump. Note that the sham treated stump had healed over with pigmented skin with a centrally located scar tissue plug (P). This photograph was taken three months post amputation. This limb showed no further change in appearance until the time of sacrifice (3 months, 3 weeks later). The cathodally stimulated regenerate in *(B)* was photographed six months, three weeks after amputation. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool., 200*:403, 1977.)

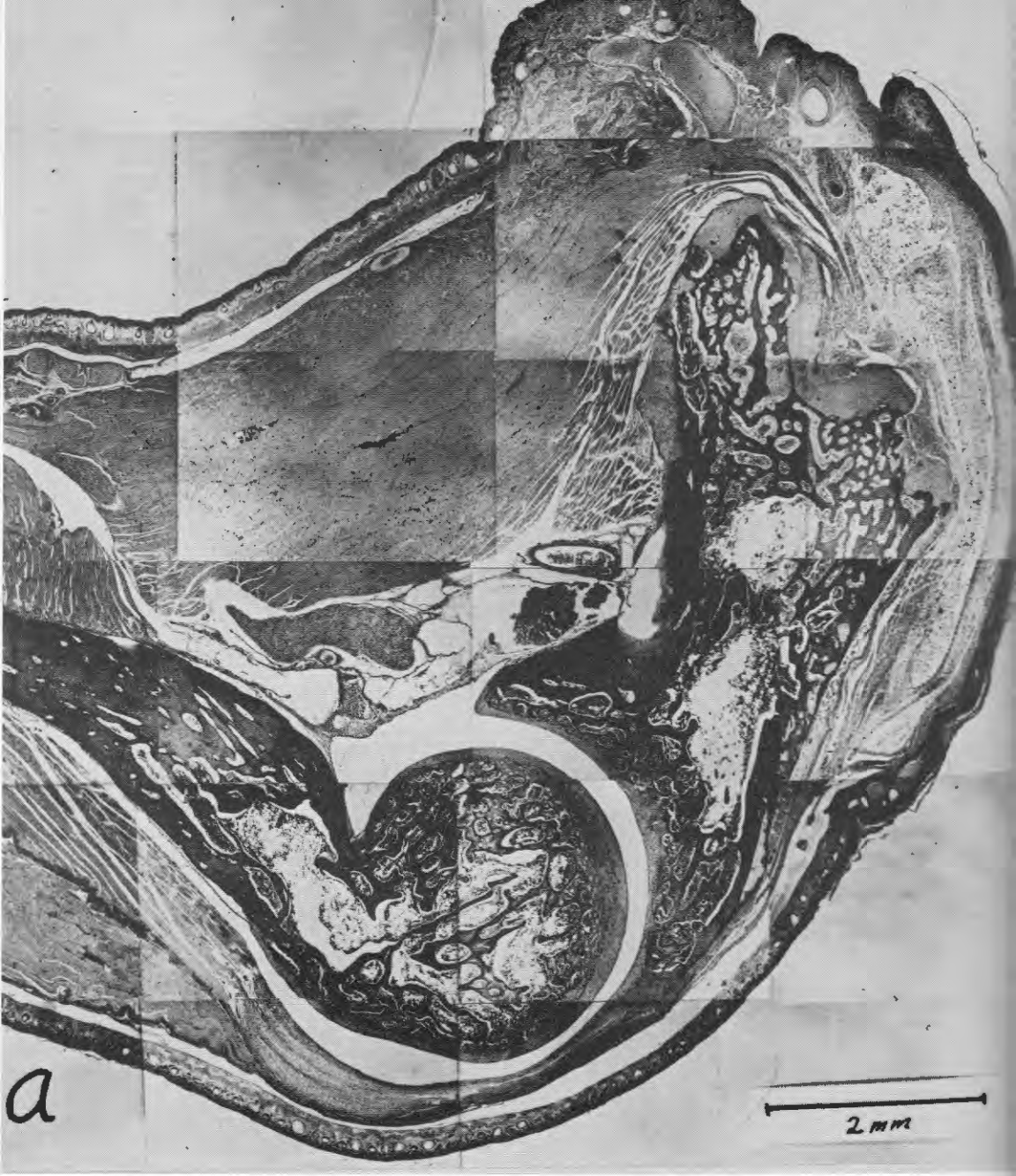
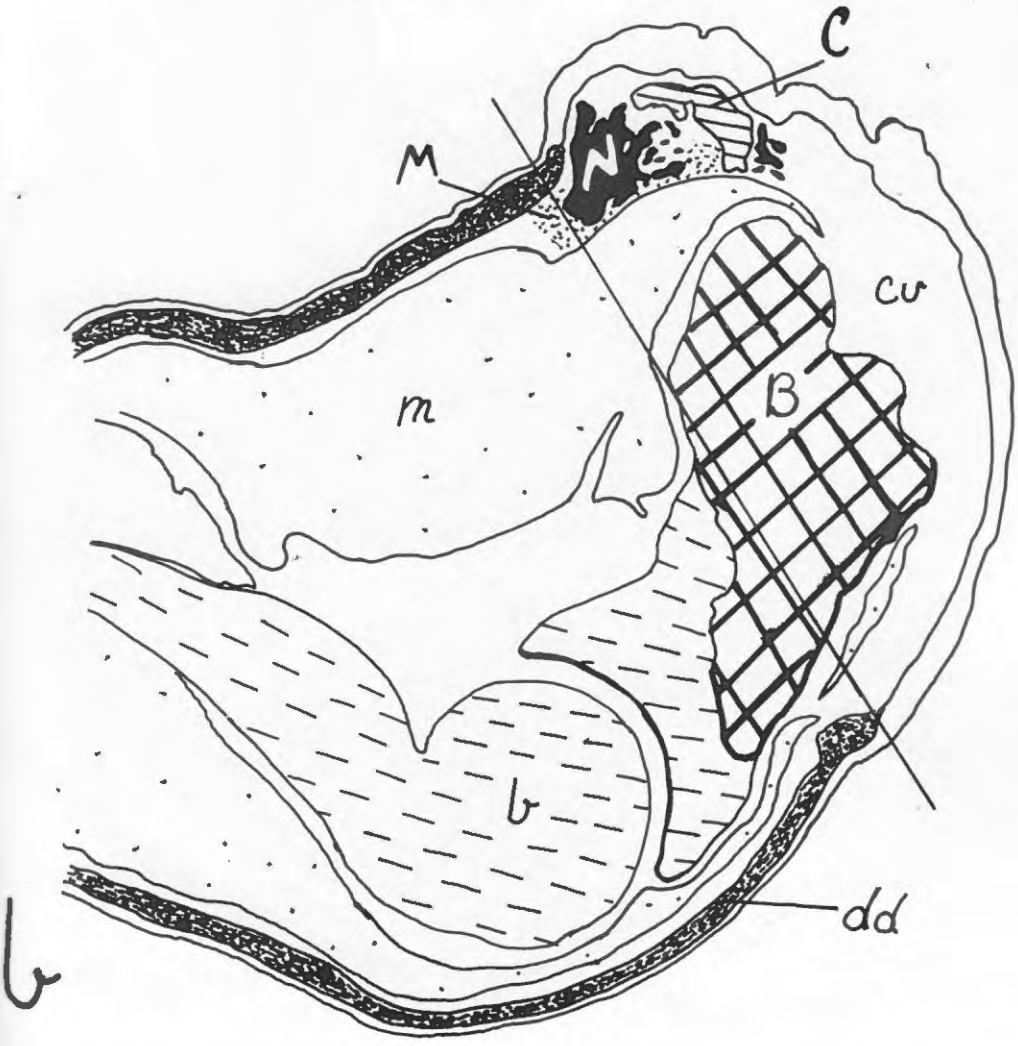


