Chapter 8

MODULATION OF NEWT LIMB REGENERATION BY ELECTROMAGNETICALLY INDUCED LOW LEVEL PULSATING CURRENT

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Introduction

THE ABILITY of certain amphibia to regenerate organs and appendages has stimulated considerable research (see Goss, 1972, for a recent review). Limb regeneration in the newt has been examined using classical biochemical techniques (Morzlock, 1971; Tassava, 1969). In addition, certain tissue correlations, such as an apparent requirement for sufficient nerve population, have been reported (Singer, 1951). Following the early experiments of Rose (1945) on the induction of partial limb regeneration in nonregenerating amphibia, by external ionic changes, Smith (1967) showed that the regeneration of adult frog limbs could be initiated by the application of minute direct electrical currents using silver-platinum bimetallic implants. If careful attention is paid to current levels and positioning of the electrode at the wound surface, such implants can result in essentially perfect regenerates (Smith, 1974). These studies have been repeated using other D.C. sources (Borgens, 1977). Partial limb regeneration in the rat has been reported, using electrode implants delivering small D.C. current in the amputation site (Becker, 1972).

The physical perturbation in the above studies is both electrical and (primarily) chemical as a result of electrolysis effects (Pilla, 1974). The latter are relatively uncontrollable and for this reason implants always carry with them the possibility of infection or untoward tissue reactions. Of much more fundamental importance is the fact that D.C. current contains, by definition, significantly less selective electrical "information" than time varying current (Pilla, 1972, 1974).

In order to assess the mode of action of purely electrical influences on cell and tissue function, it has been proposed that current functions can be designed to perturb nonfaradaic electrochemical phenomena (such as ion binding and membrane transport) at cell surfaces and junctions (Pilla, 1972, 1974, 1977). That these electrochemical events may be involved in cellular regulation is suggested by results using electromagnetically induced current pulses to modulate the following: Ca^{++} uptake in

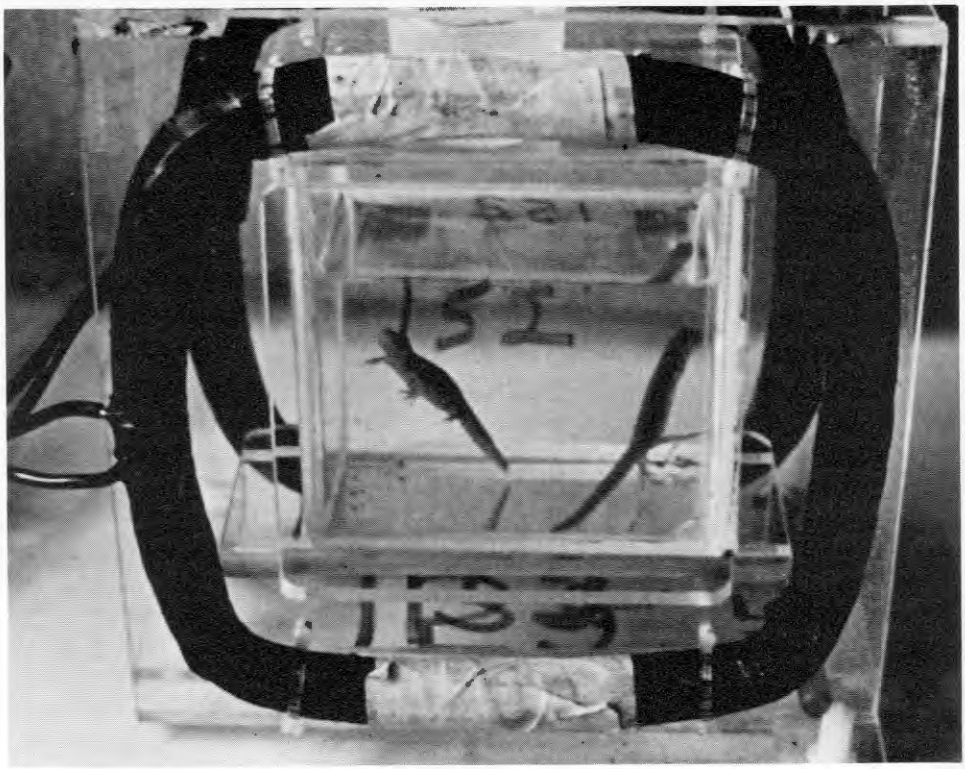

Figure 8-1. Photograph of the experimental apparatus shown in this study. The newts are shown in a plastic aquarium configured to fit between two air gap coils, which are magnetically aiding and large enough to provide a spatially homogeneous magnetic field within the aquarium. The coils are powered by units supplied by Electro-Biology Inc., Fairfield, N.J. to provide the induced current waveforms shown in Figure 8-2.

embryonic chick limb explants (Colacicco, 1979; Monet, 1979); DNA synthesis in isolated bone cells (Shteyer, 1979); DNA uncoiling in amphibian nucleated RBCs (Chiabrera, 1978); rate of radial fracture healing in the rat (Christel, 1979); membrane transport phenomena (Pilla, 1977, 1979); and the healing of recalcitrant bone fractures (Bassett, 1977, 1978, 1979; Pilla, 1979). This study was designed to further test the generality of the electrochemical information transfer approach by using electromagnetically induced currents similar to those used in the above studies to alter the course of naturally occurring amphibian limb regeneration.

