The Electrical Control System Regulating Fracture Healing in Amphibians

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Not a great deal of thought has been given to the factors that initiate, guide and control the various processes of bone repair.

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The essential element in all wound healing is cellular in nature. In simple, nonregenerative healing it is primarily a process of fibroblastic proliferation leading to fibrosis and scar formation. In regenerative healing there is a new growth of cells of the types necessary to replace the damaged or missing tissue or organ. This is accomplished by a variety of mechanisms all characterized, however, by the appearance of a regenerative blastema, a mass of undifferentiated cells which subsequently undergo differentiation and growth. This blastema may arise from: proliferation of prexisting (stem) cell line,[55] dedifferentiation of preexisting mature cells at the site,[34] or migration to the site of multipotent blast type cells.[40]

Regenerative processes vary markedly in their level of competency from that exhibited by the planarian worms which are capable of regenerating a new total organism from a fragment as small as one fifth of the original,[70] to that residual in the mammals which is limited to the regeneration of certain tissues only, notably skin and bone. In the latter case, the commonest example is fracture healing[41] in which a process of periosteal stem cell proliferation is reported to produce the blastema.[63]

The initiating and controlling factors for regenerative growth processes in general are unknown.[54] Competent healing or replacement of damaged or destroyed areas may be considered to be one of the basic requirements for living organisms, and this ability must have appeared very early in the evolution of living organisms or may have been present from the onset. It is not unreasonable to postulate that such an ability long antedated any type of self-replicating ability.[67] The process of fracture healing should then be viewed in this much larger context rather than isolated from the remainder of healing processes simply because the end point is an ossified tissue. In this light, the basic event in fracture healing is the cellular process leading to the initial soft callus—the regenerative blastema.

The question that immediately arises is that phrased by Pritchard and quoted above. This has been the subject of much speculation in the past and a variety of agents have been proposed but never confirmed.[50] More recently, Urist[64, 66] has reported on a series of ingenious experiments indicating the ability of the fracture callus, and particularly demineralized bone matrix to induce an osteoblastic response from mesenchymal cells. The demineralized matrix is believed to produce the "bone induction principle" in combination with competent mesenchymal cells resulting in transformation of the mes-

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enchymal cells into osteoblasts[65]; the relationship of these observations to fracture healing remains to be shown.

In 1961, Becker,[6] reported on a series of experiments dealing with amphibian limb regeneration in which certain electrical events at the amputation site related to the central nervous system were postulated to be controlling factors. This thesis has subsequently been expanded in two directions. The electrical properties of the central nervous system and their relationship to growth processes have been investigated in greater detail,[5, 7–10, 12, 13] and bone growth, particularly as exemplified by Wolff's law, has been related to electrical phenomena produced by stressed bone.[3, 14] The electrogenic properties of bone have been described as inherent in the solid state organization of the bone matrix.[15, 16, 42, 43]

With this background, it seemed desirable to attack the problem of fracture healing from the viewpoint of the electrical properties of the bone matrix and their possible relationship to the stimulation of the cellular response. This paper reports the result of such an investigation of fracture healing in several classes of vertebrates, particularly the amphibia. This investigation has provided even firmer ties between fracture healing and regeneration in general, and has enabled us to identify the factors responsible for the initiation and control of this process.

The observations made will be reported in 3 sections, although this does not necessarily reflect the actual sequence of experimentation. First, a study of histologic and cytologic events in the fracture healing sequence was made utilizing light and electron microscopy in an attempt to identify with precision the cellular source of the regeneration blastema. Secondly, the electrical phenomena subsequent to fracture were measured both on the bone and the periosteum. Thirdly, the cells responsible for providing the blastema were isolated from normal, nonfractured animals and exposed in vitro to electrical phenomena simulating that found at the fracture site.

METHODS

Adult grass frogs, Rana pipiens obtained commercially or Rana pipiens and Rana clamitans collected locally, were used as the primary experimental animals. This amphibian was chosen for several reasons. First, our prior experiences with this animal indicated that they were capable of clinically healing a long bone fracture in 14–21 days. Secondly, the tibia and fibula in the frog are fused into a single bone, the tibiofibularis, which in the adult is quite long (averaging 5 cm in these species) and remarkably straight and uniform. We found that a standard, noncomminuted fracture of the mid-shaft of this bone could be produced manually without rupture of the periosteum in a large percentage of trials. Thirdly, the length of the frogs' hind limb permitted plaster immobilization where long-term observations were necessary. Lastly, the histologic appearance of the frog tibiofibularis is typical of vertebrate long bones except for a somewhat thinner periosteoum than in mammalia.

This choice of experimental animal permitted us to evaluate the rapid healing of a standardized fracture.* The animals were easily maintained in continuously running tap water at a mean temperature of 50–60 F. Anesthesia was obtained by immersion in 10 per cent ethanol or a 1–5 per cent Tricaine (Sandoz) solution. For some short-term observations and for final observations on long-term experiments, the animals were pithed. The ensuing spinal shock lasts 5–7 minutes and is replaced by a relatively normal physiologic state, (except for hypereflexia) distal to the cord transection. We found no significant differences between electrical potentials at the fracture site in parallel experiments between animals anesthetized and animals pithed 7 minutes or more prior to measurement. It must be stressed that the animals should be in optimum physiologic state—not infected or parasitized; and animals maintained in, or obtained from, cold storage are unsatisfactory.

A. FRACTURE HEALING SEQUENCE IN FROGS

METHODS

Adult frogs of the previously mentioned species were selected at random and a fracture of the tibiofibularis was produced by manual

* All data in this paper refer to fractures in which the periosteum remained intact, unless otherwise noted.

angulation. After the appropriate time interval the animal was sacrificed by pithing and the fracture bone dissected free from the adjacent soft tissue of the leg. Specimens were taken at the time of fracture and at 15 minute intervals for 2 hours; then daily for 2 weeks, at 2-day intervals for an additional week, and weekly for 3 more weeks. The fractured limb was immobilized in a plaster cast for the duration of time between fracture and sacrifice, except in the cases of the specimens removed the first day. Precautions were taken during dissection to minimize distortion of the fracture and disruption of the early callus.

Specimens removed were transferred immediately to Bouin's solution and fixed for a minimum of 24 hours. Decalcification was carried out in nitric acid prior to embedding for routine histologic sectioning. Sections were stained with hematoxylin and eosin, or with van Gieson stain for collagen.

For ultra structural examination fractures of corresponding duration were dissected and a portion of the hematoma or callus adjacent to the bone end was removed and fixed immediately in glutaraldehyde. The tissue was then post-fixed in osmic acid and embedded in maraglass. Examination was carried out with a modified RCA EMU 3D microscope.

Observations

In general, histologic examination confirmed a consistent pattern to the fractures. Comminution was minimal and the periosteum appeared to be intact in most of the early fractures (Fig. 1 A). Initially, the periosteum was lifted from the major fragments for varying distances from the fracture site and bleeding into this enclosed space produced a typical fracture hematoma. This hematoma at first seemed to be a random collection of nucleated red cells which then became organized in a loose network of fibrinous material.* Within 2 hours characteristic nucleated red cells were seen attached to bone by the fibrin clot, predominantly around the fractured ends (Fig. 1 B). During the subsequent week the thin fibrin strands of the red cell clot became thicker and took on faint staining characteristics of collagen. The red cells themselves within this stroma appeared swollen and pleomorphic with small round nuclei and minimal or variable cytoplasm (Fig. 1 C). Where the cytoplasm could be delineated it was less dense than normal and often elongated or irregular in outline.

During this period the periosteum remained relatively thin and quiescent in appearance with only an occasional mitosis evident on extensive examination of multiple sections (Fig. 1 D). Therefore, during the the first week the initial step in the healing process was that of formation of a hematoma and organization of the cellular elements of that hematoma—nucleated red cells and a small white cell component—into a spongy network affixed to the bone on one side and to the undersurface of the elevated periosteum or opposing bone fragment on the other.

During the second and third week after fracture the cellular elements in the hematoma took on more of a pattern of fracture callus. Cells became quite variable in size and staining characteristics as the surrounding stroma became more prominent. At 3 weeks the remaining cells once more had a uniform appearance, embedded in a fibrous or cartilagenous matrix with some remaining zones of transition between this stage and the early stage (Fig. 1 E).

From 3 to 6 weeks after fracture, osteoid began to appear in the mature fracture callus. Portions of the mature new bone appeared irregularly, but most consistently at the fractured ends of bone at the site of initial attachment of the fibrinous hematoma (Fig. 1 G). Portions of the mature callus had a fibrous characteristic with a linear pattern of orientation in a cap about the raw fracture ends (Fig. 1 F). Other areas of callus were definitely cartilagenous in nature without specific orientation, the cells corresponding to the hypertrophic type of cell seen in enchondral ossification in the epiphyseal plate. No mitoses were seen in the chondrocytes, nor any cells typical of the proliferative zone in rapidly growing epiphyseal cartilage. Vascular channels within

* All classes of vertebrates except for the mammalia have nucleated red cells as the final stage in hematopoiesis.

