DIVISION OF INSTRUMENTATION

A METHOD FOR PRODUCING CELLULAR DEDIFFERENTIATION BY MEANS OF VERY SMALL ELECTRICAL CURRENTS*

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In a phylogenetic study of the mechanisms involved in the healing of fractures, we have determined that in the amphibia this process involves primarily the dedifferentiation of the nucleated erythrocytes in the fracture hematoma into cells with fibroblastic potential (FIGURE 1). This mechanism is quite at variance with current concepts of fracture healing; however, these have been largely determined by experimentation on mammalian species, in which fracture healing is accomplished primarily by a process of periosteal stem cell proliferation. It should be noted that of all vertebrates the mammals alone have nonnucleated erythrocytes and that their periosteum is considerably thicker than that of other vertebrates. Since fracture healing is basically a process of tissue regeneration, both stem cell proliferation and mature nucleated red cell dedifferentiation are equally competent to produce the necessary blastema. The question to which we addressed ourselves was, "What is the nature of the trigger or induction stimulus that initiates the cellular event—in this case, the dedifferentiation of the mature erythrocyte?" Previous studies on another bone growth phenomenon — the growth response to mechanical stress — have indicated that this process was governed by a control system based upon specific solid state properties of the bone matrix itself.[1,2] In brief, the collagen fiber-apatite crystal relationship in the matrix constitutes a semiconducting PN junction diode[3-5] which acts as a transducer, converting

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mechanical stress into an electrical signal with polarity related to the direction of the stress and amplitude proportional to the magnitude. The current so produced causes migration and alignment of tropocollagen molecules along lines of maximum stress and, in mammals, produces an osteoblastic reaction. This cellular response can be reproduced experimentally in dogs by a direct current source with rather specific current levels and with a nonuniform field pattern.[6] A search was therefore begun for similar electrical activity associated with fractures.

Our standard experiment involved subperiosteal fracture of the midshaft of the tibiofibularis in the adult grassfrog, *Rana pipiens*. Using salt bridge Ag-AgCl electrodes, specific electrical patterns were noted in each case both on the intact periosteum and on the underlying bone fragments (FIGURE 2). Immediately following the fracture, the periosteum directly over the fracture site becomes highly negative with respect to the remainder of the shaft periosteum. Injury to the periosteum alone without underlying fracture produces the same electrical pattern but with magnitudes less than one-half as great. The potential existing at this site, with respect to a reference point 1 cm proximal prior to any fracture or injury, was also uniformly negative but never exceeded 1 mv in magnitude. The postfracture periosteal potentials change rapidly during the first two hours and then demonstrate a slow change over the next five to seven days. Immediately following fracture, the bone surface itself adjacent to the fracture site becomes positive with respect to a point 1 cm proximal on the shaft. Measurements prior to fracture, using the same reference points on the intact shaft, demonstrated uniformly a modest negativity again not exceeding 1 mv. The postfracture bone potentials also changed during the first few hours following fracture, demonstrating the highest positivity at approximately one hour. Changes subsequent to two hours were not determined because of the possibility of active metabolic activity within the bone or residual from the hematoma that would introduce error. Nerve section experiments indicated that the periosteal potentials were, at least in part, neurally related. The origin of the bone potentials can only be speculated upon at this time; however, stress sensitive materials that are stressed to failure frequently show prolonged periods of residual polarization. It is evident from the change distribution present at one hour (FIGURE 3) that the electrical field pattern within the hematoma is complex and certainly nonuniform.

The possibility was considered that the electrical phenomenon accompanying the fracture was the trigger stimulus initiating the process of erythrocyte dedifferentiation. The fact that the target cell, in this case, was the red cell enabled us to perform a variety of experiments in vitro with few technical difficulties. Fresh heart blood obtained from sacrificed adult frogs was diluted with balanced amphibian Ringer's solution and exposed to a variety of electrical stimuli in small enclosed chambers. Several types of chambers were prepared with a variety of electrodes producing uniform or nonuniform fields within the chamber (FIGURE 4). A 1.5 volt dry-cell battery, 3 meg ohm variable resistor, 10 meg ohm fixed resistor, and a Hewlett-Packard 425A microammeter were placed in series with the test chamber. The chambers themselves were situated directly upon the stage of an inverted biological microscope and the cells