Materials and Methods

Randomly selected pairs of adult *Notophthalmus v. viridescens* were anesthetized with 1 : 1000 tricaine methane sulfonate (Finguel-Ayerst Laboratories) solution. Both forelimbs were then amputated at mid-forearm. The wound surfaces were dusted with Mycostatin® (Squibb)/sulfadiazine

(Searle) powder, and the animals placed on wet paper toweling until they revived. Following recovery from anesthesia, the pairs were placed in small (9 × 9 × 6 cm) Plexiglass® aquaria filled with Holtfreter's solution diluted 1 : 100 with aerated distilled water (artificial pond water, APW). The animals were fed strips of beef liver twice weekly, and the APW in the aquaria was changed three times weekly. The frequent water changes were necessitated by tbe small volume of the aquaria. The aquaria were placed between pairs of 10 × 10 cm square essentially Helmholtz-aiding coils spaced 6.5 cm apart. Loose-fitting weighted covers were then applied to the aquaria to prevent the escape of the animals. Figure 8-1 illustrates the configuration of the experimental device. Except for the controls, the coils were then energized for twenty-four hours daily with the aid of pulse generators supplied by Electro-Biology Inc. The induced current waveforms were of two basic types as shown in Figure 8-2. Both single pulse (a, Fig. 8-2) and pulse burst (b, Fig. 8-2) waveforms were utilized in this preliminary survey in order to assess the effect of interrelated waveform parameters as described by frequency spectral analysis, which takes into

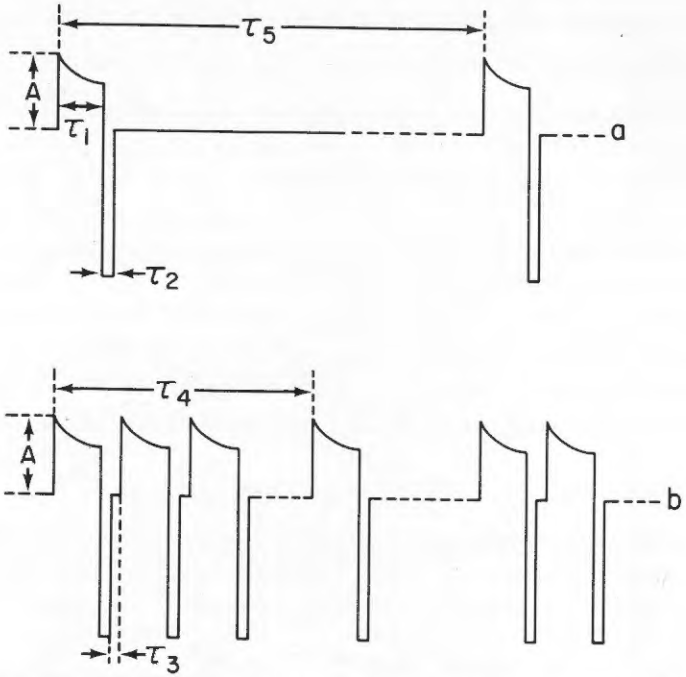

Figure 8-2. Schematic representation of the two basic waveform types employed in this study. The single pulse signal shown in "a" was configured as described in Table 8-I for waveform 1. The remaining three waveforms were pulse bursts as shown in "b." The amplitude A, utilized for calibration of the main polarity portion (τ_1) of each waveform is in arbitrary units referred to a specific probe coil (65 turns, #36 wire, 0.5 cm diameter).

account the kinetics and irreversibility of these nonfaradaic processes (Pilla, 1980). Thus, for each waveform, main and opposite polarity pulse durations (τ_1, τ_2; Fig. 2), single or burst mode (τ_3, τ_4; Fig. 8-2) and repetition rate (τ_5; Fig. 8-5) can be taken into account along with the nonlinear membrane behavior to assess the effective perturbation that reaches the cell/tissue complex. For this preliminary study four waveforms were employed as described in Table 8-I. Laplace plane analysis (real axis frequency analysis, see Pilla, 1970, 1972a) of waveforms 1, 2 and 3, using a 10 percent nonlinearity factor in the cell surface process, are shown in Figure 8-3. As can be seen, these waveforms have spectra designed to provide an overall examination of the importance of high ($>10^3$ rad/sec), low (<1 rad/sec), and mid ($\approx 10^2$ rad/sec) frequency ranges. Waveform 4 differs from waveform 3 only in τ_2 (see Fig. 8-2), with the result that their frequency spectra differ only in amplitude over the whole frequency range. The electrochemical model dependent spectral analysis for these waveforms is shown in Figure 8-4; waveform 1 is also included for comparison. Induced current levels in homogeneous conducting electrolytes hoth with and without cellular pathways have been measured (Pilla, 1980) and are between 10 $\mu A/cm^2$ for waveforms with amplitude A = 1 (see Table 8-I) and 1 $\mu A/cm^2$ for A = 0.1 at the peak of the main polarity portion of the waveform. Knowledge of the total current affecting the cells