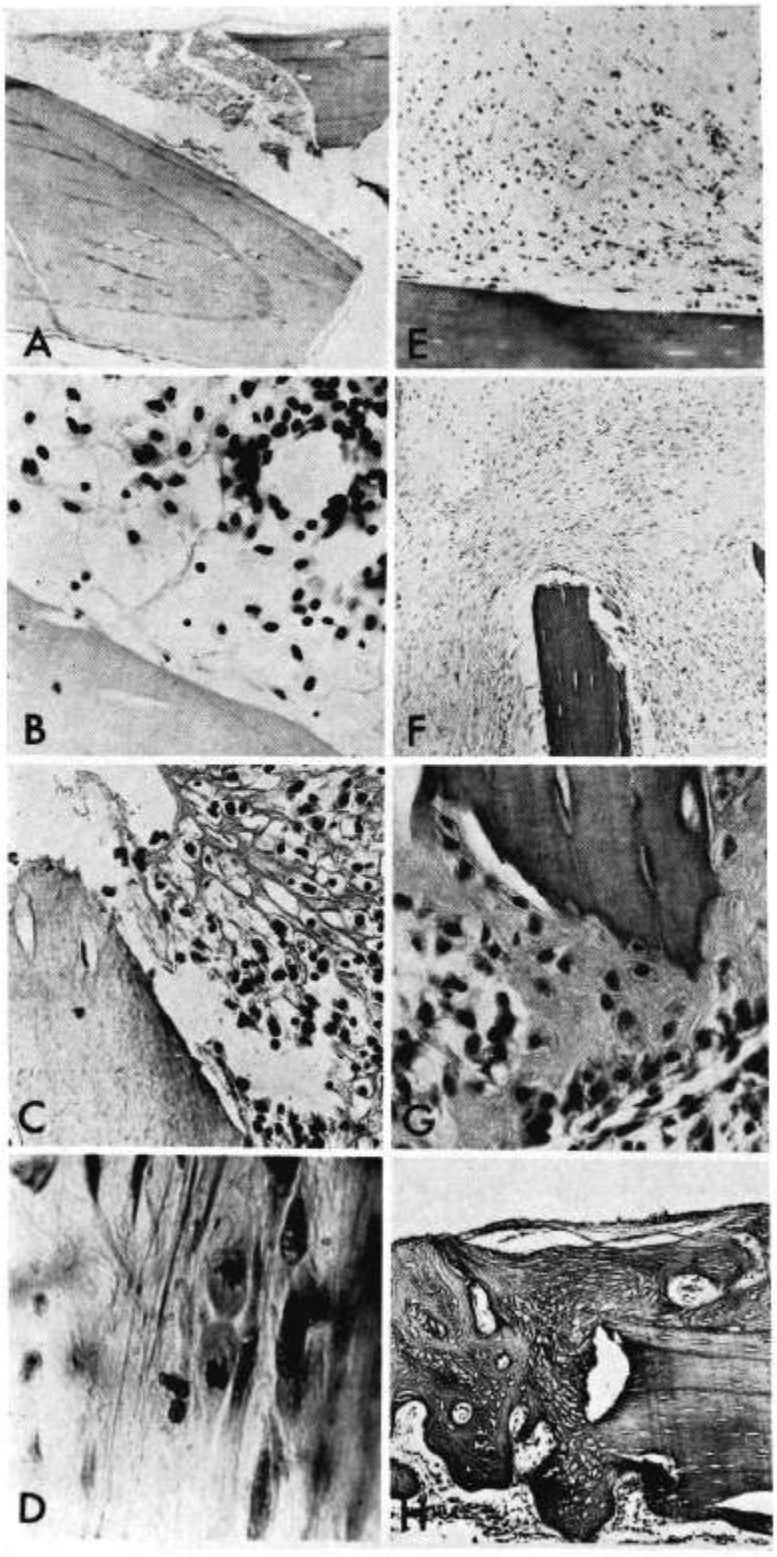


FIG. 1. A, Early fracture demonstrating intact periosteum with formation of fracture hematoma between bone ends and adjacent to periosteum (×50). B, Early hematoma with characteristic nucleated red cells enmeshed in fibrin clot adhering to fractured end of bone (×500). C, Later stage hematoma showing cellular pleomorphism and thickening of matrix between cellular elements (×500). D, Section through periosteum showing mitotic activity. This was an infrequent finding during the entire healing process (×750). E, Later stage fracture callus showing transitional zone between pleomorphic areas on the right and uniform cartilagenous callus on the left (×150). F, Late callus showing arrangement of collagen fibers in a specific orientation about bone ends. An area of transformation into cartilage is present in the upper left corner (×150). C, Early ossification taking place adjacent to bone end at the site of initial attaching of hematoma. Bone formation on the periosteal surface appears simultaneously or slightly later (×500). H, Healed fracture showing typical continuity between old and new bone with restoration of marrow space (×150).

the fracture were relatively scarce at early stages of healing.

In the final stage of healing bony bridges formed in the matrix of the callus, joining bone fragments. Some gaps were bridged directly by bone, whereas other areas were joined by an irregular trabecular network. As bone matured, vascular spaces developed in the interstices of the new bone and conversion to a more typical marrow space began (Fig. 1 H).

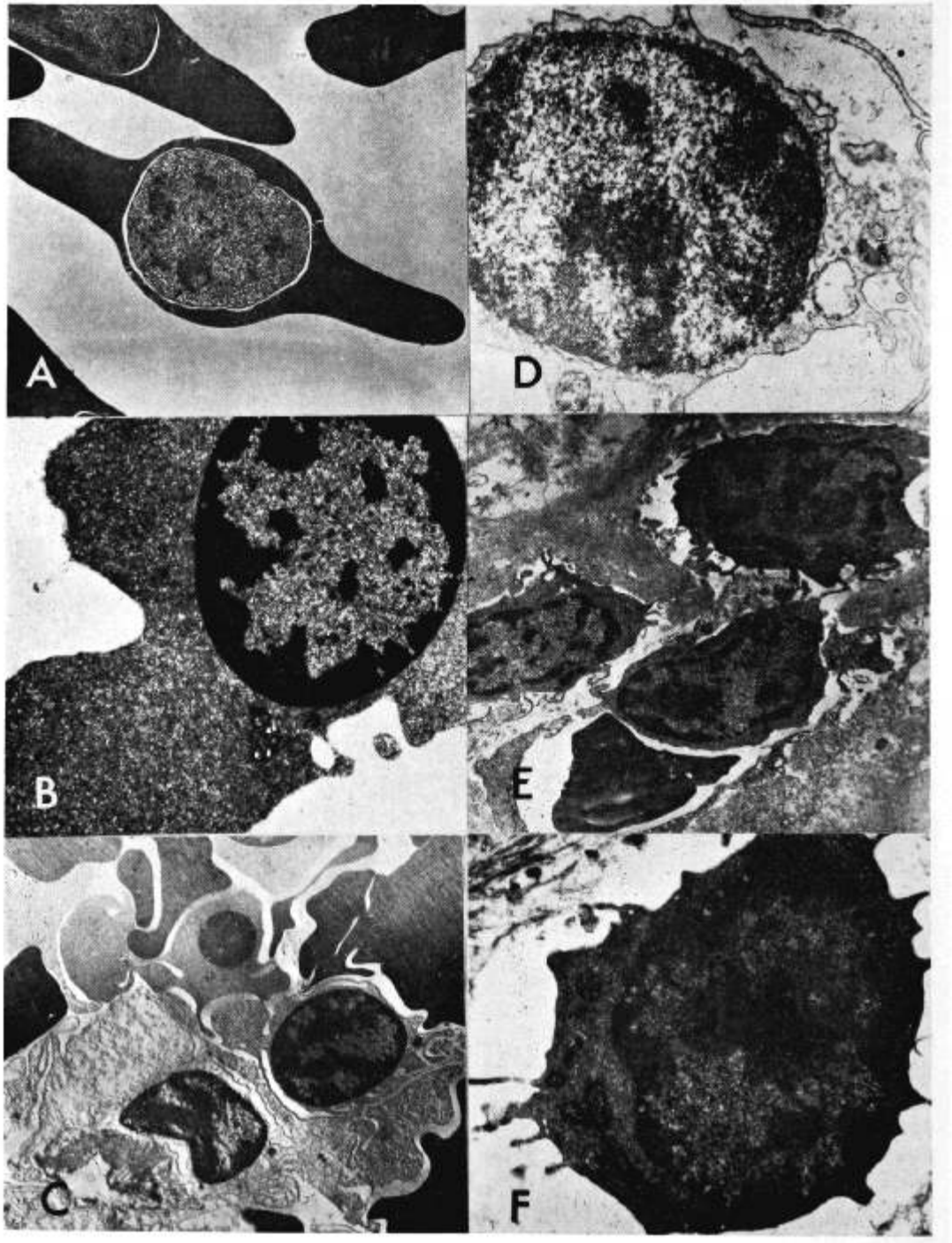
Once bridging of the fracture site with bone took place, healing continued with thickening and maturation of the bony trabeculae. Dissection of 120 commercially obtained frogs yielded 3 healed fractures which had occurred in a natural state. All naturally healed fractures showed strong bone union with varying degrees of remodeling evident. There was no histologic difference between healed fractures in the frog bone and the description of healed fractures in other animals, including mammals.