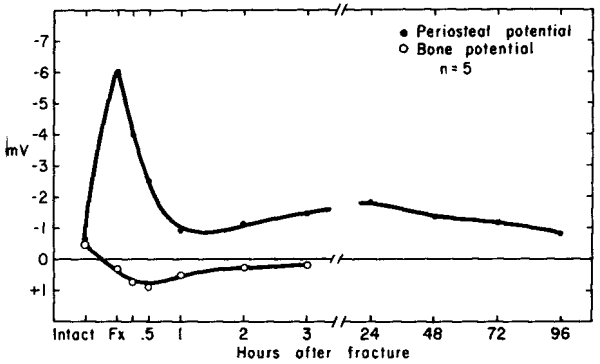


FIGURE 2. Electrical events at the fracture site in *Rana Pipiens*. All measurements (periosteal and bone) were made with reference to a point 1 cm proximal on the shaft of the tibiofibularis. For bone determinations, the periosteum was incised and the hematoma removed; recording electrode placement was 1 mm proximal to the actual fracture line to avoid possible errors introduced by contact with marrow elements.

were under direct visualization at all times. Utilizing the variable resistor, current levels between 10 $\mu\mu$A and 50 mμA were obtainable with reasonable precision. Polarization effects were minimal with the platinum wire electrodes and the silver plate electrodes and only slightly greater with the silver paint electrodes. Polarization could be minimized by reducing the contact area between the electrode and the solution. In no case was any attempt made to duplicate the fracture field; however, except for the silver plate electrode chamber (FIGURE 4D) the fields produced were highly nonuniform.

In the initial experiment utilizing the uniform field chamber, no cellular effects were noted in the current range between 20 $\mu\mu$A and 50 mμA with

FIGURE 1(A & B). Representative cells obtained from a three-hour fracture hematoma as seen by regular illumination (A) and in phase contrast (B). Both views are of the same area at 600x magnification with the cells suspended in balanced amphibian Ringer's solution. Relatively normal erythrocytes are seen at the left of the field with altered cells in the remainder. The alterations range from prominence of the nuclei and rounding of the overall cell shape to round cells with clear cytoplasm and nuclei with darkly staining clumps. Holtfreter[7] describes two basic stages in erythroblast differentiation, first the acquisition of hemoglobin and second the transformation of the spherical cell into the flat elliptical type, with a number of intermediate steps (amoeboid movement, crenated membrane, and so forth). All types have been identified in fracture hematomas with the age of the hematoma correlating with the stage of dedifferentiated cell observed. The round clear cells with prominent nuclei are apparently the end stage of the sequence. After the dedifferentiation process has passed through the initial stage of nuclear change (refractility), a phase shift of the nucleus occurs and appears to indicate the stage of irreversibility of the process. All of the altered cells are confined to the hematoma and do not appear in the circulating blood. Hematomas were removed and suspended in amphibian Ringer's solution and observed unstained either with normal or phase contrast illumination. Standard histological techniques (fixation, staining and dehydration) cannot be used to identify these cell types, although such preparation clearly indicates that the erythrocytes have changed into another type of cell.