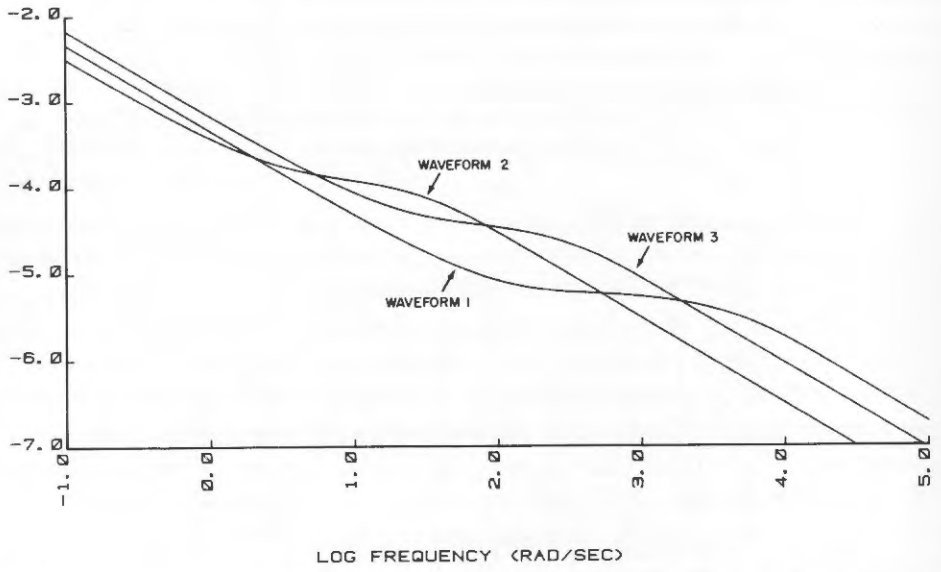

Figure 8-3. Frequency spectra (via real axis Laplace Transformation; Pilla, 1972) for waveforms 1, 2, and 3 (curves are so labeled). Note that the low frequency portion (<10 rad/sec where rad = $2\pi \times$ frequency) appears to be the region over which effective perturbation levels correlate with the noted alteration in regeneration. The voltage scale is arbitrary and useful only for waveform comparison.

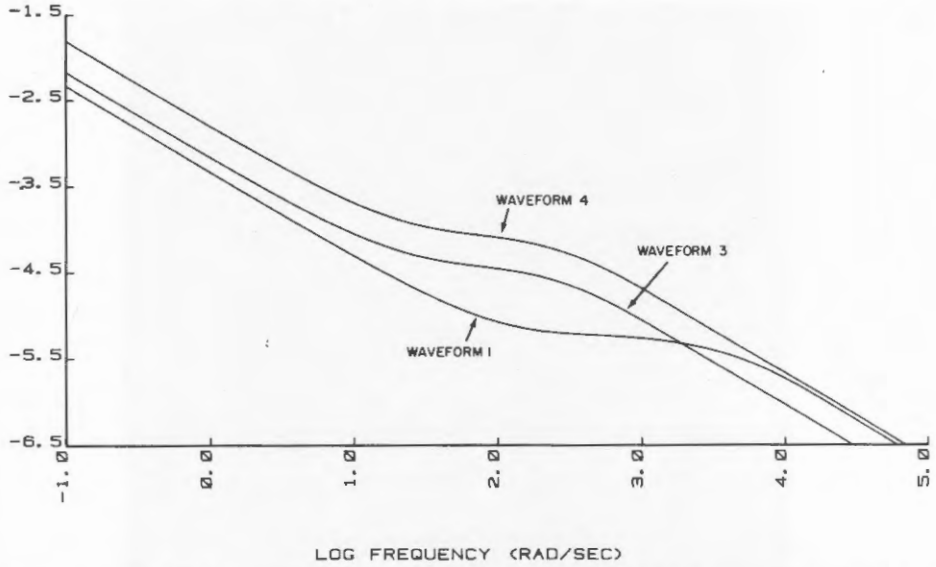

Figure 8-4. Real axis frequency spectra for waveforms 1, 3, and 4 (curves are so labeled). Note the difference between 3 and 4 is a constant amplitude shift over the whole frequency region. This provides further confirmation that perturbation levels which inhibit or accelerate regeneration appear to be determined by the low frequency region. The voltage scale is arbitrary and useful only for waveform comparison.

in this regenerating tissue must await a measure of their impedance. It is certain, however, that this quantity will be less than that for tissue fluids alone because of the low membrane/electrolyte impedance ratio (Cole, 1968; Pilla, 1974, 1977).

At the end of twenty-one days of treatment, the animals were removed, reanesthetized, and decapitated. The forelimbs were removed and photographed with a Wild® M-5 stereomicroscope, then fixed in Bouin's solution. After confirmation of photographic exposure, the limbs were decalcified, embedded in low M.P. (53°C) paraffin, and sectioned serially at 10 micrometers in the frontal plane. The sections were mounted on glass

TABLE 8-I
INDUCED CURRENT WAVEFORMS FOR NEWT LIMB REGENERATION*

Waveform	τ_1(μsec)	τ_2(μsec)	τ_3(μsec)	τ_4(msec)	τ_5(Hz)	*A*
1	325	11	0	0	72	1
2	250	3	5	50	2	.1
3	200	22	6	5	15	1
4	200	6	9	5	15	1

* Refer to Figure 8-2 for an explanation of all timing designations. Amplitude, A, refers to peak of main polarity portion of given waveform and is in arbitrary units.