The results of histologic studies in this case did not support the theory that cellular proliferation from the periosteum is responsible for forming the fracture callus and bringing about bone healing. At no time in the sequence of events was marked proliferative activity apparent in the cells of the periosteal layer. The earliest evidence of bone formation was in the areas adjacent to the bone—not immediately subadjacent to the periosteum. Some might argue that with stripping of the periosteum, a few cells are left behind which serve to inaugurate bone formation. However, this does not fit with the observations that earliest bone appeared at the fractured ends where periosteum had never been present. Trabeculae as they formed did not parallel the periosteal layer or the bone surface but most often followed a radial or perpendicular direction to both planes. These observations lead to the speculation that a more fundamental component of the fracture hematoma, the red cell or other nucleated circulating cell, might play a major role in the healing of a broken bone.

Electron microscopic studies were performed on the fracture hematoma and subsequent callus to define cellular changes that might be going on within this area. As controls, samples of heart blood from nonfractured frogs were examined to ascertain the typical pattern of circulating red cells (Fig. 2 A). The cells were uniform in appearance, elliptical in shape with dense cytoplasm and a similarly dense nucleus, either uniform or with some variable clumping of chromatin. An occasional organelle could be seen within the cytoplasm, but in general it was homogeneous and amorphous throughout with the staining characteristics attributable to contained hemoglobin. The cell membrane was smooth with some gross irregularities related to packing of cells.

Specimens of fracture hematoma taken 2 hours after fracture revealed many red cells characteristic of normal blood but, in addition, many cells which appeared to be undergoing a definite change. Chromatin clumping with the heaviest distribution around the margin of the nucleus was most commonly seen (Fig. 2 B). Also prominent was a decrease in density of the cytoplasm which became more granular and less homogeneous. This was interpreted as a loss of hemoglobin into the surrounding extracellular space, either through diffusion or pinocytosis (Fig. 2 C). In some cells the hemoglobin seemed to be concentrating in localized areas, possibly prior to being lost from the cell. The affected cells themselves appeared rounded in many cases rather than ellipitical and with some increased irregularity of the cell membrane. Coincidental with loss of hemoglobin was a decrease in size of the cells with the smallest cells seen having little or no hemoglobin remnants. A significant change in the number of mitochondria could not be detected in the 2 hour sample.

Specimens from a hematoma at 3 days showed persistence of some typical red blood cells, but most cells in the hematoma were altered to some degree (Fig. 2 D). The cell membrane often exhibited long cytoplasmic extensions and the cells varied in overall size with configuration varying from round to



irregularly ellipitical. Some cells were vacuolated and others, in addition to some vacuolition, showed increased numbers of mitochondria. Distinct Golgi apparatus and endoplasmic reticulum was lacking at this stage. Clumping of chromatin in the nucleous remained prominent while the material supporting the cells appeared to be fibrous in nature but without dense cross-banding.

Cells from the fracture area at seven days were embedded in a dense stroma of thin fibers and amorphous material (Fig. 2 E). The nuclei had a well-organized chromatin pattern and the cytoplasm had condensed with evidence of mitochondria and dense bodies. The cells at this stage appeared to contain the necessary components for the production and transport of protein and polysaccharides. Later, at 3 weeks, cells from the well-defined fracture callus were obviously mature and typical of chondrocytes in lacunae surrounded by a fibrillar matrix (Fig. 2 F).

DISCUSSION

This serial histologic and electron microscopic study of the healing long bone fracture in the frog led to two observations. First, the healing process, i.e., the blastema, is not derived solely from the periosteum, although this tissue may make some contribution, but is more directly a product of the fracture hematoma. Secondly, the method by which the hematoma contributes to healing is through alteration of a previously circulating cell, in this case the nucleated red cell trapped in the hematoma, into a cell capable of producing collagen and ground substance essential in the reconstitution of bone. There is no evidence of massive invasion by granulation tissue or capillary buds. There are no giant cells or large numbers of macrophages ordinarily associated with removal of cellular debris. The red cells do not present a static picture, but undergo

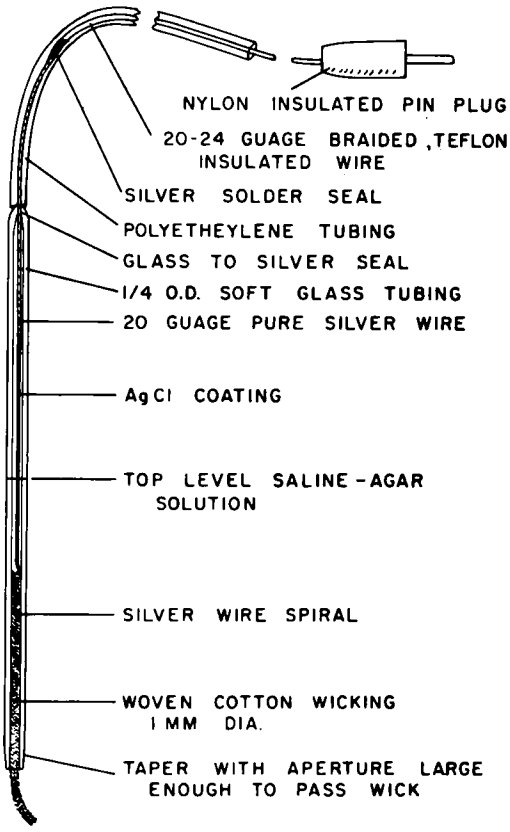


FIG. 3. Silver-silver chloride electrode of the type used to determine electrical potentials of bone and periosteum. The electrodes are stable, have little inter-electrode potential and are nondamaging to tissues. In use, both the recording and the indifferent electrodes were of this type, electrode pairs being chosen for the lowest inter-electrode potential. Each electrode contacted approximately 1 mm^2 surface area of the substrate being measured. These electrodes must be stored with the wick ends totally immersed in saline solution.

FIG. 2. A, Electron micrograph of normal frog blood cells obtained from heart showing typical elliptical dense nucleated red cells with an occasional cytoplasmic vacuole or organelle. B, Cell from 2 hour fracture hematoma showing peripheral clumping of chromatin in the nucleus, irregularity in cell outline and less homogeneous density of the cytoplasm. C, Specimen from hematoma 2 hours after fracture. Marked variation in characteristics of red cells is apparent, particularly with reference to cytoplasmic density and chromatin clumping of the nuclei. D, Cell from 3-day hematoma showing irregular outline, marked loss of cytoplasmic density and irregular clumping of chromatin in nucleus. E,Section from 7-day hematoma showing dense matrix enclosing viable cells with well organized nuclei and irregular cytoplasmic outline. Small organelles and dense bodies can be detected within the cytoplasm as evidence of cell activity. F, Representative cell from callus at 3 weeks showing well differentiated cells within a fibrocartilaginous matrix and bearing no resemblance to nucleated red cell.

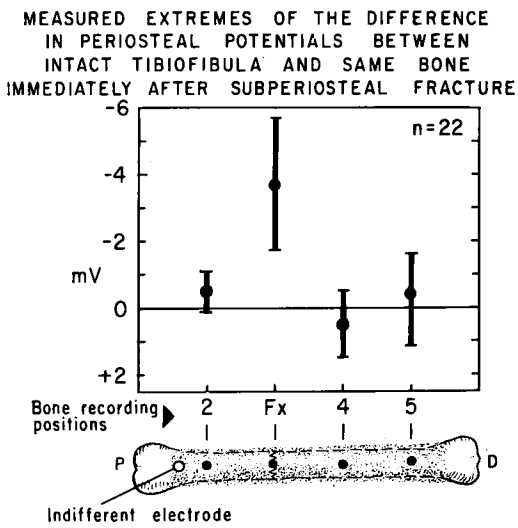


FIG. 4. Means and extremes of 22 determinations of the periosteal potentials along the shaft of the fractured tibiofibularis. These measurements were taken within 5 minutes of fracture by the methods described in the text.

a series of changes apparent in histologic sections and demonstrable in electron micrographs. Whether these changes lead to cell death or represent a process of dedifferentiation and redifferentiation can only be surmised from serial observations such as these. However, the relative absence of mitoses and capillary invasion support a process involving change within the cell population present at the fracture site in the hematoma.