Figure 7-9. A low magnification photomicrograph *(A)* and tracing *(B)* of the cathode stimulated frog stump seen in Figure 7-8 at nine months, one week after amputation. N = regenerated nerve; M = regenerated muscle; m = original muscle; c = cartilage island; B = regenerated bone; b = original bone; dd = dense dermis; cv = loose connective tissue and vascular tissue. The line indicates the approximate level of amputation. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp Zool., 200:*403, 1977.)



large amounts of callus and connective tissue capped the end of the severed bone (Fig. 7-10) (see Schotté and Wilber, 1958; and Rose, 1944). Though undoubtedly some of this artificially supplied current is also shunted through the lymph spaces beneath the skin, the fact that the stump electrode was pulled tightly into the stump musculature insured that much of the current had to traverse this area to complete the circuit. In addition to cathodally stimulated experimentals and sham-treated controls, we also treated frog's stumps with distally positive current. In response to such treatments, we observed a remarkable deterioration of the forearm stump (Fig. 7-11). It appears that the polarity of current flow through the limb stump needs to be proximal-distal in direction, not only

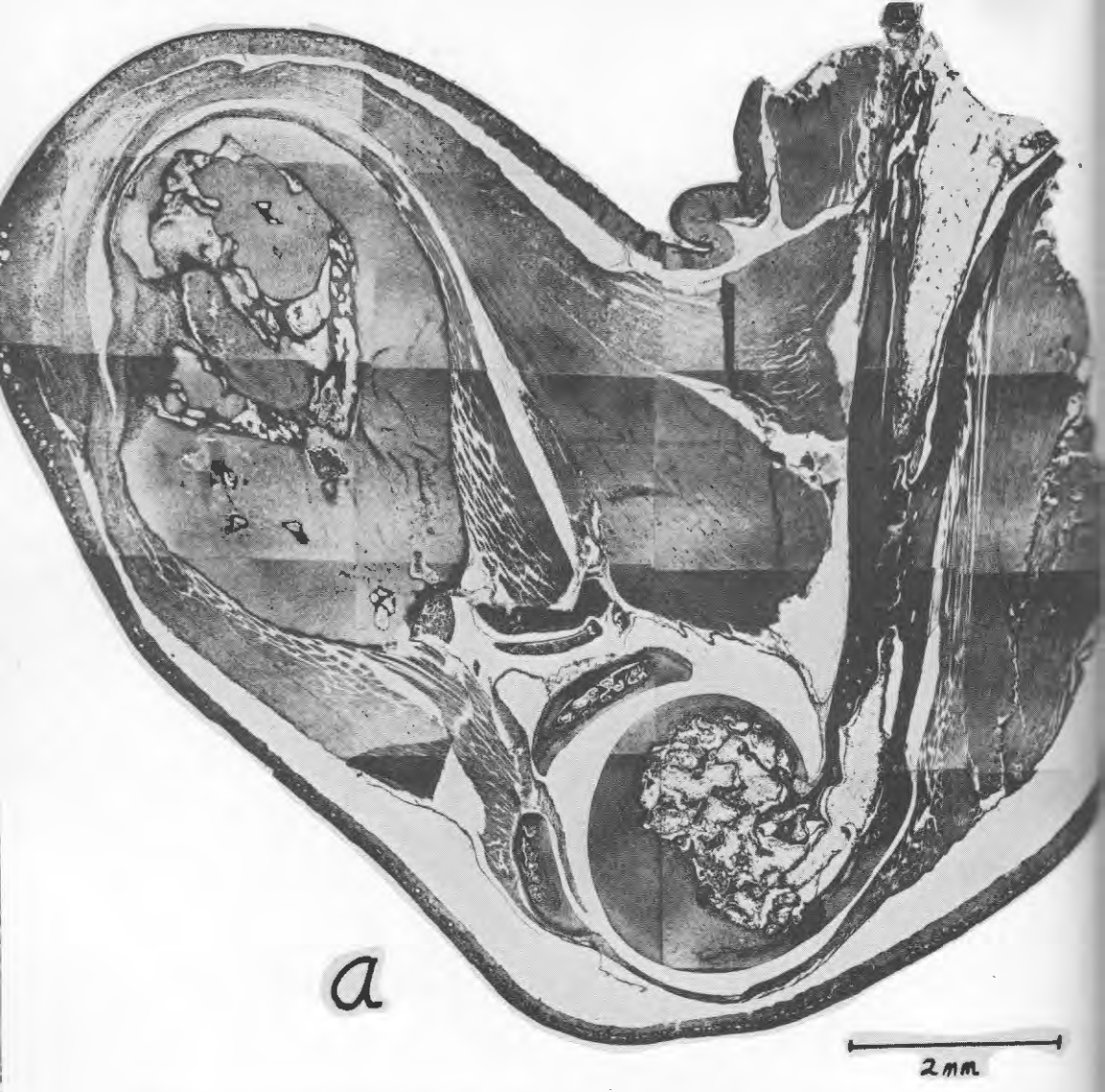
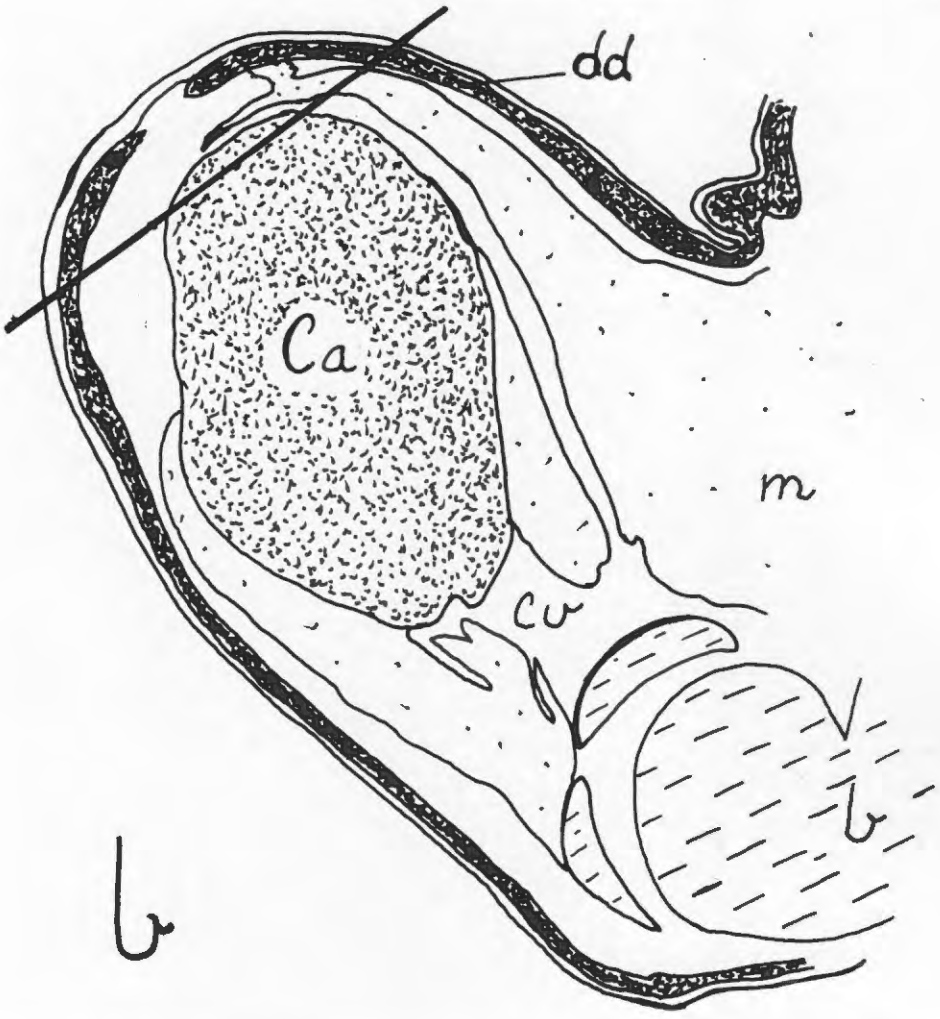