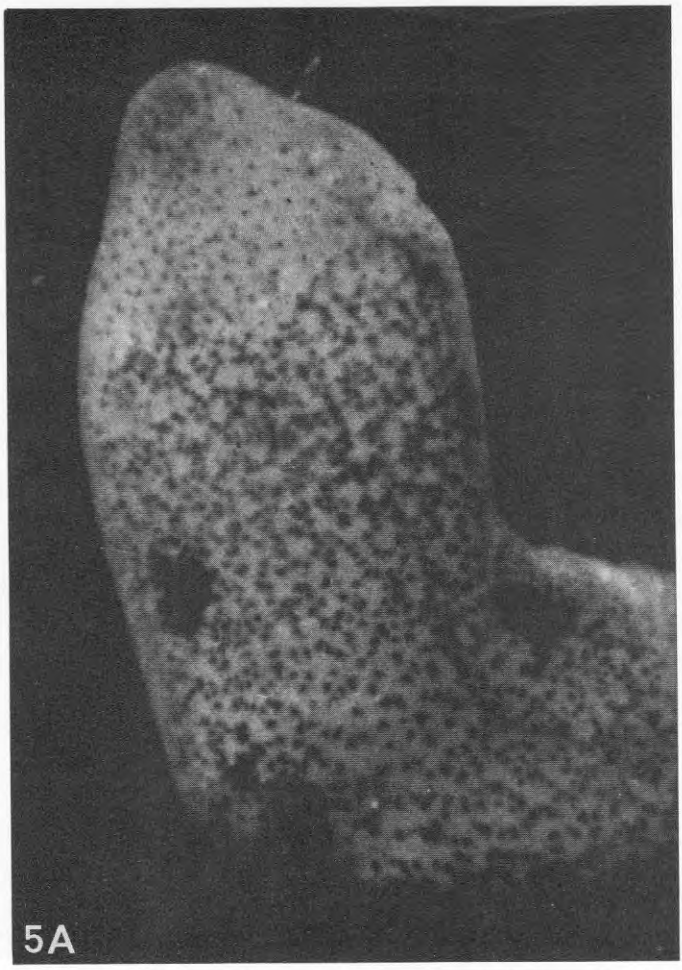

Figure 8-5A. Control — This is the appearance of typical control animal's limb after twenty-one days. The regeneration blastema is a cone shaped lump extending from the end of the amputated limb. ×20

slides and stained with Harris' hematoxylin and eosin. Appropriate sections were examined and photographed using a Zeiss® photomicroscope 1.

Results and Discussion

The results obtained in this study were assessed on the basis of standard morphological states of the regenerating forelimb. The nomenclature employed reflects quite closely the geometric situation at animal sacrifice after twenty-one days. A summary of all results obtained is shown in Table 8-II. As can be seen, the degree of regeneration depended strongly on induced current waveform characteristics. The actual physical state of the limb after twenty-one days is shown in the photographs of Figure 8-5 for

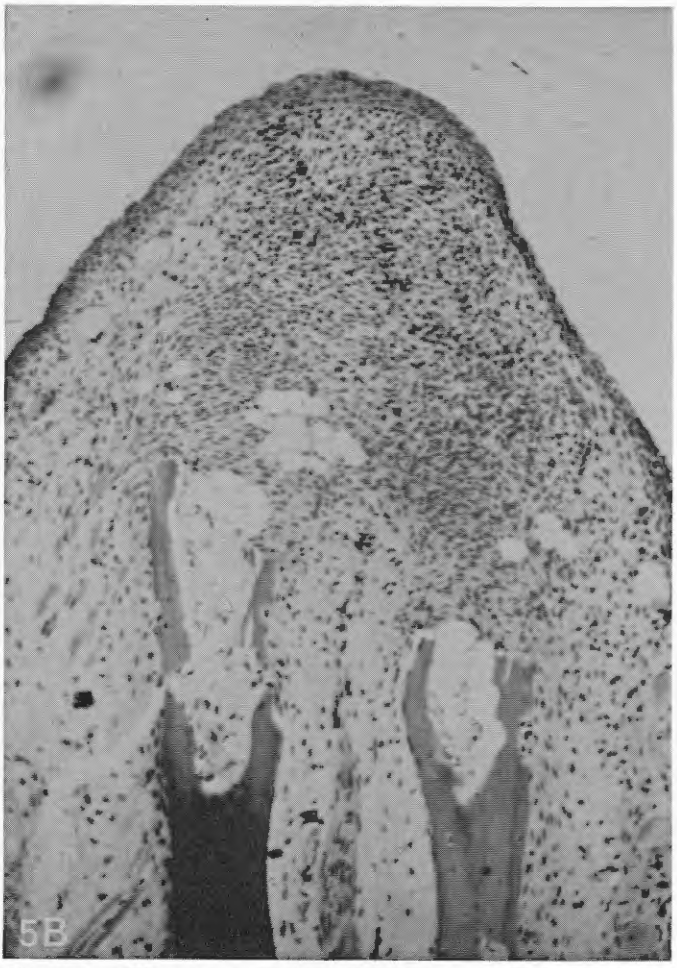

Figure 8-5B. Control — This histological section demonstrates the typical appearance of a cone stage blastema. There are many mesenchymal-type cells collected beneath tbe wound surface, the overlying epithelium is thickened, and the limb bones are being severely eroded to release osteocytes. ×30

typical control animals and for waveforms 1, 3, and 4 in Figures 8-6, 8-7, and 8-8.

Controls

The majority of limbs in this group had progressed to the cone stage (Fig. 8-5A) with only one animal exhibiting development to the palette stage. Control limbs would normally progress to the fingerbud stage in approximately forty-five days, allowing a relative assessment to be made. The internal structure corresponding to a typical limb in the cone stage is shown in Figure 8-5B.