Since a change such as this must involve a marked alteration in metabolic activity, from a cell that is practically inert to a highly active one, correspondingly major alterations must occur in the cytoplasmic content of RNA. We have made some preliminary observations of this, utilizing acridine orange staining.[60] and tritiated uridine autoradiography on the fracture hematoma. While details will be presented in a later paper, we can at this time report that many cells in the early hematoma stages demonstrate a well-developed orange fluorescence in the cytoplasm with acridine orange indicative of an increase in RNA content (Fig. 17).

Injection of the fracture hematoma with tritiated uridine (1 μc/g total body weight) at 2, 4 and 6 hours past fracture resulted in a cytoplasmic grain count approximately 10x background in the 4 and 6 hour samples upon sacrifice one hour after injection. This observation is also interpreted as an increase in RNA content of the hematoma erythrocytes.

As a result of the above observations, therefore, a sequence of fracture healing in the amphibia is proposed which varies from the classical descriptions.[20, 48, 49,71]

Phase 1. Hematoma formation immediately following fracture with fibrin clot surrounding bone ends and limited by periosteum or surrounding muscle.

Phase 2. Dedifferentiation of a proportion of cells within the hematoma into a primitive cell type.

Phase 3. Redifferentiation into connective tissue cells capable of synthesizing collagen and ground substance.

Phase 4. Enchondral ossification within the matrix of the mature fracture callus or blastema with the earliest appearance directly adjacent to the fractured surfaces.

These phases proceed at different rates, the fracture area presenting a mixed picture of early, late cartilagenous, fibrous and ossifying callus within the same specimen. Periosteum and osteogenic cells within the bone and marrow may play an important role in the healing process but not the sole or even a dominant part according to these observations. The feasibility of this thesis was substantiated by our subsequent work involving measurements of electrical potentials in bone secondary to fracture, and by stimulation of frog red cells in vitro with minute electrical currents as reported in the following sections.

B. ELECTRICAL POTENTIALS ON BONE AND PERIOSTEUM SECONDARY TO FRACTURE

METHODS

A self-contained type of silver-silver chloride, salt bridge electrode was used (Fig. 3) with electrode pairs chosen for minimal interelectrode potential. Contact with the preparation was through the integral cotton wicking

saturated with amphibian Ringer's solution. Potentials were measured with a Hewlett-Packard model 425A microvoltammeter with an input of 1 meg ohm. In all cases, unless otherwise noted in the illustration, the indifferent electrode was placed on the proximal metaphyseal portion of the tibiofibularis after careful surgical exposure. The recording electrode was then placed either over the fracture site, or on one of the 4 standard measuring positions along the shaft of the bone. The polarity of the site contacted by the recording electrode with reference to the indifferent electrode was obtained by this method. For periosteal measurements the periosteum was left intact and both electrodes were on its outer surface. For bone measurements the periosteum was reflected from the proximal fragment sufficiently to permit both electrodes to contact bone only. In all cases, hemostasis was always secured and contact of the electrodes with free blood avoided.

To ensure reproducible electrode placement both electrodes were secured in rapidly adjustable semi-micro manipulators (Harvard Instrument Company). Each data point recorded was the average of 5 separate recordings made from the same measurement coordinate points within a 2 minute time interval. Electrode zero was checked between each single measurement. In the illustrations the mean of this type observation from a number of different animals is recorded. The number of animals in each set of experiments is also indicated (n). Figure 4 gives the means and extremes for a typical experiment; in all other experiments the variation in measurements was within these limits. We found the greatest cause of lack of reproducibility was in specimen preparation, particularly free blood in the tissues due to poor dissection technic or rupture of the periosteum at time of fracture. All preparations of this type were excluded from the series reported.

Observations

I. Periosteal Potentials

Measurements on the periosteum before fracture indicated a minor longitudinal electrical gradient that was distally negative with a maximum value between proximal and distal metaphysis of approximately 1 mv. Immediately following fracture, the periosteum directly over the fracture site becomes highly negative with an average value of 7 mv. The entire voltage gradient appears to shift in a negative direction, however, positive going potentials are located about halfway between the fracture site and the metaphysis at each end (Fig. 4). This suggests a balanced dipole extending in both directions from the fracture site with the periosteum superior to the fracture site negative and with relative positive areas located about 1 cm from it both proximally and distally. During the next hour there is a steady decline in all of the field values, however, the pattern of the balanced dipole appeared to persist (Fig. 5). During the following 7 days the fracture site again became more negative than before fracture with a peak value at about 4 days. However, the maximum negativity during this time was less than half the value measured immediately following fracture and no dipole configuration was

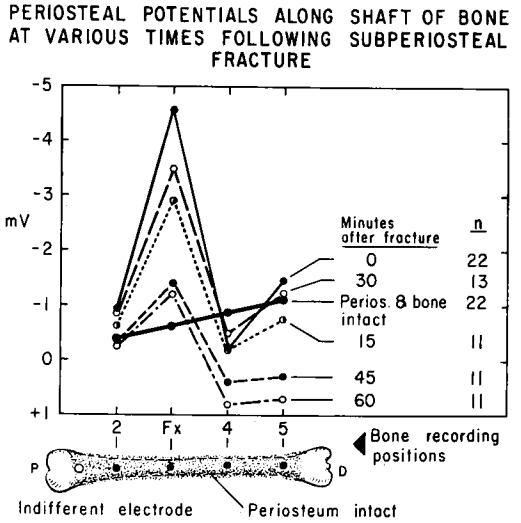


FIG. 5. Changes in the electrical field pattern along the shaft of the fractured tibiofibularis over 1 hour. The periosteum was intact and the potentials represent periosteal potentials only. Each time interval was determined on a separate set of experimental animals, the number of animals in each set is indicated under N. In order to prevent artifacts due to exposure and drying, each time period determination was made by first producing the fracture, waiting until 5 minutes before the desired time and then surgically exposing the periosteum. The heavy solid line represents the normal, slightly distally negative, electrical field present prior to any fracture. The open circle represents the indifferent electrode near the proximal end.

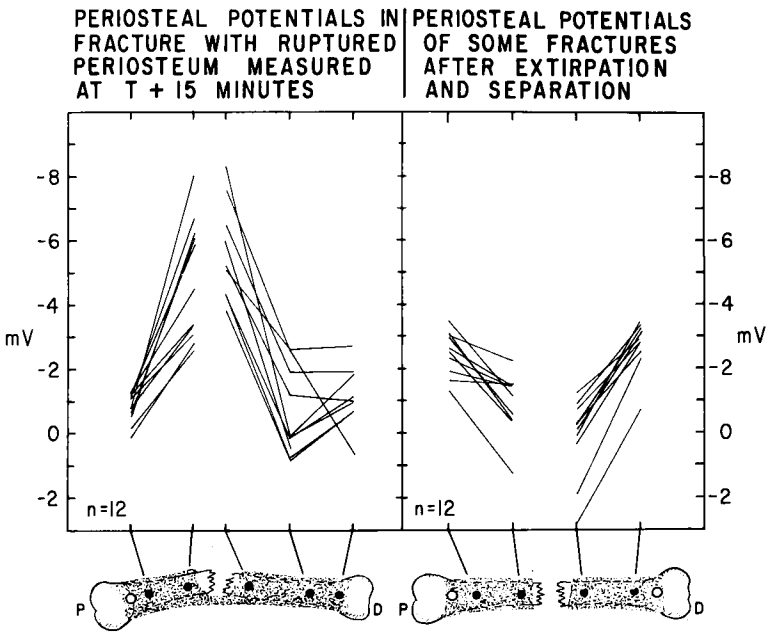


FIG. 6. Periosteal potential pattern (left) along shaft of fracture with ruptured periosteum. The measurement points at the fracture site were displaced 1 mm proximally and distally to avoid as much current of injury from the cut periosteal edge as possible. The potentials at these sites are markedly higher than that directly over the fracture site with periosteum intact.

Right, Same series, but following amputation and separation of the fracture fragments. The amputation procedure effectively produces complete denervation of the specimen, the measurements being taken within 2 minutes following the procedure. While there is a concomitant lack of circulation produced by the procedure, it was felt that the marked alteration in polarity was the result of the total nerve section.

discernible. By the seventh day the periosteal potential at the fracture site generally returned to a normal value and the longitudinal gradient found before fracture was restored. A series of acute experiments were done in which the periosteum over the fracture site was ruptured (under direct visualization) by increasing the angulation of the fracture. Preparations in which excessive bleeding occurred were discarded. Measurements were then made with the indifferent electrode placed on the proximal metaphysis, the gradient between the metaphysis and a point on the periosteum (1 mm back from the ruptured edge to minimize currents of injury) nearest to the fracture site was measured. The periosteal potential between these 2 points appeared to be higher than when the periosteum was not ruptured by the fracture ends (Fig. 6 A). The lower leg was then completely amputated at the knee joint and the fracture surgically separated. Approximately 2 minutes elapsed between this surgical procedure and the next measurements along the periosteum in the same fashion as before. This procedure consistently produced a reversal in the preexisting voltage gradient with the periosteum adjacent to the fracture site now positive in respect to the point intermediate between it and the respective metaphysis (Fig. 6 B).