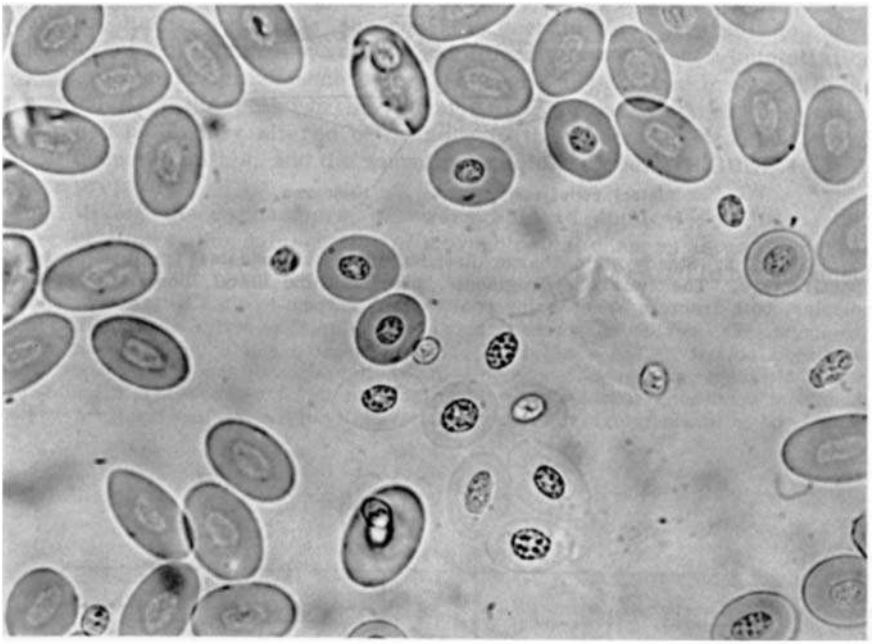
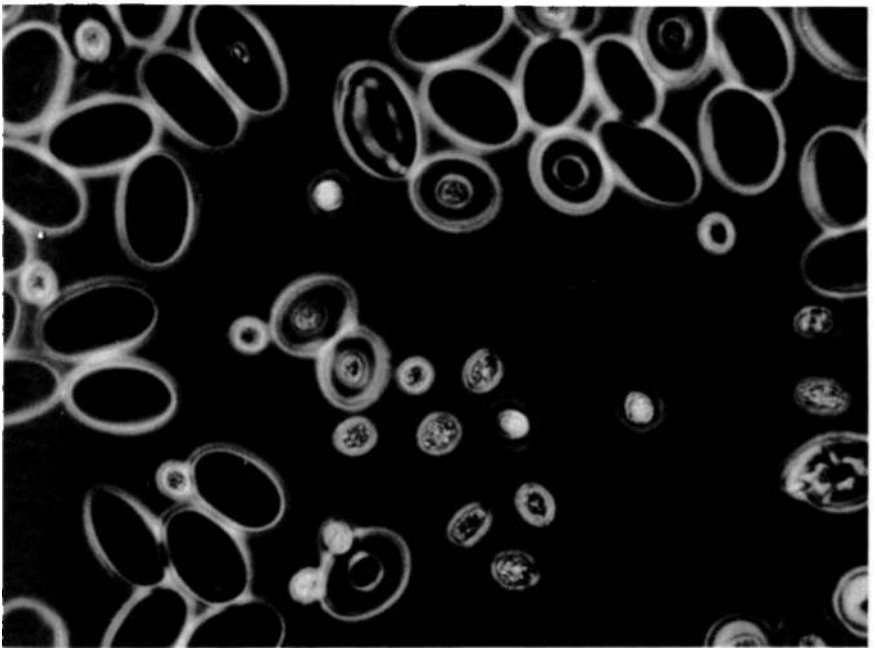


FIGURE 1A

FIGURE 1B



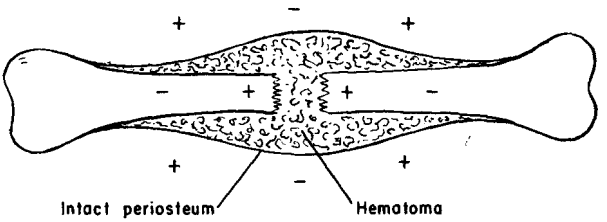


FIGURE 3. Steady state charge distribution of subperiosteal fracture one hour postfracture. The two bone fragments, proximal and distal, constitute opposed dipoles, both fractured ends being positive with respect to sites 1 cm away on each fragment. The periosteal potential may also be viewed as a similar dipole but with reversed polarity. The exact field pattern generated within the hematoma under these circumstances would vary with the configuration of the hematoma and with boundary conditions that are largely unknown at this time; however, it would obviously be quite nonuniform in the immediate vicinity of the fracture itself.

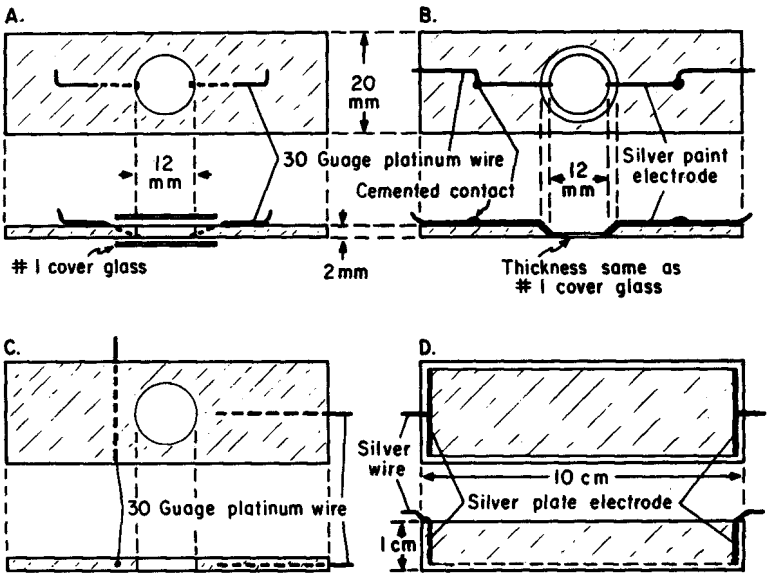


FIGURE 4. Various types of chambers used for exposure of amphibian erythrocytes to electrical currents.