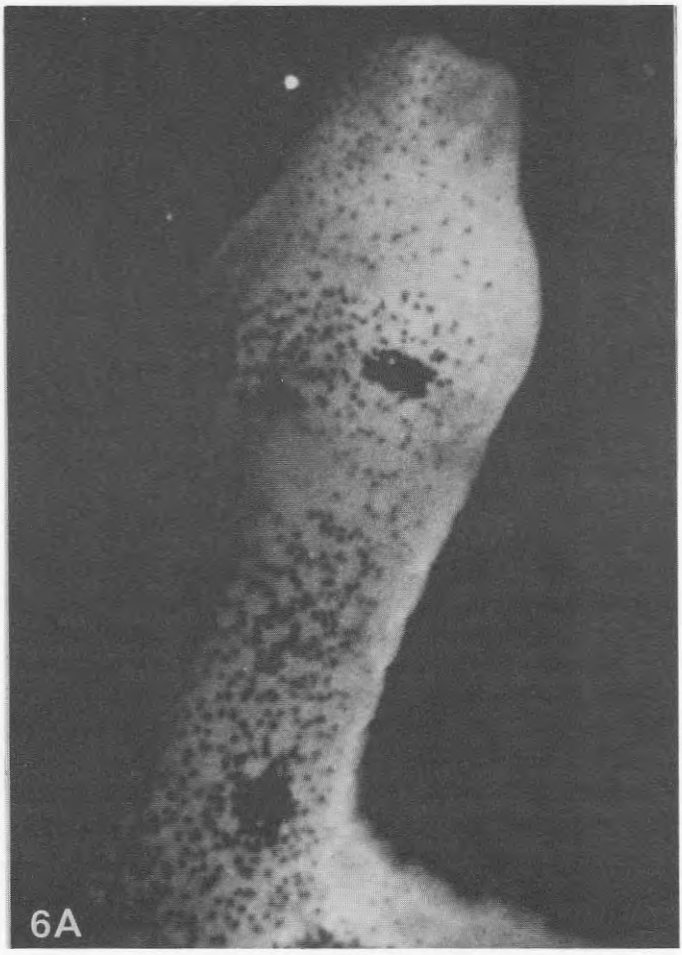

Figure 8-6A. Waveform 1 — This is the typical type of regenerate formed with the use of this waveform. The regenerate is morphologically advanced but considerably smaller than a normal fingerbud stage, and there are only three fingerbuds. ×20

Waveform 1

These animals showed a very interesting and pronounced response to this stimulus. As may be seen in Figure 8-6A, differentiation was remarkably speeded. Upon further examination, it was apparent that the regeneration was not entirely normal, however. The regenerates in this series were invariably quite small, and usually hypomorphic, in that they ordinarily developed only three, rather than four fingerbuds. The internal organization of the regenerates was appropriately scaled to the external appearance, as shown in Figure 8-6B. When digit IV was missing (digits I, II, and III were never deleted), so were its corresponding carpal elements. Despite the small size of the regenerates and the missing digit, they were

Figure 8-6B. Waveform 1 — This is typical of the history of a waveform 1 regenerate. The advanced morphology and finger cartilage rudiments are clearly visible. ×20

normal in polarity. We never observed any alteration of normal polarity in any of the treated animals in which polarity could be determined.

Waveform 2

The results of treating amputated limbs with these pulse trains was somewhat variable. Some animals responded as though they were treated with waveform 1, achieving the hypomorphic fingerbud stage, while others were essentially similar to controls. Comparison of the frequency spectrum of this signal with that of waveform 1 (Fig. 8-3) shows that they are most closely matched in amplitude over the lower portion of the frequency spectrum (<10 Hz) exhibiting a difference in real perturbation levels at 10^{-1} Hz (i.e. on the vertical axis of Fig. 8-3) of approximately 50 percent (waveform 1 > waveform 2).

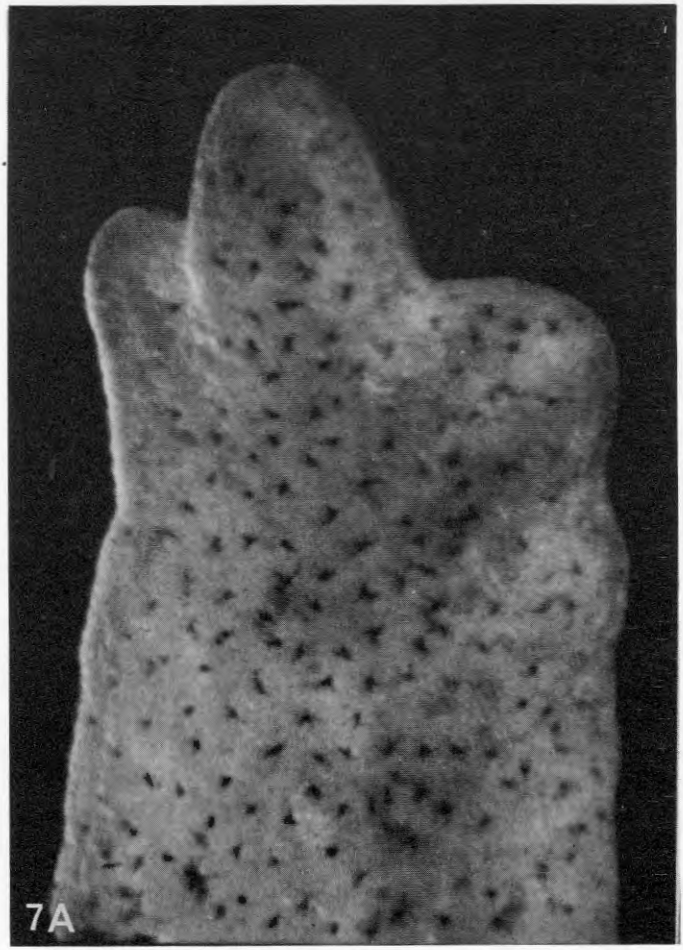

Figure 8-7A. Waveform 3 — This whole specimen photo illustrates the remarkable acceleration of development achieved with the use of this waveform. The regenerate seems to be typical of what one would normally see at about forty-five to fifty days without treatment. It is normal in size and has four clearly visible fingerbuds. ×20

Waveforms 3 and 4

The difference in results obtained using these two waveforms was most dramatic. As seen in Figure 8-7A, the regeneration obtained using waveform 3 corresponded to the fingerbud stage. However in comparison to the hypomorphic limbs achieved with waveforms 1 and 2, this regenerate was normal in all respects (see Fig. 8-7B). Examination of the frequency spectrum of waveform 3 (Fig. 8-3) shows that the closest amplitude correlation with waveforms 1 and 2 occurs in the low frequency region. The effective perturbation level of this signal at 10^{-1} Hz is approximately 100 percent higher than waveform 2 and 45 percent above waveform 1. Ampli-