Figure 7-10. A low magnification photomicrograph *(A)* and tracing *(B)* of a sham treated frog stump six months, three weeks after amputation. Ca = callus; b = original bone; m = original muscle; dd = dense dermis; cv = loose connective tissue and vascular tissue. The line indicates the approximate level of amputation. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool., 200:*403, 1977.)

to initiate regeneration but also it may be necessary to maintain the basic structural integrity of the tissues within the stump itself.

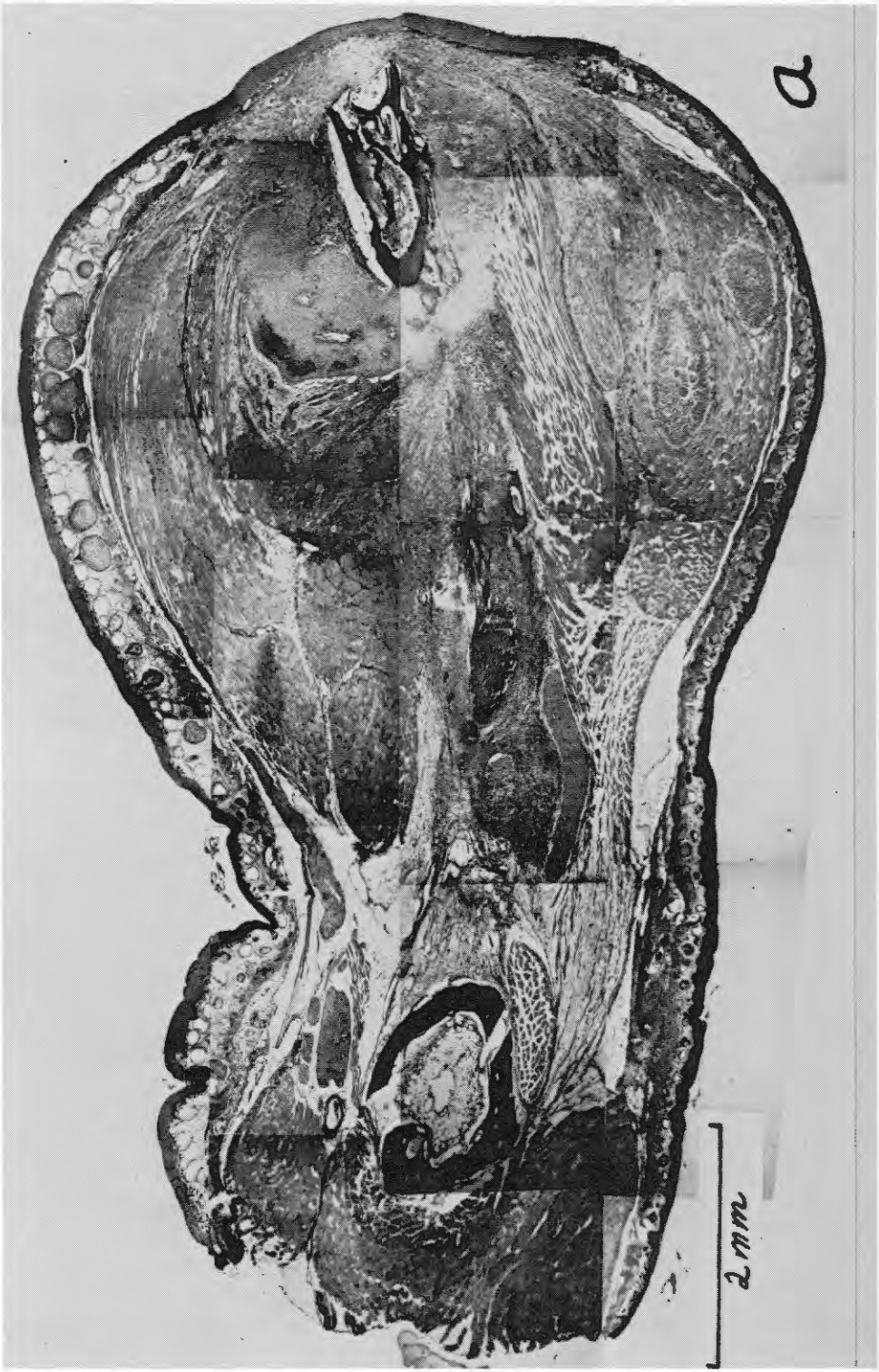
Other Methods of Inducing Regeneration in Adult Frogs