A. Lucite cell with imbedded platinum wire electrodes.
B. Glass cell with silver paint electrodes.
C. Lucite cell with platinum wire electrodes not in contact with the fluid chamber.
D. Lucite cell with silver plate electrodes at either end.

Chamber D produced a reasonably uniform electrical field pattern except in the immediate vicinity of the electrodes; all other chambers produced a highly nonuniform field with maximum nonuniformity in vicinity of each electrode.

two-hour exposure to fresh red cells at every 100 $\mu\mu$A level. Occasionally, cells of Holtfreter's type 4b[7] could be seen in close proximity to one of the electrodes; in no case, regardless of the time of current administration or the presence or absence of oxygen, were any changes produced in the remainder of the cells in the chamber. It appeared that the occasional presence of slightly altered cells close to the electrodes was due to local field nonuniformities, and the nonuniform field chambers (FIGURE 4A & B) were prepared.

Utilizing these chambers, we were able to duplicate, within certain current limits, the same series of morphological changes as was observed at the fracture site (FIGURE 5). The phenomenon appeared to be dependent

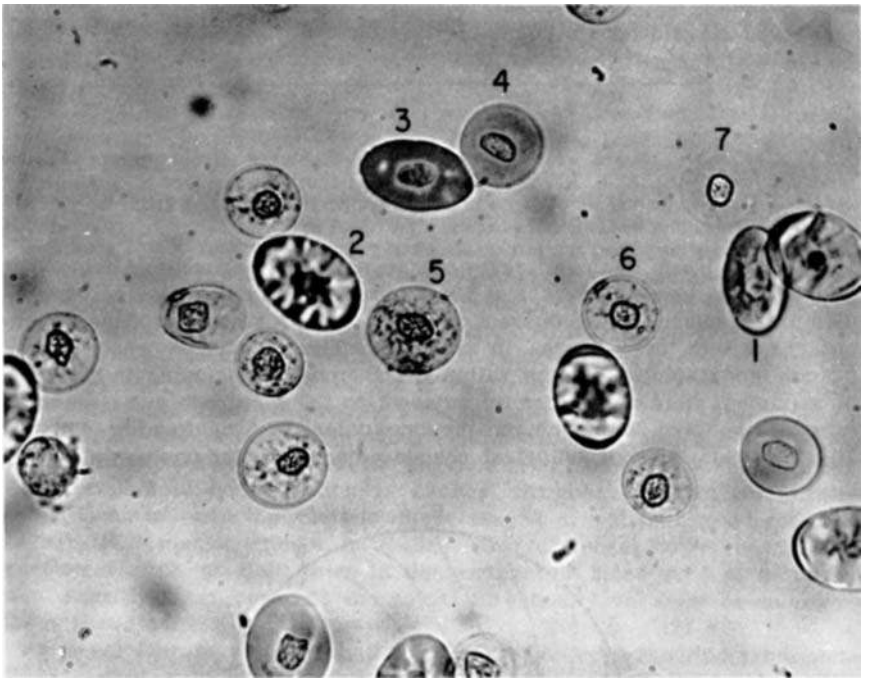


FIGURE 5. Typical cellular alterations produced in nonuniform field chambers with effective levels of current flow. While each area in the chamber follows an overall sequence of changes, the individual cells differ in their sensitivity to the electrical stimulus, possibly on a basis of cellular age. The view above was selected to demonstrate as wide a variety of cell types as possible. The numbering refers to the sequence of morphological change, although a few steps are missing in this view. The sequence of cellular changes noted closely parallels that reported by Holtfreter[7] for the maturational sequence from erythroblast to erythrocyte in amphibia.

upon a number of variables: the degree of field nonuniformity, the total current level, and the time of exposure. If one equates the efficiency of the electrical factors to the time required to produce a complete sequence of morphological changes in a zone within 1 mm of each electrode, a re-

lationship with the total current level can be established (FIGURE 6). With the chambers described, the optimum current level was in the vicinity