To evaluate the effect of periosteal injury alone without attendant fracture, a series of 12 animals was prepared and the periosteum exposed. The periosteum over the usual site of fracture was then forcibly compressed with a smooth forceps sufficiently to produce obvious damage (subperiostel hematoma) and the potential appearing between this site and the standard metaphyseal reference point was determined. There was an immediate increase in negativity, although averaging half the magnitude of that appearing on the intact periosteum in conjunction with a fracture. This negativity returned to its original pre-injury value within 3 hours.

II. Bone Potentials

A distally negative electrical gradient was noted on the intact bone, similar to but of less magnitude than that described for the

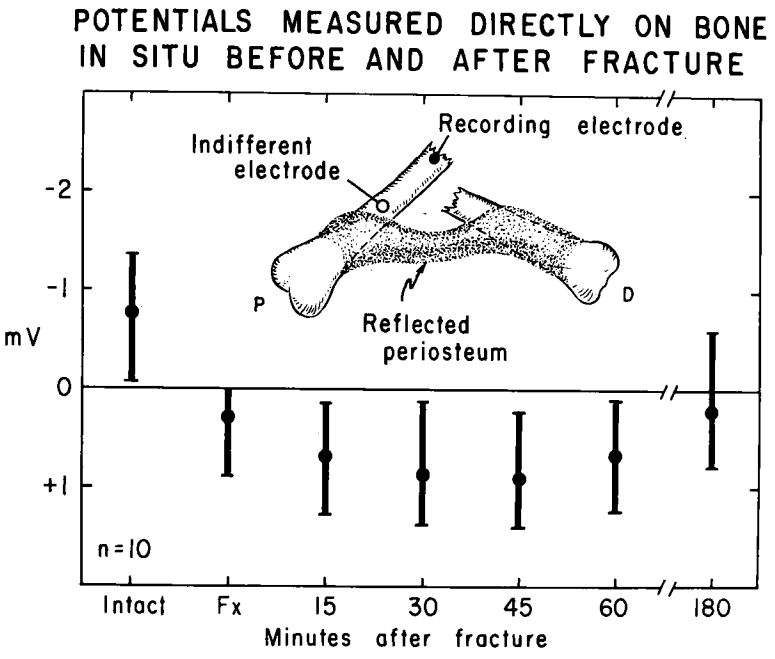


FIG. 7. Means and extremes of measurements directly on bone before and after midshaft fracture of the tibiofibularis. There is the appearance of a positive polarity at the fracture site which increases with time to a maximum at 45 minutes postfracture. The recording electrode was placed 1 mm proximal to the actual fracture site to prevent contact with marrow elements and to obviate the possible higher currents of injury at the fracture surface. All specimens with comminuted fractures or with large hematomas were excluded from this series.

intact periosteum. Immediately following fracture, the bone surface adjacent to the fracture site became positive with respect to the bone surface of the proximal metaphyseal area. This positivity subsequently increases, reaching a maximum of approximately 2 mv in one hour and returning to approximately its original value within 2 hours (Fig. 7). The potentials recorded immediately and for the following hour are considered to be most likely associated with the bone itself, later measurements were not made because of the difficulty in clearing the hematoma from the bone surfaces.

DISCUSSION

It now appears firmly established that bone, as well as tendon collagen, produces electrical potentials under stress.[3, 31, 58] However, the source of these potentials remains in dispute.[11] All of these studies have utilized bending or compressional stresses and the resultant potentials have been relatively transient. Stressing certain materials to failure (fracture) results in the persistence of residual stress within the material which is slowly relieved following the actual breakage. Bonfield and Li[18, 19] conclude from studies on the physical parameters of deformation and fracture in bone that stress related phenomena would be present for an appreciable period of time following fracture. Therefore, it is not unreasonable to expect that electrical potentials would be present on the bone for such periods as we have observed following fracture. Friedenberg and Brighton[29] have reported potentials following fracture which in part, paralleled those observed by us. They noted an area of negativity in skin potentials over tibial fracture sites in humans and rabbits. These potentials persisted until shortly before clinical healing became apparent. It would appear quite likely that this skin negativity reflects the periosteal negativity observed by us in the amphibia. It should be noted that in our experiments the actual bone positivity at the fracture site was unobserved through the intact periosteum. The source of the periosteal potentials is not directly related to either the electrogenic properties of the bone or solely to injury potentials in the periosteum itself. The prompt alteration in these potentials following the interruption of

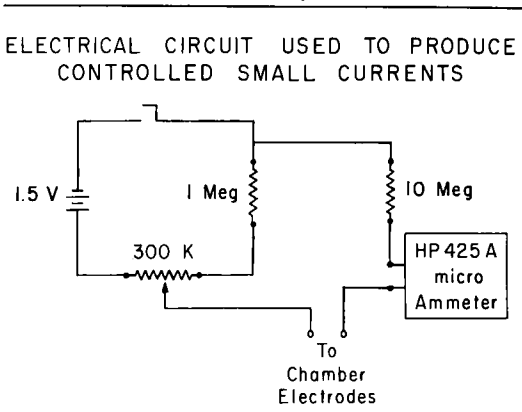


FIG. 8. Schematic drawing of the circuit used to produce controlled small currents. Standard components were used in this simple voltage divider network. Because the current level was continuously monitored by the microammeter in series with the chamber, there was no need for precision components.

all nerve pathways to the limb indicates that they probably are neurally related. The rapidity of this effect precludes it being produced by the attendant interruption in blood supply.

The positive potentials on the fractured ends of the bone with respect to the remainder of the shaft, both proximal and distal, produce an opposed dipole pair within the fracture hematoma. The resultant electrical field within the hematoma would be markedly nonuniform[39] and would be subject to further perturbation by the opposing pattern of potentials on the periosteal surface. If we assume that the resultant current flow is generated solely by the electrogenic properties of the bone matrix alone, we can establish a minimum level of current traversing a 1 mm^2 area of hematoma immediately adjacent to the fracture site.

From another study,[42] we have determined that the resistance across 1 cm of bone matrix is approximately 1 x 10^{10} to 1 x 10^{11} ohms. Using this figure and the observed voltage of 2 mv, Ohm's law indicates a current level of between 2 and 2,000 micro-micro amperes ($\mu\mu$A) mm^2 through portions of the hematoma at the time of maximum bone potential.

C. EFFECT OF ELECTRICAL CURRENT ON NORMAL AMPHIBIAN ERYTHROCYTES

In accord with our original thesis that the electrical phenomena accompanying fracture was the direct stimulus for the cellular response and since we had determined that the nucleated erythrocyte was the responding cell, we exposed normal frog erythrocytes to electrical fields and currents simulating those produced by the fracture.

METHODS

Much of the methodology utilized was previously described in a preliminary report.[17] In brief, chambers of various sizes and shapes were constructed from glass or lucite and various configurations of nonreactive metallic electrodes applied.* Normal amphibian heart blood was obtained by direct puncture and diluted 1:800 with amphibian Ringer's solution to facilitate observation before instilling in the chamber. Currents were derived from a battery powered voltage divider network (Fig. 8) and measured with a Hewlett-Packard 425A microvoltammeter in series with the chamber. The alternating currents experiments utilized the same divider network but substituted a Hewlett-Packard 202A low-frequency function generator for the battery. The chambers were of a size that fit the stage of an inverted biologic microscope (Unitron model BN 13 with Zeiss optics). The cells were continuously observed at magnifications from 250X to 1250X in both bright field and phase contrast illumination. Chambers could be emptied by micropipette at any desired time and smears of the cells made and stained by the usual Geimsa-Jenner technics.

OBSERVATIONS

Many of the observations made with direct current administration were reported by

* The purity of the metal used for the electrodes is critical and only 99.9 per cent pure material should be employed. Washing the chamber and electrodes in ethanol should be avoided, since this produces unstable electrodes with high interelectrode potentials. The efficiency of the electrodes declines with prolonged usage, possibly due to accumulation of polarization products on their surfaces. The polarization potentials of the electrodes should be tested each time before current is applied, because if interelectrode current exceeds approximately 500 $\mu\mu$A, spurious results will be noted. The cells will become round, develop prominent granularity to the cytoplasm, and fail to show a phase shift in the nucleus).