The amount of limb regeneration in adult frogs achieved by these electrical treatments has been modest indeed; however, it has been comparable to the regeneration achieved by other methods. Moreover, the per-



spective that is provided by our experiments on the electrical controls of limb regeneration allow us to make some interesting suggestions about what all of these different techniques used to initiate limb regeneration in frogs may have in common.

It is often cited that S. Meryl Rose was able to initiate limb regeneration in adult frogs by chronically irritating their limb stumps with saturated solutions of NaCl. Of three published experiments (1942, 1944, and 1945), the most informative (and best illustrated) is the 1944 paper in which Rose reports that the best limb regeneration was achieved through applications of 0.3% (52 mM) NaCl to the animals. In fact, quite remarkable regeneration of amputated toes was also achieved with this concentration of aqueous NaCl; saturated salt solutions were not even reported to have been used in this regard.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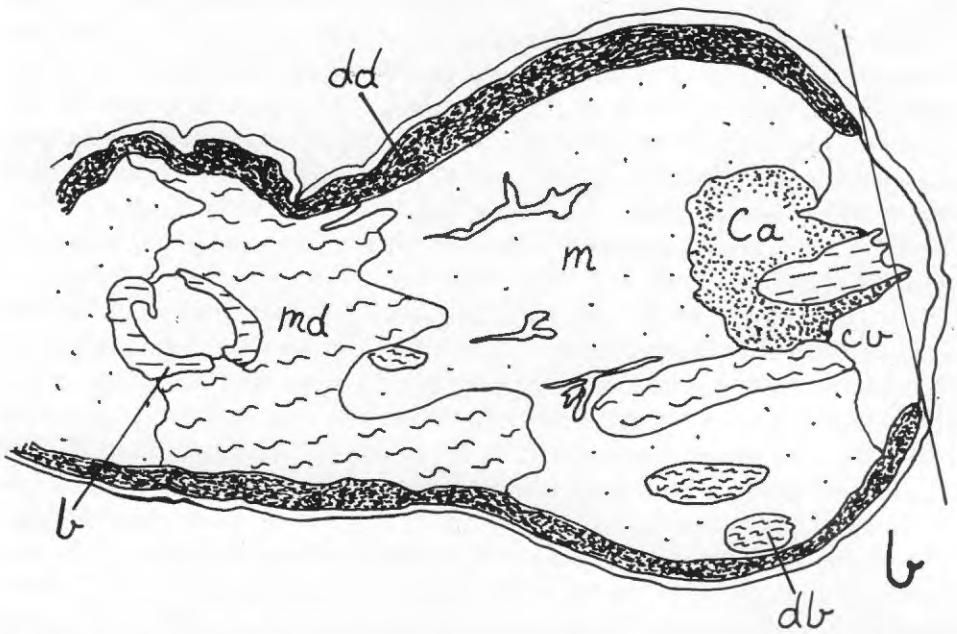

Figure 7-11. A low magnification photomicrograph *(A)* and tracing *(B)* of an anode treated frog stump four months after amputation. Ca = callus; b = original bone; cv = loose connective tissue and vascular tissue; m = original muscle; md = muscle degeneration area; db = pockets of cellular debris; dd = dense dermis. The distal remnant of the radio ulna was not connected to the more proximal segments in this limb. The line indicates the approximate level of amputation. (From R. B. Borgens, J. W. Vanable, Jr., and L. F. Jaffe, *J. Exp. Zool., 200:*403, 1977.)

Polezhaev (1972) also made various applications to the stumps of adult frogs to traumatize them and prolong the period of "dedifferentiation" of stump tissue. He reported that aqueous solutions of 0.8% $NaHCO_3$ (95 mM in Na^+) was effective in promoting regeneration. This concentration of Na^+ is about that found in the body fluids of amphibians; the most effective concentrations that Rose reported were even less than this. These solutions can hardly be called irritating, and it is difficult to see how they would prolong the period of tissue destruction. However, they are 50 to 100-times more concentrated in Na^+ than is pondwater. Used as a bathing medium, they would greatly increase the production of current produced by the skin, and therefore, the amount of current traversing the core tissues of the stump (where natural levels are low due to lymph space shunting.)

Possibly, the most complete regeneration yet initiated in adult frogs was achieved by Oscar Schotté. Schotté implanted supernumerary adrenal glands into *Rana clamitans* and suggested that the results observed were

due to higher levels of systemic glucocorticoids (Schotté and Wilber, 1958). Schotté suggested that these hormones inhibited the premature scar formation that occurs in adult frog stumps, thus permitting the limb to begin to regenerate. This is a very reasonable suggestion; however, I would point out that both mineral corticoids (also produced by the adrenals) and glucocorticoids are known to increase the output of the amphibian skin Na^+ pump (Bishop et al., 1961; Myers et al., 1961). Perhaps all of these hormonal effects acted in concert to produce the remarkable degree of regeneration that Schotté observed.