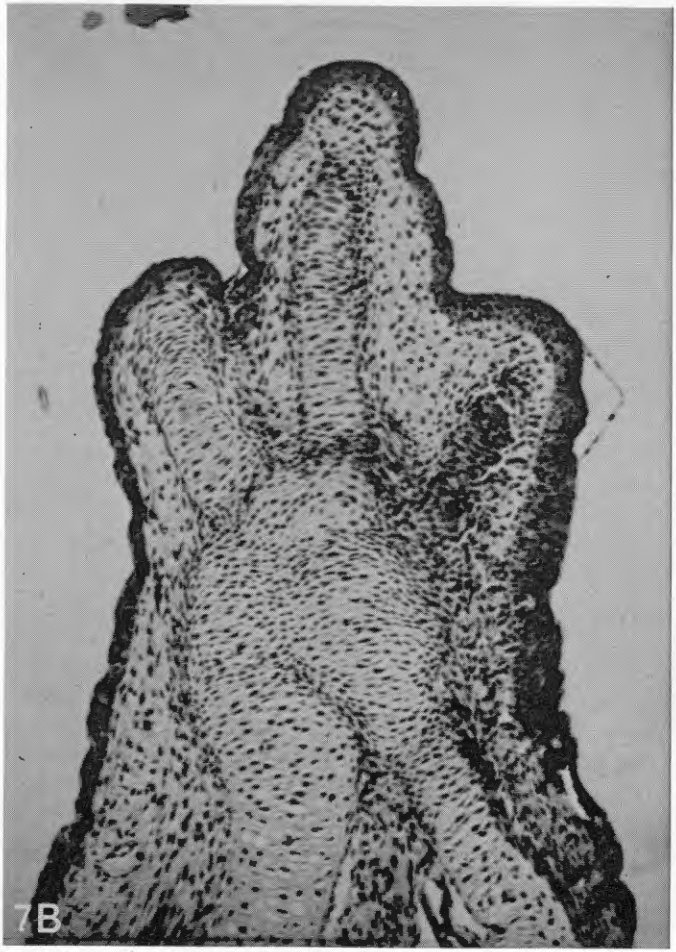

Figure 8-7B. Waveform 3 — This section illustrates the inner structure of a regenerate (like that in Fig. 8-7A). The advanced state of development is obvious. The fingerhud cartilages are well formed, and the position of the carpo-metacarpal joints is being delimited. The fourth fingerbud is out of the plane of this section. It extended to the right and into the page. ×20

tude differences among these signals over the remainder of the frequency range follow no discernible trend with respect to elicited regeneration.

The results of treatment with waveform 4 were dramatically opposed to those obtained with the other waveforms. As seen in Figure 8-8A, regeneration was essentially stopped entirely. In most cases the wound surface was covered with an epithelium that never thickened into the typical apical cap seen in the initial stages of regeneration. The internal structure of these limbs (Fig. 8-8B) was typical apical cap seen in the initial stages of regeneration. The internal structure of these limbs (Fig. 8-8B) was typical of nonregenerating amputation sites: some rather dense connective tissue

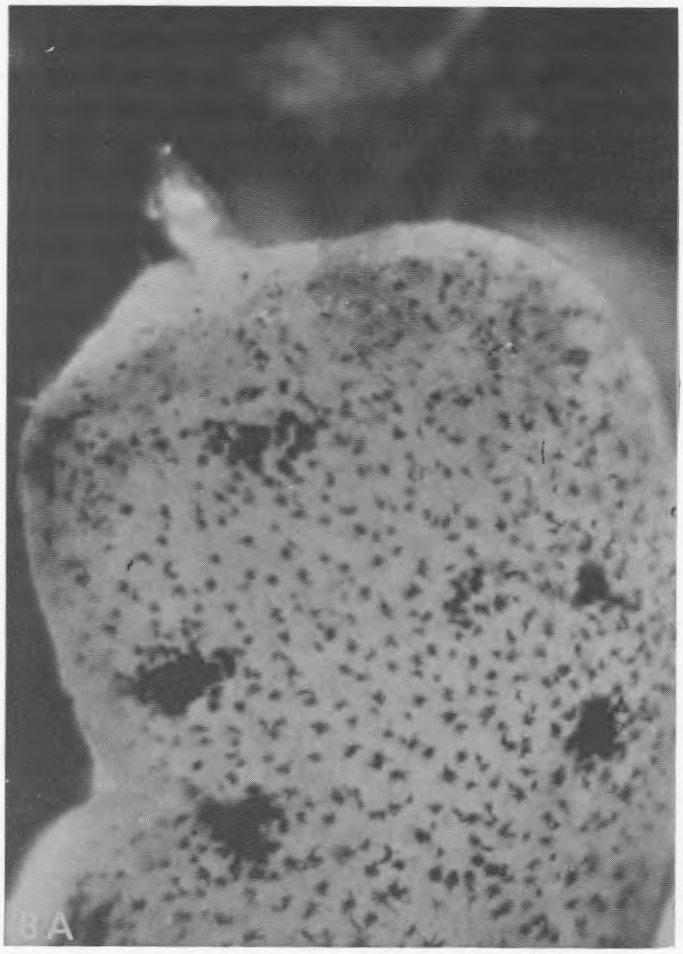

Figure 8-8A. Waveform 4 — This remarkable photograph illustrates the almost complete arrest of development that was typical of limbs treated with the type 4 apparatus. There has been no visible outgrowth whatsoever. The scrap of bone extending from the tip of the limb should long ago have been severed by osteoclasts at its base and discarded. ×20

beneath the epithelium covering the small amounts of cartilage. Only one of the sixteen limbs developed what might reasonably be called a blastema, and that did not progress beyond the mound stage. Most interesting is the comparison of the frequency spectra of signals 1, 3, and 4 as shown in Figure 8-4. Once again an amplitude correlation appears to exist only in the low frequency region where waveform 4 is three times the effective perturbation level of signal 3 and four times the amplitude of signal 1. (Note that signals 1 and 4 are essentially amplitude matched in the high frequency region, $>10^3$ rad/sec.)