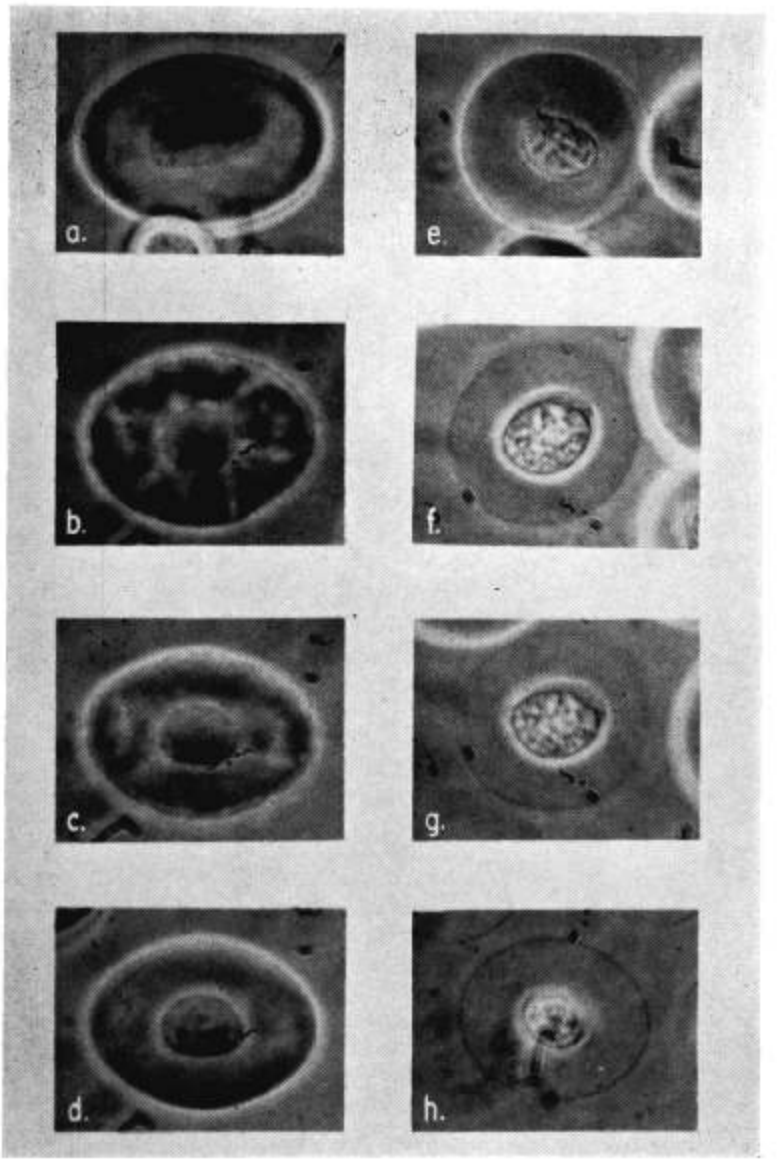


FIG. 9. Phase contrast photomicrographs at 1250X of a single cell exposed to a total current of 200 $\mu\mu$A in the standard chamber. The time interval between the separate exposures is approximately 3 minutes. In addition to the obvious loss of hemoglobin by pinocytosis and the major morphological alterations in the nucleus, one should note the phase shift in the nucleus, beginning with exposure d. We have observed that prior to this stage, a cell can revert to its previous morphology if the electrical current is stopped, but once this nuclear phase shift occurs, the sequence goes to completion even in the absence of an applied electrical field.

Becker and Murray.[17] In brief, we have observed that at total current levels from 1–1,000 $\mu\mu$A in chambers producing nonuniform electrical field patterns, the red cells could be observed to undergo a series of morphologic alterations, similar to those described by Holtfreter[35] for the maturational sequence. A sequential series of phase contrast photomicrographs at 1250X of a single cell undergoing this alteration is reproduced in Figure 9. To compare these in vitro alterations with those in the actual hematoma, a series of fracture hematomas were prepared by suspension in amphibian Ringer's solution and also observed by phase contrast microscopy (Fig. 10). The series of changes in individual cells paralleled those observed in vitro but due to the mixing in suspension, erythrocytes in various stages of alteration could be observed in each field.

Smears of cells removed from the in vitro chambers and stained by the Geimsa-Jenner method were evaluated and the cell alterations were found to parallel those reported

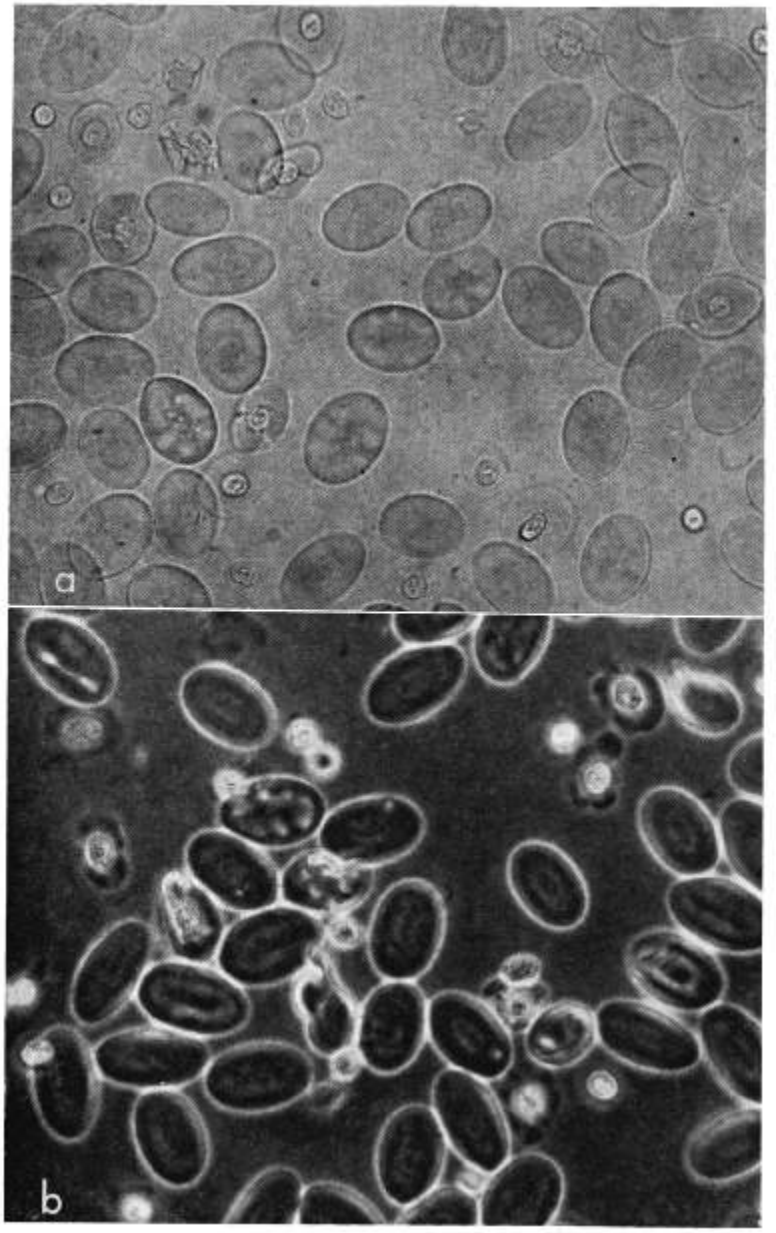


FIG. 10 A–B. Four hour fracture hematoma, suspended in amphibian Ringer's solution. A, bright field; B, phase contrast. (Both ×400.) The similarity between the altered cell types in these views and those in Fig. 9 is evident. In the phase contrast illustration, a few amoeboid forms are visible and the phase shift in the nucleus is evident. A variety of forms is observed in various stages of change due to the fact that the entire hematoma cell population does not change at the same rate (distance from fracture surface varies, etc.). The altered forms that we observed are seen randomly distributed in the sample and not clumped into groups as would be expected if they actually represented fragments of hematopoietic marrow.

by Jordan and Spiedel[38] for the maturational sequence in amphibian erythrocytes (Fig. 11). A few differences were noted, probably because the sequence was proceeding in the direction of dedifferentiation, thus necessitating the discharge of the hemoglobin from the cytoplasm apparently by a process of reverse pinocytosis. In addition, a stage appeared early in the dedifferentiation sequence when the nucleus became refractile under direct observation and lost the normal staining characteristics to Geimsa-Jenner. Later stages in the sequence demonstrated staining reactions typical of differentiation, i.e., basophilia of cytoplasm, appearance of nucleoli, etc. Some additional details have been observed of a minor nature, and a schematic diagram of the entire sequence of morphologic alterations observed in the chambers with phase microscopy is illustrated in Figure 12.