In 1954, Marcus Singer published a paper that later became a famous experiment in the field of limb regeneration. Singer hyperinnervated the forearm stumps of post metamorphic frogs by deviating the sciatic nerve of the leg to the amputated forelimb. These stumps began to regenerate and ultimately produced hypomorphic regenerates. Singer had earlier established that for limb regeneration to occur a critical relationship between forelimb innervation and stump surface area must exist (Singer, 1947). Not only are nerves necessary, but they are necessary in sufficient quantity. There is a certain low threshold cross-sectional area of nerve fibers per cross-sectional area of stump tissue that is required to support limb regeneration in Urodeles (Singer, 1965). Nonregenerating vertebrates (the adult frog, the lizard, and the mouse) are naturally deficient in this "axoplasmic ratio." Thus, when frog stumps were hyperinnervated, then regeneration was initiated (Singer, 1954).

As part of our analysis of the effect of weak steady fields in promoting limb regeneration in adult frogs, we determined the relative amounts of newly regenerated tissue in the regenerated structure (Borgens et al., 1977a). We were astonished to learn that one of the most predominant regenerate tissues was nerve, sometimes occupying 20 percent of the volume of newly regenerated tissue. Therefore, a plausible hypothesis is that the steady field imposed across frog stumps increased the amount, and possibly the rate, of nerve growth into the presumptive blastema, helping to initiate regeneration. We have also observed such induced "hyperinnervation" in the electrically enhanced regeneration of Xenopus stumps (Borgens et al., 1979c).

Possible Targets of the Electric Current

In concluding this discussion of the electrical controls of limb regeneration, it would be appropriate to discuss what the possible targets of the current might be. The most plausible targets would be cells that would be unusually long in a direction parallel to the field, or cells and tissues that have an unusually high electrical resistance. Muscle cells and especially nerve cells are elongate and would be especially sensitive to very shallow fields, since a particular density of current would produce a relatively large

voltage drop along their extended surface. The wound epithelium is an example of a tissue that is resistive enough to the flow of current to produce a relatively large voltage drop across itself. After the wound epithelium stratifies to about fifteen cells thick, it is many times referred to as the apical cap epithelium. Moreover, this epidermis has been shown to be a critical component of regeneration (Thornton, 1968).

However, of these possible candidates, we lean towards nerve as the critical target of the natural and applied fields. As I discussed, extraordinary amounts of nerve were observed in the electrically induced Anuran regenerates. Additionally, recent experiments have confirmed earlier (and somewhat controversial) reports that the direction of nerve growth can be influenced by small steady fields. When weak electrical fields are imposed across cultures of explanted chick dorsal root ganglia or individual (and developing) Xenopus neuroblasts, the direction, the rate, and the amount of growth of the emerging neurites can be influenced (Jaffe and Poo, 1979; Robinson and McCaig, 1980). This accelerated growth occurs towards the cathode in the applied fields. It is important to note that this polarity of effect is the same as would account for enhanced growth of nerves into the terminal regions of amphibian stumps in response to endogenous or artificially enhanced electric fields (compare Fig. 7-6A and 7-6B).

Therefore, we have suggested that the role of the natural electrical field in Urodele stumps is to insure adequate inervation to the appendage for regeneration to occur (Borgens et al., 1977a, 1979d). According to this hypothesis, denervated limbs fail to regenerate because the *targets* of the endogenous fields are not present (although an ample amount of stump current is present*).

Altogether, we feel that, in some way, naturally occurring steady fields and nerve tissue each share a role in the regrowth of an amphibian limb, and that each is necessary but not sufficient for such regeneration to occur.

Studies into the electrical controls of amphibian regeneration have been very rewarding, yet there is still much to learn. Our attempts at demonstrating the relevance of the stump currents to the regrowth of the limb are only a modest first step. We also need to more directly test the possible relationship between nerves and the endogenous stump current. A thorough study of the electrical character of regenerating tadpole limbs would help our understanding of the role stump currents might play in the loss of regenerative ability during metamorphosis. However, in many respects,

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* I note one recent report in which regeneration is claimed to have been initiated in denervated newt stumps by electrical means (Rose, 1978). The animals in this study were denervated only once. This has been shown sometimes to be insufficient to permanently inhibit regeneration since nerves will grow back into the stump after a week or so (Singer, 1952). Moreover, the histology presented in this study clearly shows nerve within the regenerating limbs themselves.

the amphibian limb system is too complicated to gain insight into how steady fields might affect the individual cells themselves. For this reason, Melvin Cohen, Lionel Jaffe, and I have been looking into the possible role of injury current in the regeneration of identifiable neurons within the spinal cord of the lamprey eel larvae. There are several advantages to this preparation: giant reticulospinal neurons, the Müller and Mauthner cells (Fig. 7-12), found in the brain of the eel larvae project giant axons (ca 40 μm) down the length of the spinal cord (which can be up to 10 cm long).