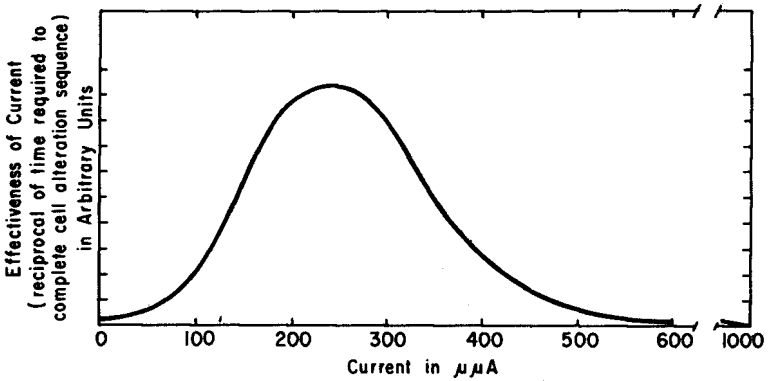


FIGURE 6. Relative efficiency of various levels of direct current utilizing standard chambers A & B. The reciprocal of the time required to complete the morphological sequence of changes in a zone approximately 1 mm from the positive electrode is used as the index of efficiency (vertical axis).

of 200-300 $\mu\mu$ A with efficency decreasing both above and below that. Interestingly, levels in excess of 5 mμA produced no morphological changes within six hours, while current as low as 10 $\mu\mu$A could produce definite changes within three hours. In all cases, cellular changes proceeded in a precise geographical fashion within the chambers, beginning in the zones of maximum field nonuniformity adjacent to the electrode and from there extending outward, with time, into the zones of lesser nonuniformity (FIGURE 7). The rate of morphological change adjacent to the positive electrode

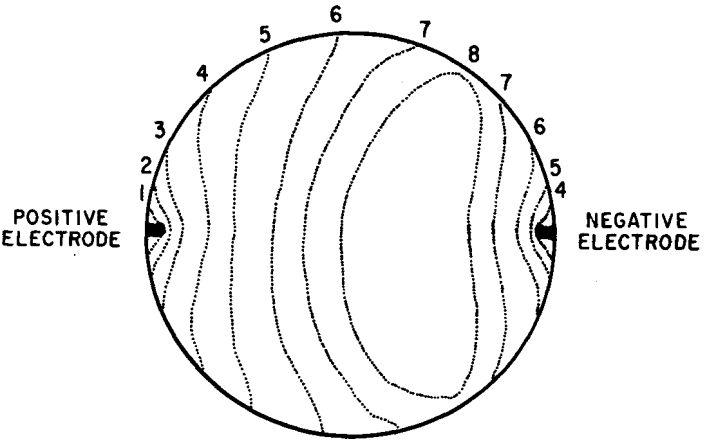


FIGURE 7. Spatial distribution of cellular changes produced by effective current levels in chambers A & B. The numbering refers to the time sequence of observed changes. Note that changes are seen in zone 3 around the positive electrode before they are noted in the area adjacent to the negative electrode.

was faster than at the negative. Initial changes at the positive electrode preceded those at the negative by five to ten minutes (at 200 $\mu\mu$A), with the sequence completed in this area before it was in the corresponding area at the negative electrode. The electrical factors seemed to be in the nature of a "trigger" stimulus, for if the current were shut off after cells had progressed to the stage of refractile or phase-shifted nuclei, these cells would continue to progress through the full sequence. Apparently direct current is necessary to produce the phenomenon for alternating currents (sine wave) at 1 kc with power equivalent to 200 $\mu\mu$A produced no discernible morphological alterations after two hours of exposure. The changes have no relationship to anoxia or to the material from which the chamber was fashioned, and normal erythrocytes placed in either glass or plastic chambers, with or without enclosed air bubbles, demonstrated no morphological changes after twenty-four hours if not exposed to any electrical factors.

In an attempt to determine whether a nonuniform field with a current flow was necessary, a chamber was made with a nonuniform field and no contact between the electrodes and the solution. (FIGURE 4C). Without the chamber cutout, this type of electrode arrangement would produce a nonuniform field with the nonuniformity changing in a linear fashion. However, the chamber cutout produces a major perturbation in the field and, when filled with a conducting solution, the field lines concentrate onto the chamber in an almost radial fashion. Despite the low voltage, it was impossible to avoid a very small (1 $\mu\mu$A) leakage current during the experiment. Cellular changes began at the extreme periphery of the chamber area and extended inward for a maximum distance of 0.1 mm; within this zone, the changes proceeded to completion in two to three hours. Continuous current for twenty-four hours produced a slight inward extension of this zone but did not alter cells in the center of the chamber. The exact field configuration in such a chamber is dependent upon the proportions between the various elements and cannot be easily predicted. Actual field measurements in a 10x analog chamber, however, indicated a concentration of field lines in the periphery. Since the 1 $\mu\mu$A current was actually total current, the actual threshold level must be somewhat lower than this.