The results given above clearly show that the *rate* of new forelimb

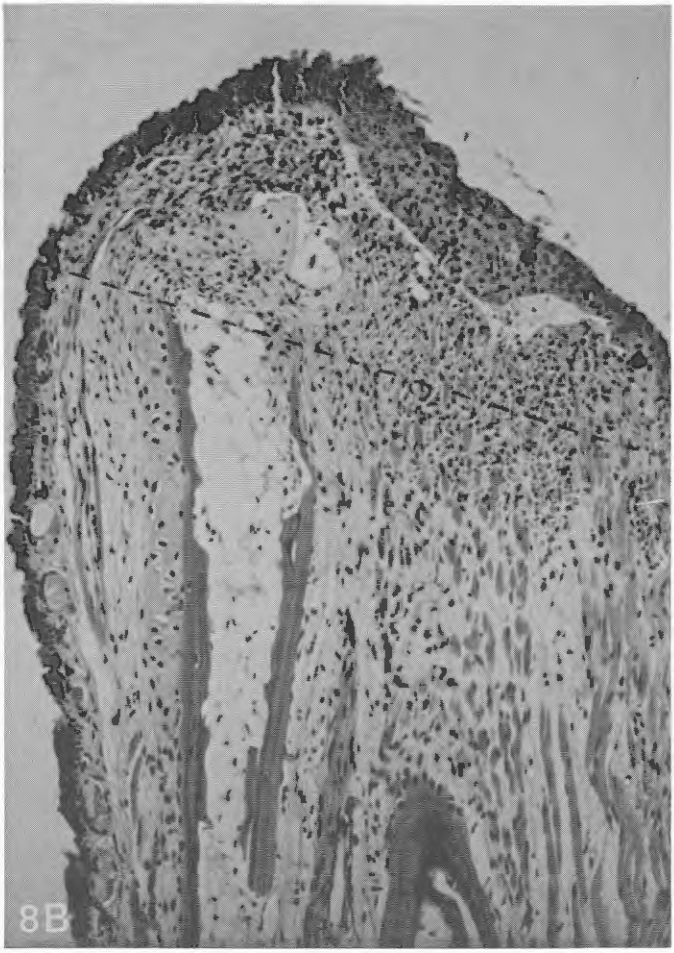

Figure 8-8B. Waveform 4 — This photograph of an histological section illustrates the *maximum* regeneration achieved using this waveform. The original plane of amputation is marked by the dotted line. It is evident that very little outgrowth has taken place, and that practically no absorption of the bone has occurred. Regeneration has been inhibited almost totally. ×20

regeneration is significantly altered using electromagnetically induced low level pulsating current. The original choice of waveform parameters was based on the assumption that the potential dependence *and* kinetics of specific charge interactions at the surfaces and junctions of *any* living cell represented the general class of physical (electrochemical) processes by which the perturbation employed in this study could modulate cell regulation (Pilla 1972, 1974). This approach allowed the prediction of an efficient set of parameters for the basic waveform (pulse width, τ_1, Fig. 8-2; repetition rate, τ_5, Fig. 8-2). In addition it was considered that the elec-

trochemical nature of the processes to be modulated could grossly define the average current density required to cause sufficient alteration in the average population of charged species (e.g. ions) at key regulatory sites on the cell membrane. However, even if the assumptions concerning the nature of the physical process are correct it is impossible to know *a priori* its actual kinetics (both rate *and* reversibility). This relates to the average net perturbation per pulse, taking into account the timing and amplitude characteristics of both polarities (see Fig. 8-2). Finally, for any given potential dependence and kinetics the question of optimal dose, for example rate acceleration, had to be established by effective (frequency domain) amplitude sweeps over one order of magnitude within the predicted real-time range (see e.g. Pilla, 1980a).

In view of the above, the choice of waveforms utilized in this study was made to ascertain the following:

a. whether the kinetics and reversibility of the perturbed regulatory process would place restrictions upon the timing (and amplitude) characteristics of the opposite polarity portion of the main waveform (τ_2, Fig. 8-2).
b. whether a long time average or a periodic perturbation was important in the context of the overall regulatory loop kinetics.
c. the manner in which a and b interrelated to achieve a doselike effect with respect to rate modulation.

The answer to *a* is clearly evident when the frequency spectra and results obtained for waveforms 3 and 4 are compared (*see* Fig. 8-4 and Table 8-II). This shows that both polarity pulse durations (τ_1 and τ_2, Fig. 8-2) are within the kinetic reversibility range to determine a net perturbation per pulse. The net amplitude difference for these waveforms occurs over the whole frequency range so that the answer to *b* had to be determined by utilizing waveforms 1 and 2. Correlation of results obtained with these perturbations with their frequency spectra appears to indicate that the low frequen-

TABLE 8-II
EFFECT OF INDUCED CURRENT WAVEFORM ON THE RATE OF NEWT LIMB REGENERATION
Gross Morphology

Waveform	*No Regen.*	*Mound*	*Cone*	*Palette*	*Fingerbud*	*N*
1	0	0	1	5	10	16
2	0	0	4	7	5	16
3	0	2	0	10	12	24
4	14	2	0	0	0	16
Control	0	3	20	1	0	24

cy portion of the spectrum is most relevant. Indeed this is true for all waveforms utilized in this study, indicating that both the basic waveshape and repetition rate, for a given main polarity amplitude, appear to define the net relevant perturbation of cell function when low frequency amplitudes (e.g. along the vertical axis of Figs. 8-3 and 8-4) are compared. The answer to *c*, therefore, appears to be that a quantitative relation between real time perturbation and biological effect for this system requires that *all* waveform characteristics be taken into account, as expected for a system exhibiting only small nonlinearities.