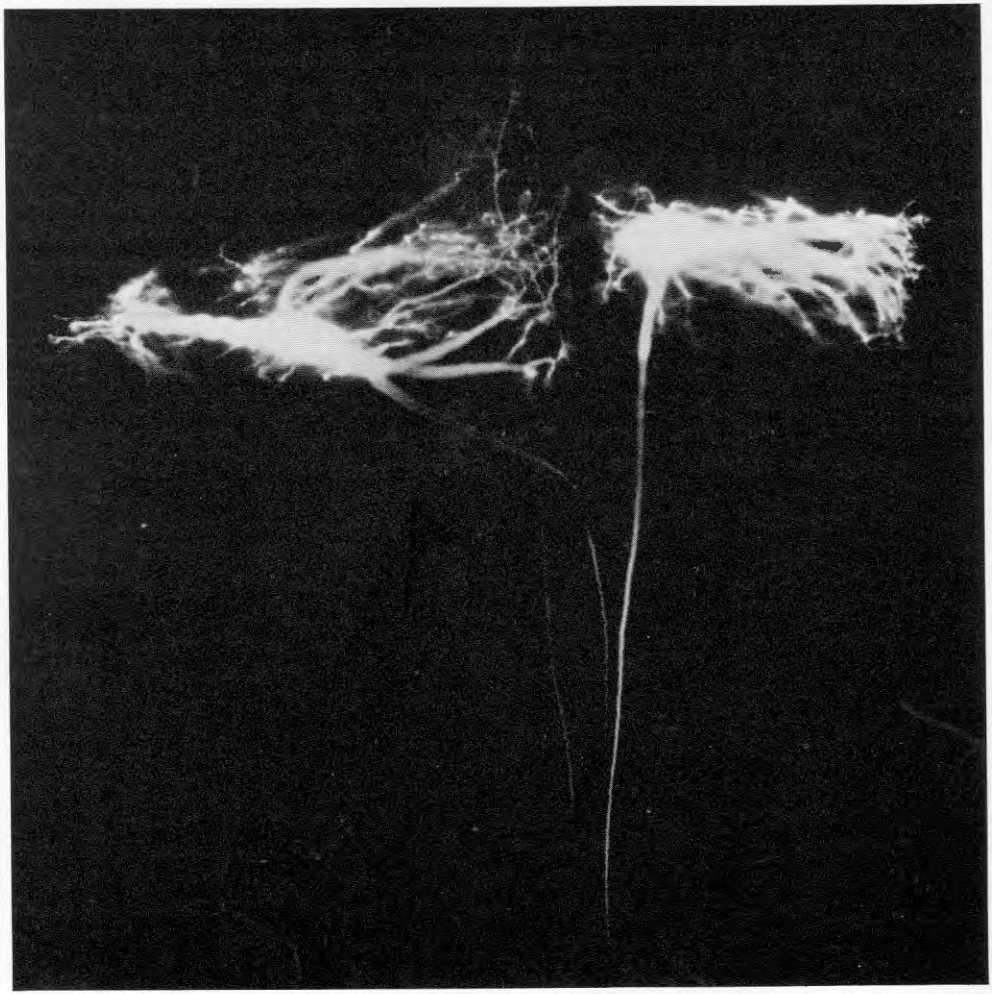

Figure 7-12. A darkfield photomicrograph of two reticulo spinal neurons in the brain of a lamprey eel larvae. A Mauthner cell (with the decussating axon) is on the left; a Müller cell on the right. The third axon visible is from another dye-injected cell body out of the photographic field. The neurons were filled by iontophoretic injection of the fluorescent dye, Lucifer Yellow. After injection, the entire CNS was fixed, cleared, and photographed. (The cell bodies are about 80 μm in diameter.)

These cells can be identified individually at various locations within the brain and cord, and are known to have limited powers of regeneration (Rovainen, 1967; Wood and Cohen, 1979). Since the spinal cord is not intrinsically vascularized, receiving its nourishment by diffusion, explanted CNS preparations do very well in simple organ culture for periods up to a week or more. The viability of these preparations can be easily checked by conventional electrophysiological procedures.

Large Currents Enter the Severed Lamprey Spinal Cord

We kept excised CNS preparations in organ culture and measured the electrical currents with the vibrating probe produced by a complete transection of the cord. Immediately after the cord has been completely severed, immense currents (0.6 to 0.8 milliamp/cm^2) enter the cut face of both proximal and distal segments. In this exploratory study, we continued to study (and reported on) only the proximal segment of the spinal cord, that in continuity with the brain (Fig. 7-13). These currents fall to a quarter of their initial value within the first hour post transection. Over the next two days they generally decline from about 100 μA/cm^2 to about 4 μA/cm^2. This level of 4 μA/cm^2 persisted for the next four days of measurement

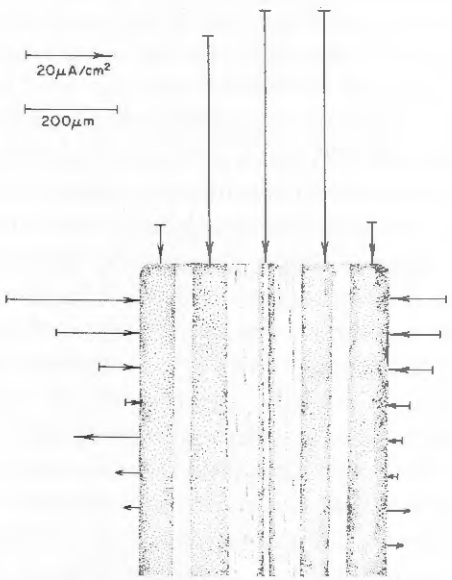

Figure 7-13. A scan of the perpendicular component of ionic current entering or leaving the cut end of a lamprey eel spinal cord, transected four hours previously. This diagram is of the cut end of the proximal segment, which is in continuity with the brain. The giant axons are shown as light bands parallel to the long axis of the cord. The lengths of the arrows are proportional to the current density entering or leaving the spinal cord. (From R. B. Borgens, L. F. Jaffe, and M. J. Cohen, *Proc. Natl. Acad. Sci. USA*, 77:1209, 1980.)

showing no further decline. We discontinued the measurements at this time (6 days post transection) knowing the preparations were viable and electrophysiologically active (Borgens et al., 1980). Indeed, the currents may even persist much longer.

Soon after transection, the density of the current entering the cord appears uniform. By four hours after transection, a measure of nonuniformity becomes apparent. There are sharp peaks of current density opposite the cut ends of individual Mauthner axons, and also opposite the mid ventral bundle of cut Müller axons. By moving the probe less than one axon diameter to either side of this peak, the current fell off by 20 to 50 percent. This nonuniformity suggests that the current actually enters the cut ends of, at least, the giant fibers.

We also made a modest first attempt at discovering what ions are carrying this current. We systematically replaced the major ions in the bathing medium and observed the responses. It appears that about 60 percent of the injury current is carried by Na^+, and that much of the balance may be carried by Ca^{++}. The other ions tested (K^+, Mg^+, and Cl^-) appeared to be, at best, only minor components (Borgens et al., 1980). Additional calculations determined that, over the six days that we measured currents entering the cut end of the spinal cord, this influx represented about 6 coulombs per cm^2 of total charge. Since Na^+ and CA^{++} are found in only minute quantities within the axoplasm, this ionic current should result in a rather prolonged and radical alteration in the ionic compositions of the axoplasm in the terminal areas of the severed nerve. The effects of this incurrent, then, should fall into at least two categories: ionic effects and possibly voltage mediated effects. It is known that the integrity of structural elements (neurotubules and microtubules) within axoplasm are very sensitive to the level of free cytoplasmic calcium (Schlaepfer and Hassler, 1978; Schlaepfer and Bunge, 1978).The influx of calcium due to the injury should, we believe, be primarily responsible for the rapid deterioration of the axoplasm observed near the site of injury (Zelena et al., 1968). Even the growth phase of the regenerating nerves may involve the long-term, more steady component of the current we have measured. Actin assembly, which occurs in a variety of elongating cells, is known to be under ionic control (Tilney, 1979). Additionally, currents on the order of 30 to 300 $\mu A/cm^2$ flowing within the cut axon should also produce voltage gradients on the order of 1 to 30 mV/mm. Such gradients should be large enough to partition some components within the injured nerve. For example, negatively charged macromolecules would be expected to be driven towards the cut end. We have asked if self-electrophoresis may even play some role in the gross accumulation of organelles at both ends of a severed or crushed axon soon after injury. Terminal accumulations of mitochondria, for example, are known to result from a *movement* of these organelles,

and not from a *synthesis* near the cut end (Zelena et al., 1968; Zelena, 1969).