It is possible that these represent details

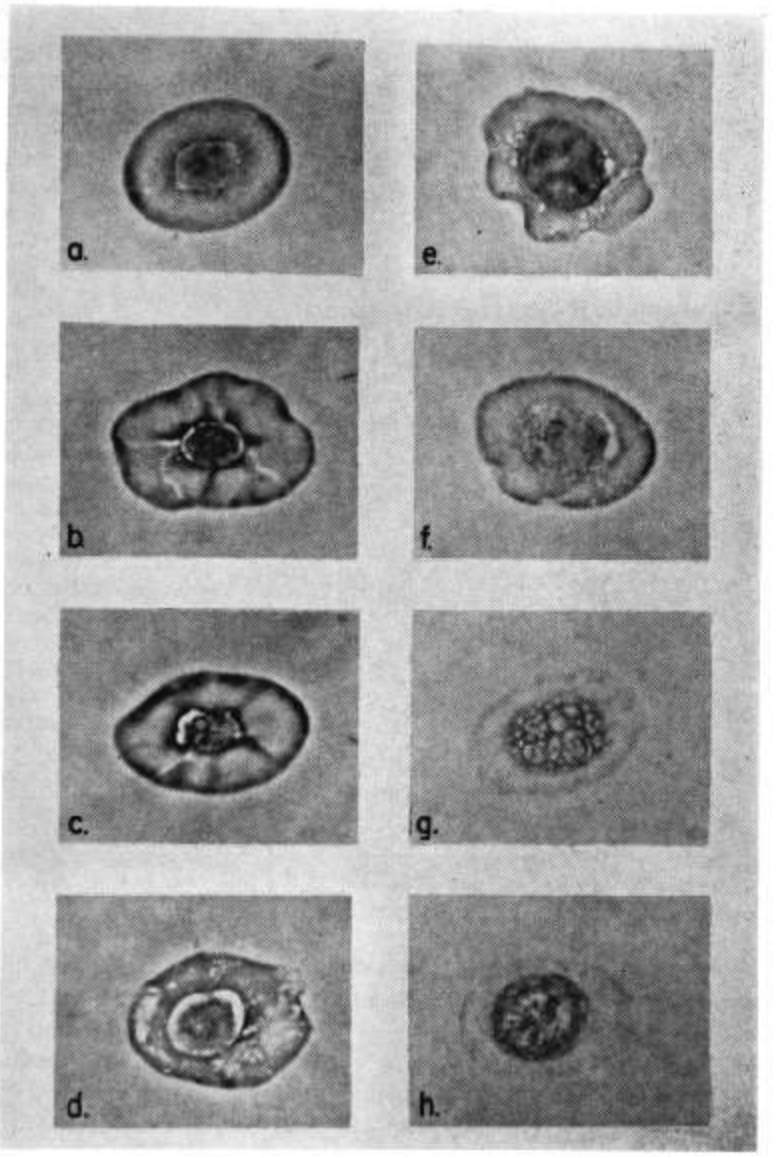


FIG. 11. The sequence of morphologic alterations as seen with Geimsa-Jenner stain and phase contrast optics at ×1250. These cells (except a) were harvested from chambers which had been exposed to electrical currents for sufficient periods of time to produce the complete alteration of approximately 50 per cent of the total cells. (a) is from a normal blood smear prepared by standard technic and similarly stained. The sequence shown is somewhat arbitrary in that only representative types were selected, the actual smears include an infinite series with many interval stages between those shown. These cell types have not been observed in normal amphibian peripheral blood smears except for an occasional example of stage b.

in the maturation process that have not been previously observed. Janus green vital staining was used to determine whether there was an increase in mitochondria in the altered cells and while results with this method are notoriously inconsistent, when positive staining was obtained the mitochondrial count in these cells varied from 4 to more than 20 per cell. The normal erythrocyte usually contained 1–3 mitochondria per cell by this method.

While most of these observations have been made on frog heart blood, parallel experiments were done on several specimens of goldfish, snake, turtle, and salamander heart blood. In each case, similar morphologic alterations were noted, although the time requirements demonstrated a class difference, the fish cells proceeding more rapidly and the reptilian cells less rapidly than amphibian cells at the same current density.

It should be noted that there was an upper limit to the effective range of total currents for the size chambers utilized; in general, values exceeding 1,000 $\mu\mu$A were either ineffective in producing the change or the

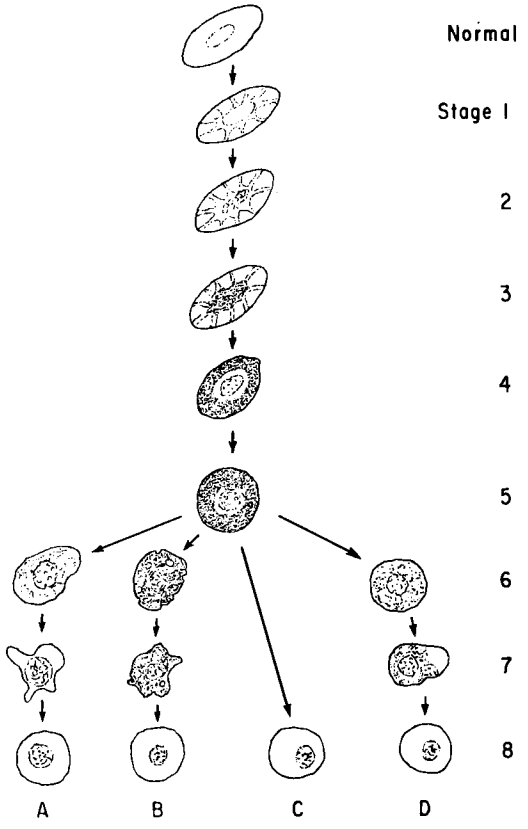


FIG. 12. Artist's rendering of the observed sequence of morphologic alterations in the chambers. The initial changes noted Stage 1 consists of radial condensations of the cytoplasm. Stage 2 is characterized by nuclear condensation; Stage 3 is prominent in the nucleus becomes glassy and refractile, lacking in nuclear detail. The phase shift in the nucleus occurs at Stage 4, which also demonstrates the beginning of cytoplasmic vacuolization and irregularities in the cell membrane. All cells seem to progress to Stage 5 where they demonstrate spherical shape, uniform cytoplasmic staining and a prominent bright phase nucleus, following which they may follow several different pathways, some of which are frankly amoeboid (Sequence A). However, these constitute less than 5 per cent of the total cells. Another small portion, Sequence B, shows prominent vacuoles and very irregular cell membrane, but no amoeboid movement. Sequence C is the most common with gradual clearing of the cytoplasm either by irregular vacuolization or by the appearance of a perinuclear clear zone which progresses radially outward. An occasional preparation will show a large number of Sequence D types which demonstrate typical lobopodia and are also motile, although to a lesser degree than Sequence A types. The final result in each sequence is a rather large, clear cell (with occasional cytoplasmic granulation) and a prominent nucleus when viewed in vivo (Fig. 9 G). We feel that this is represented in the Geimsa-Jenner preparation, Fig. 11, by illustration h.

changes proceeded at a very slow rate. The most effective total current was in the range of 300–700 $\mu\mu$A.

In chambers not having direct contact with the electrodes and constructed to optimize a linear type nonuniform field (Fig. 13), total current levels of approximately 50 $\mu\mu$A were obtained due to current leakage through the lucite. The uniformity of the field pattern in these chambers and the observed progression of cell changes enabled us to measure and calculate the voltage and current levels to which individual cells were exposed. The voltage gradient was uniform throughout the chamber and all cells were exposed to approximately a 0.3 mv drop across their diameter simultaneous with the onset of the current. The current density at the chamber apex approached 50 $\mu\mu$A/mm^2, while at the widest portion of the chamber it approximated 4 $\mu\mu$A/mm^2. The cell alterations appeared first at the chamber apex and gradually spread to include the entire chamber. Complete morphologic alterations at the apex occurred in 10–15 minutes, while cells at the widest portion of the chamber altered more slowly and required 4–5 hours to complete the change. Using chamber configurations with electrodes separated more widely, lower leakage currents could be produced and complete cell alterations were observed at current densities as low as 1$\mu\mu$A/mm^2. Since the cells were all exposed to the same voltage drop, the time limiting factor must be either field density or current density. In any event, the measured potentials at the fracture site in vivo, and the calculated current densities are well within the range of the effective levels produced by the fracture in vivo.

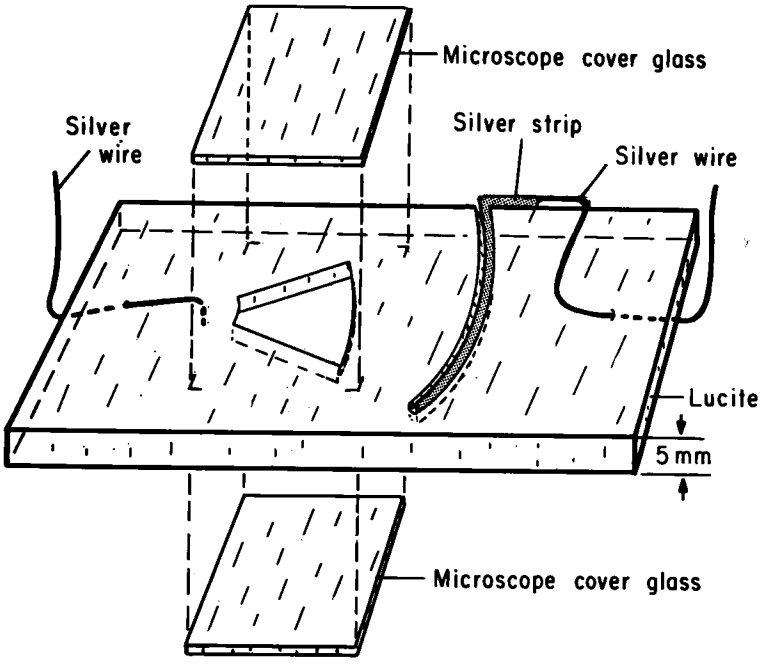


FIG. 13. Chamber used to produce a linearly changing nonuniform field. The chamber is constructed of lucite with a single point electrode projecting through it to its inferior surface. A curved plate electrode, extending similarly to its inferior surface is radiused to the point electrode. A chamber is cut out which subtends a segment of the circular sector formed by the 2 electrodes. In use, a microscope cover glass is attached with silicone or vaseline to the upper and lower surfaces of the chamber. The electrodes do not contact the solution and the current passed is limited to leakage current through the lucite.