The preliminary results obtained in this study, when placed in the context of those achieved in the other biological systems reviewed here, appear to indicate the generality of the electrochemial information transfer concept. It is intriguing to consider that a similar dose relationship, expressed via electrochemical model dependent spectral analysis, exists in every study. Of even greater interest is the fact that rate increase occurs over the identical range of low frequency amplitudes reported here. Whether or not this means that a regulatory process basic to all cells is involved can only be ascertained through detailed biochemical analysis. These are ongoing for some cell systems (Gary, 1980).

Summary

In order to assess the applicability of electrochemical information transfer to regeneration processes, pairs of adult newts (Notophthalmus viridescens) on which mid-forearm amputations had been performed were subjected to induced low level pulsating currents for twenty-one days. The current waveforms were designed to kinetically couple to non-faradaic electrochemical processes at the cell surface. The four signals utilized in this study were correlated to the degree of alteration of limb regeneration hy examination of their electrochemical model dependent frequency spectra. Results obtained seem to indicate that effective perturbation levels corresponding to a net average change (e.g., rectification via nonlinear membrane behavior) in charge interactions at a regulatory site were required. Pulses inducing 1 to 10 $\mu A/cm^2$ peak current densities could he designed to give results ranging from inhibition through hypomorphic and finally normal fingerbud stage as compared to the cone stage prevalent among the control animals. Maximum acceleration of differentiation was 200 percent.

REFERENCES

1. Bassett, C. A. L.; Mitchell, S. N.; Norton, L.; Caulo, N.; and Gaston, S. R.: In *Electrical Properties of Bone and Cartilage*, Brighton, C. T.; Black, J.; and Pollack, S. R., eds. Grune and Stratton, New York, 1979, p. 605.
2. Bassett, C. A. L.; Mitchell, S.; Norton, L.; and Pilla, A. A.: *Acta Orthoped. Belg., 40:*706, 1978.

3. Bassett, C. A. L.; Pilla, A. A; and Pawluk, R. J.: *Clin. Orthop. 124:*117, 1977.
4. Becker, R. O.: *Nature, 235:*109, 1972.
5. Borgens, R. B.; Vanable, J.W. Jr.; and Jaffe, L. F.: *J. Exp. Zool., 200:*403, 1977.
6. Chiabrera, A.; Hinsenkamp, M.; Pilla, A. A.; Ryaby, J.T.; Ponta, D.; Beltrame, F.; Grattarole, M.; and Nicolini, C.: *J. Histochem. and Cytochem., 27:*375, 1979.
7. Christel, P.; Cerf, G.; and Pilla, A. A.: 2nd Meeting of the European Society of Biomechanics, Strasbourg, France, Sept. 1979; *International Journal of Biomechanics,* in press.
8. Colacicco, G. and Pilla, A. A.: 5th International Symposium on Bioelectrochemistry, Weimar, East Germany, Sept. 1979.
9. Cole, K. C.: *Membranes, Ions and Impulses.* University of California Press, Berkeley, 1968.
10. Goss, R.: *Principles of Regeneration.* Academic Press, New York, 1969.
11. Monet, J. D.; Assailly, J.; Goureau, Y.; and Pilla, A. A.: *J. Electrochem. Soc. 126:*142C, 1979.
12. Morzlock, F. V. and Stocum, D. L.: *Dev. Biol. 24:*106, 1971.
13. Pilla, A. A.: Bioelectrochemistry: Ions, Surfaces, Membranes, *Adv. in Chem. 188:*126, 1980.
14. Pilla, A. A.: In *Proceedings, 1st U.S.-Australian Workshop on Bioelectrochemistry,* Keyser, H., ed. Plenum, New York, 1980a.
15. Pilla, A. A.: In *Electrical Properties of Bone and Cartilage,* Brighton, C. T.; Black, J.; and Pollack, S. R., eds. Grune and Stratton, New York, 1979, p. 455.
16. Pilla, A. A. and Margules, G. S.: *J. Electrochem. Soc., 124:*1697, 1977.
17. Pilla, A. A.: *Ann. N.Y. Acad. Sci., 238:*149, 1974.
18. Pilla, A. A.: In *Proceedings 7th Intersociety Energy Conversion and Engineering Conference,* San Diego, 1972. Amer. Chem. Soc., Washington, D.C., p. 761.
19. Pilla, A. A.: In *Computers in Chemistry and Instrumentation: Electrochemistry,* Mattson, J. S.; MacDonald, H. C.; and Mark, H. B. Jr., eds. Marcel Dekker, New York, 1972a, p. 139.
20. Pilla, A. A.: *J. Electrochem. Soc., 117:*467, 1970.
21. Rose, S. J.: *J. Morphol., 77:*119, 1945.
22. Shteyer, A.; Norton, L. A.; Pilla, A. A.; and Rodan, G.: 5th International Symposium on Bioelectrochemistry, Weimar, East Germany, Sept. 1979.
23. Singer, M.: *Proc. Soc. Exptl. Biol. Med., 76:*413, 1951.
24. Sisken, B. F.; Lafferty, J. F.; Fowler, I.; and Pilla, A. A.: *J. Electrochem. Soc., 126:*142C, 1979.
25. Smith, S. D.: *Ann. N.Y. Acad. Sci., 238:*500, 1974.
26. Smith, S. D.: *Anat. Rec. 158:*89, 1967.
27. Tassava, R. A.: *J. Exp. Zool., 170:*33, 1969.