Finally, these large currents of injury are not restricted to the giant fiber system in lampreys. Ernesto Roederer at Yale has used suction electrodes and a conventional microampmeter to measure comparable currents flowing into the cut face of a central nervous system connective in the cricket. Such connectives contain several giant axons comparable in size to those found in lamprey spinal cord. Altogether, we feel that these injury currents are a general feature of injured nerve, and they may be involved as a signal or as a mediator of the events that subsequently occur after the injury.

It is certain that injury to cells or tissues can induce a substantial, immediate, and prolonged flow of electric current. I have discussed the possible roles of such current in the regrowth of Urodele limbs and in the regeneration of reticulospinal neurons in lamprey eel larvae. Mammalian skin can generate strong wound currents also, and it is suggested that this current flow may be involved with the ability of children to regenerate the tips of their fingers (Illingworth and Barker, 1980), and of skin wounds to heal (Barker et al., 1980). It is apparent that the classical (and in many ways, forgotten) observations of wound potentials in a variety of animals and their tissues may bear important clues to the control of wound healing and regeneration, indeed even the overall responses of cells to injury.

Summary

1. Salamanders and newts, animals well known for their regenerative powers, drive strong steady current (20-100 $\mu A/cm^2$) out the ends of their forelimb stumps. This outcurrent begins immediately after amputation, and declines to very low levels at about the time of overt blastema formation. The source of this current is tbe well known "Na^+ battery" of amphibian skin.
2. When one inhibits or reduces the flow of this stump current (in Urodeles) by two different means, the normal regeneration of these limbs is severely interfered with, many times completely inhibited.
3. Adult frogs, in particular, Ranids, possess a similar "skin battery" to Urodeles. However, most of the skin-generated current is shunted through the subdermal lymph spaces that exist in Anurans, but not in Urodeles. The result of this lymph space shunting is that the density of currents traversing the stump's core tissues is greatly reduced. Since the blastema arises from these tissues, this may be a critical factor in the frog's lack of regenerative ability.
4. If one artificially supplements the level of current traversing the core tissues in frogs (by using an indwelling battery system), then the regeneration of the limb is initiated. The resulting structures are atypical in

appearance and internal organization; however, true multitissue regeneration has taken place.

5. Large currents of injury enter the transected spinal cord of lamprey eel larvae. Our measurements suggest that such currents actually enter the cut neurons themselves. These currents are extremely large (on the order of 1 milliamp/cm^2) immediately after transection and by the second day fall to a much reduced but stable level (about 4 μA/cm^2) that lasts for at least six days post transection. I suggest such injury current may be involved with the overall responses of nerves to injury.

REFERENCES

Barker, A. T.; Vanable, J. W., Jr.; and Jaffe, L. F.: Electrical fields within the skin near wounds in guinea pigs (in preparation).

Becker, R. O.: The bioelectric field pattern in the salamander and its simulation by an electronic analog. *IRE Trans. Med. Electronics ME, 7:*202, 1960.

Becker, R. O.: The bioelectric factors in amphibian limb regeneration. *J. Bone Joint Surg., 43A:*643, 1961.

Benos, D. J. and Mandel, L. J.: Irreversible inhibition of sodium-entry sites in frog skin by a photosensitive amiloride analog. *Science, 199:*1205, 1978.

Bishop, W. R.; Mumbach, M. W.; and Scheer, B. T.: Interrenal control of active sodium transport across frog skin. *Am. J. Physiol., 200:*451, 1961.

Borgens, R. B.; Vanable, J. W., Jr.; and Jaffe, L. F.: Bioelectricity and regeneration. I. Initiation of frog limb regeneration by minute currents. *J. Exp. Zool., 200:*403, 1977a.

Borgens, R. B.; Vanable, J. W., Jr.; and Jaffe, L. F.: Bioelectricity and regeneration: large currents leave the stumps of regenerating newt limbs. *Proc. Natl. Acad. Sci. USA, 74:*4528, 1977b.

Borgens, R. B.; Vanable, J. W., Jr.; and Jaffe, L. F.:Reduction of sodium dependent stump currents disturbs urodele limb regeneration. *J. Exp. Zool.*, 1979a, in press.

Borgens, R. B.; Vanable, J. W., Jr.; and Jaffe, L. F.: Role of subdermal current shunts in the failure of frogs to regenerate. *J. Exp. Zool.*, 1979b, in press.

Borgens, R. B.; Vanable, J. W., Jr.; and Jaffe, L. F.: Small artificial currents enhance *Xenopus* limb regeneration. *J. Exp. Zool., 207:*217, 1979c.

Borgens, R. B.; Vanable, J. W., Jr.; and Jaffe, L. F.; Bioelectricity and regeneration. *Bioscience, 29:*468, 1979d.

Borgens, R. B.; Jaffe, L. F.; and Cohen, M. J.: Large and persistent electrical currents enter the transected lamprey spinal cord. *Proc. Natl. Acad. Sci. USA, 77:*1209, 1980.

DuBois-Reymond, E.: Vorläufiger abrifs einer untersuchung über den sogenannten froschstrom und über die electromotorischen fische. *Ann. Phys. U. Chem., 58:*1, 1843.

DuBois-Reymond, E.: *Untersuchungen über tierische elecktrizität II, 2,* Reimer, Berlin, 1860.

Herlitzka, A.: Ein beitrag zur physiologie der generation. *Wilhelm Roux Archiv., 10:*126, 1910.