Several factors indicate that the observed effects resulted from some direct cellular effect of the electrical forces and were not produced by secondary phenomenon such as ionic gradients, and so forth. The establishment of a rather narrow zone of effective current levels with a cutoff at higher current levels would appear to mitigate the effect caused by ionic movements since such an effect should increase practically with increasing current. Similarly, ionic movements and gradients would certainly be produced by the uniform field chamber, yet this chamber failed to demonstrate significant morphological alterations within a wide range of current levels. While a nonuniform field is clearly a requirement for producing the phenomenon, it seems likely that this field must also be accompanied by a minimum current level as illustrated by the results obtained with the "field only" chamber (FIGURE 4D). That the effect is not a toxic one or a general effect on all cells is clearly indicated by the facts that leukocytes in the chambers demonstrated no abnormalities under any com-

bination of fields or currents and that the magnitude of the effective current range was extremely small.

The data seem to indicate that the action of the electrical factors in vitro is to produce morphological changes in a susceptible cell population identical to those observed in the same cells at the site of a fracture. The electrical factors most effective in vitro approximate those accompanying the fracture in vivo. Both have nonuniform field patterns and the current level produced by one fractured surface in vivo is calculated, on the basis of R equivalent to $1 \times 10^{10} - 1 \times 10^{11}$ ohms, to be in the range 1-1,000 $\mu\mu$ A. It would appear logical to conclude that the electrical phenomenon accompanying long bone fracture in amphibians serves as the trigger stimulus to produce the erythrocyte dedifferentiation necessary for regenerative healing in this animal. If this conclusion is tenable, one is faced with the necessity of proposing a mechanism adequate to explain the effect and to serve as the basis for further experimentation. It is evident from the data that nonuniformity of the electrical field pattern is vital to the effect. It is well known that dielectric particles suspended in a nonuniform field demonstrate dielectrophoresis on the basis of separation of surface changes induced by the nonuniformity of the field.[8] While the erythrocyte is by no means strictly analagous to a dielectric particle, it is known that it has an overall electrical surface change, and it is not unreasonable to postulate that the cell membrane has a particular pattern of electrically charged groups. In this case, exposure to a nonuniform DC field might well result in distortions of this pattern, while exposure to a similar field pattern, but one generated by an alternating current source, would not have the same result. Observations of the sequence of morphological changes seem to bear out this hypothesis. The initial change involves the membrane and peripheral cytoplasm with subsequent changes progressing into the perinuclear region and the nucleus. Once sufficient alteration of the nucleus has occurred, further steps in the sequence will automatically proceed and may involve major membrane alterations such as lobopodia or amoeboid movement. It would appear that environmental changes produce alterations of the membrane and subjacent cytoplasm which, in some fashion, transmit information to the nucleus which, in turn, produces dedifferentiation of a mature cell with a return to a relatively undifferentiated cell possessing fibroblastic properties. Whether or not these profound alterations in cell morphology and function are basically the result of genetic derepression mechanism can only be subject to speculation at this time.

References

1. BECKER, R. O., C. A. BASSETT & C. H. BACHMAN. 1964. Bioelectrical factors controlling bone structure. *In* Proceedings International Symposium Biodynamics of Bone. H. Frost, ed. Little, Brown and Company, Boston, Mass.
2. BECKER, R. O. 1966. The control system governing bone growth in response to mechanical stress. Arkansas Med. Soc. 62(10): 404-406.
3. BECKER, R. O. & F. M. BROWN. 1965. Photoelectric effects in human bone. Nature 206(4991): 1325-1328.
4. BECKER, R. O. & A. A. MARINO. 1966. Electron paramagnetic resonance spectra of bone and its major components. Nature 210(5036): 583-588.

5. BECKER, R. O. & A. A. MARINO. 1966. Evidence for direct physical bonding between the collagen fibers and apatite crystals in bone. Nature (In press.)
6. BASSETT, C. A., R. J. PAWLUK & R. O. BECKER. 1964. Effects of electric currents on bone in vivo. Nature 204(4959): 652-654.
7. HOLTFRETER, J. 1947. Morphogenesis, crenation and cytolytic reactions of the erythorocytes of amphibians. Morphol. 80: 345-367.
8. POHL, H. A. & I. HAWK. 1966. Separation of living and dead cells by dielectrophoresis. Science 152: 647-649.