Illingworth, C. M. and Berker, A. T.: Measurement of electrical currents emerging during the regeneration of amputated finger tips in children. *Clin. Phys. an Physl. Measm.*, 1980, in press.

Jaffe, L. F.: Electrical controls of development. *Ann. Rev. Biophys. Bioeng., 6:*445, 1977.

Jaffe, L. F.: Control of development by ionic currents. *Membrane Transduction Mechanisms Symp. Soc. Gen. Physiol., 33:*199, 1979.

Jaffe, L. F. and Nuccitelli, R.: An ultrasensitive vibrating probe for measuring steady extracellular currents. *J. Cell Biol., 63:*614, 1974.

Jaffe, L. F. and Poo, M. M.: Neurites grow faster towards the cathode than the anode in a steady field. *J. Exp. Zool., 209:*115, 1979.

Kirschner, L. B.: Electrolyte transport across the body surface of freshwater fish and amphibia. In *Transport Mechanisms in Epithelia.* Munksgaard, Copenhagen, 1973, p. 447.

Lassalle, B.: Characteristiques des potentiels de surface des membres du triton *Pleurodeles watlii,* Michah. *C. R. Acad. Sci. (Paris), Series D, 278:*483, 1974a.

Lassalle, B.: Origine epidermique des potentiels de surface du membre de triton *Pleurodeles watlii,* Michah. *C. R. Acad. Sci. (Paris), Series D, 278:*1055, 1974b.

Lorente de Nó, R.: Action currents of nerve in volume conductors. *Institute for Medical Research* (The Rockefeller Institute for Medical Research, N.Y.), Vol. 132, 1947.

McAfee, R. D.: Survival of *Rana pipiens* in deionized water. *Science, 178:*183, 1972.

Monroy, A.: Ricerche sulle correnti elettriche dalla superficie del corpo di Tritoni adulti normali e durante la rigenerazione degli arti e della coda. *Pubbl. Stn. Zool. Napoli, 18:*265, 1941.

Myers, R. M.; Bishop, W. R.; and Scheer, B. T.: Anterior pituitary control of active sodium transport across frog skin. *Am. J. Physiol., 202:*444, 1961.

Mescher, A. L.: Effects on adult newt limb regeneration of partial and complete skin flaps over the amputation surface. *J. Exp. Zool., 195:*117, 1976.

Polezhaev, L. V.: *Loss and Restoration of Regenerative Capacity in Tissues and Organs of Animals.* Harvard University Press, Cambridge, Mass., 1972.

Robinson, K. R. and McCaig, C.: Electrical fields, calcium gradients, and cell growth. *Ann. N.Y. Acad. Sci.,* 1980, in press.

Rose, S. M.: A method for inducing limb regeneration in adult Anura. *Soc. Exp. Biol. and Med., 49:*408, 1942.

Rose, S. M.: Methods of initiating limb regeneration in adult Anura. *J. Exp. Zool., 95:*149, 1944.

Rose, S. M.: The effect of NaCl in stimulating regeneration of limbs of frogs. *J. Morphol., 77:*119, 1945.

Rose, S. M. and Rose, F. C.: Electrical studies on normally regenerating, on x-rayed, and on denervated limb stumps of *Triturus. Growth, 38:*363, 1974.

Rose, S. M.: Regeneration in denervated limbs of salamanders after induction by applied direct currents. *Bioelectr. and Bioenerg., 5:*88, 1978.

Rovainen, C. M.: Physiological and anatomical studies on large neurons of central nervous system of the sea lamprey *(Petromyzon marinus)* I. Müller and Mauthner cells. *J. Neurophysiol., 30:*1000, 1967.

Schlaepfer, W. W. and Bunge, R. P.: Effects of calcium ion concentration on the degeneration of amputated axons in tissue culture. *J. Cell Biol., 59:*456, 1973.

Schlaepfer, W. W. and Hasler, M. B.: Characterization of the calcium-induced disruption of neurofilaments in rat peripheral nerve. *Brain Res., 168:*299, 1979.

Schotté, O. E. and Harland, M.: Amputation level and regeneration in limbs of late *Rana clamitans* tadpoles. *J. Morph., 73:*329, 1943.

Schotté, O. E. and Wilber, J. F.: Effects of adrenal transplants upon forelimb regeneration in normal and in hypophysectomized adult frogs. *J. Embryol. Exp. Morphol., 6:*247, 1958.

Singer, M.: The nervous system and regeneration of the forelimb of adult *Triturus.* VII. The relation between number of nerve fibers and surface area of amputation. *J. Exp. Zool., 104:*251, 1947.

Singer, M.: The influence of the nerve in regeneration of the amphibian extremity. *Quart. Rev. Biol., 27:*169, 1952.

Singer, M.: Induction of regeneration of the forelimb of the postmetamorphic frog by augmentation of the nerve supply. *J. Exp. Zool., 126:*419, 1954.

Singer, M.: A theory of the trophic nervous control of amphibian limb regeneration,

including a reevaluation of quantitative nerve requirements. In *Regeneration in Animals and Related Problems.* North Holland Publishing Co., Amsterdam, 1965.

Smith, S. D.: Effects of electrode placement of stimulation of adult frog limb regeneration. *Ann. N.Y. Acad. Sci., 238:*500, 1974.

Taylor, R. E., Jr. and Barker, S. B.: Transepidermas potential difference: Development in Anuran larvae. *Science, 148:*1612, 1965.

Tilney, L. G.: Actin, motility, and membranes. *Soc. of Gen. Physiol. Series: Membrane Transduction Mechanisms,* Vol. 33:163, 1979.

Thornton, C. S.: Amphibian limb regeneration. *Advances in Morphogenesis,* Vol. 7:205, 1968.

Ussing, H. H.: Transport of electrolytes and water across epithelia. *Harvey Lect., 59:*1, 1964.

Wood, M. R. and Cohen, M. J.: Synaptic regeneration in identified neurons of the lamprey spinal cord. *Science, 206:*344, 1979.

Zelena, J.: Bidirectional shift of mitochondria in axons after injury. *Symp. of the Intl. Soc. for Cell Biol.: Cellular Dynamics of the Neuron,* Vol. 8:73, 1969.

Zelena, J.; Lubińska, L.; and Gutmann, E.: Accumulation of organelles at the ends of interrupted axons. *Z. Zellforsch. Mikroskop. Anat., 91:*200, 1968.