As previously reported,[17] uniform fields with current densities of from 10–50 $\mu\mu$A/mm were ineffective in producing morphologic alterations. Alternating currents at 1 KC with 200 $\mu\mu$A density were also reported to be nonproductive of morphologic alterations. This portion of the study has subsequently been extended into the very low-frequency range. It was possible to produce the normal series of morphologic alterations with frequencies below 100 cps, although with much less efficiency than with equivalent current levels of direct current. The efficiency increased as the frequency was decreased until at 0.01 cps it was approximately 50 per cent of that at direct current (Fig. 14). The slope of the curve would appear to indicate that only a very low frequency (which in effect would be direct current) would approach the efficiency of direct current. We interpret these findings as indicating that the slower rate of change of the low frequencies can be sensed by the cells as direct current, but that the mechanism normally in operation within the cells utilized the in vivo direct current produced by the fracture.

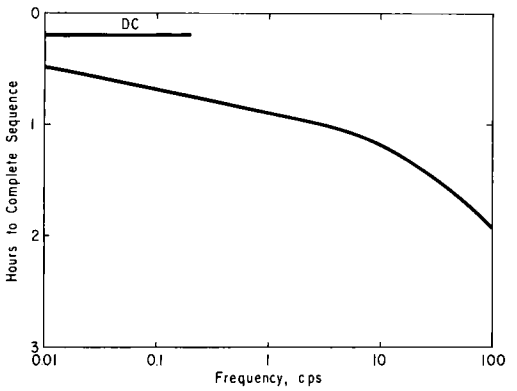


FIG. 14. Comparison of the efficiency of DC versus AC electrical fields. The efficiency is represented by the time required to complete the sequence of morphologic alterations within 1 mm of the electrodes. The current used was equal in all cases and optimal (500 $\mu\mu$A) for the chamber configuration. At DC approximately 15 minute exposure was required, while at 0.01 cps 30 minutes was required. With increasing frequency the efficiency decreased, linearly up to 10 cps and increasingly thereafter. It may be concluded that alternating currents can produce the cellular alterations but only as their frequencies are lowered to the point where they approach DC.

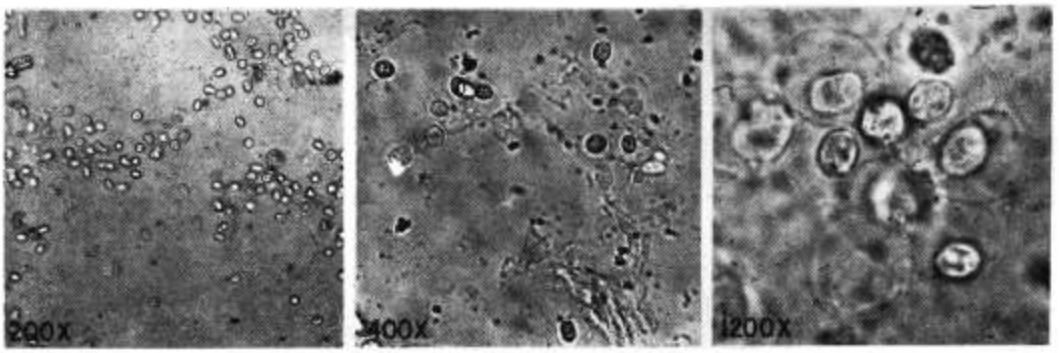


FIG. 15. Appearance of cells altered in chamber by exposure to optimal electrical fields and subsequently transplanted into cell culture medium. All illustrations were taken at 72 hours after beginning cell culture and all are bright field at the magnifications shown. The clumping and stranding are apparent at 200× and 400×. The latter demonstrates the strands of extracellular material observed at the 72 hour period. The 1200× illustration shows the persistence of cellular morphology and the alteration of the spherical outline of the cell membranes within the clumps.

We found it possible to maintain these morphologically altered cells in cell culture with various media for periods of up to 10 days. Cells having undergone the complete sequence of alteration were removed from the chamber and placed in a medium consisting of frog serum sterilized by passage through 0.8 μ millipore. These were maintained at 21 C in moist chambers with medium changes at 3 day intervals. The culture chambers permitted examination of the cells at all times with the inverted microscope. Initially, cell distribution within the chamber was random, but during the first 48–72 hours, migration took place with the formation of aggregates of the cells as clumps and strands (Fig. 15). During this period, extracellular material of an amorphous nature

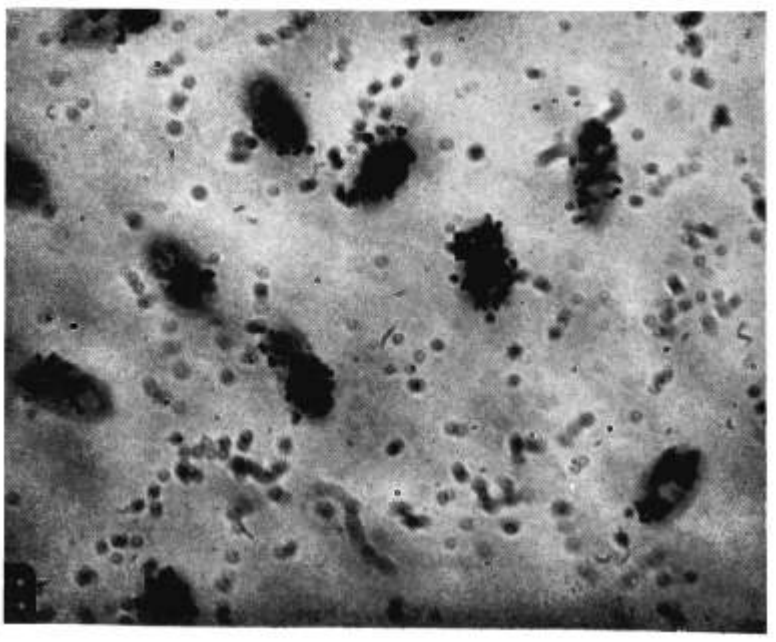


FIG. 16. Autoradiographs of uptake of mixed tritiated amino acids by altered frog erythrocytes. Normal cells were exposed to effective levels of electrical current in the lucite chambers for periods of 24 hours in amphibian Ringer's solution containing 1.0 μc/ml of the isotopic amino acids. Subsequent autoradiographs demonstrated a marked nuclear concentration of grain counts indicating a major uptake of the amino acid. Control cells incubated in the same solution for same period of time displayed no uptake in either cytoplasm or nucleus.

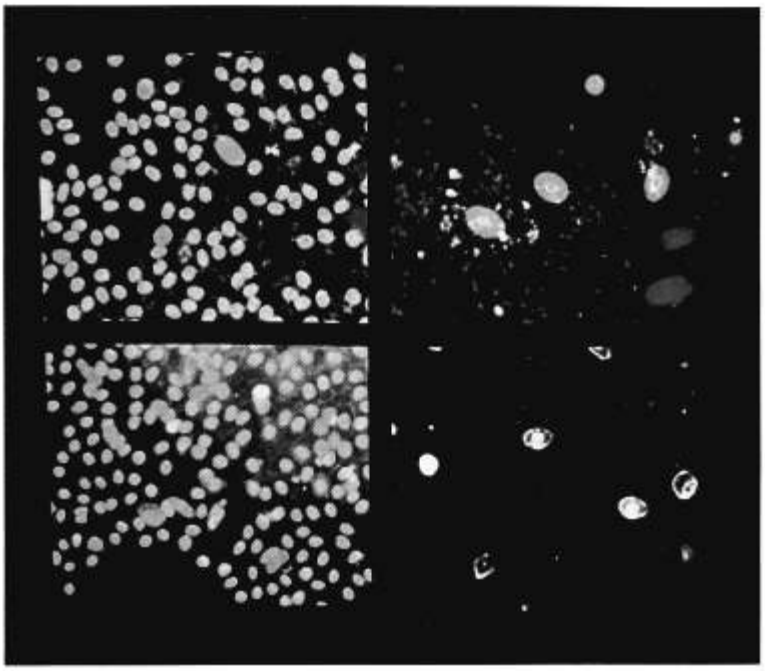
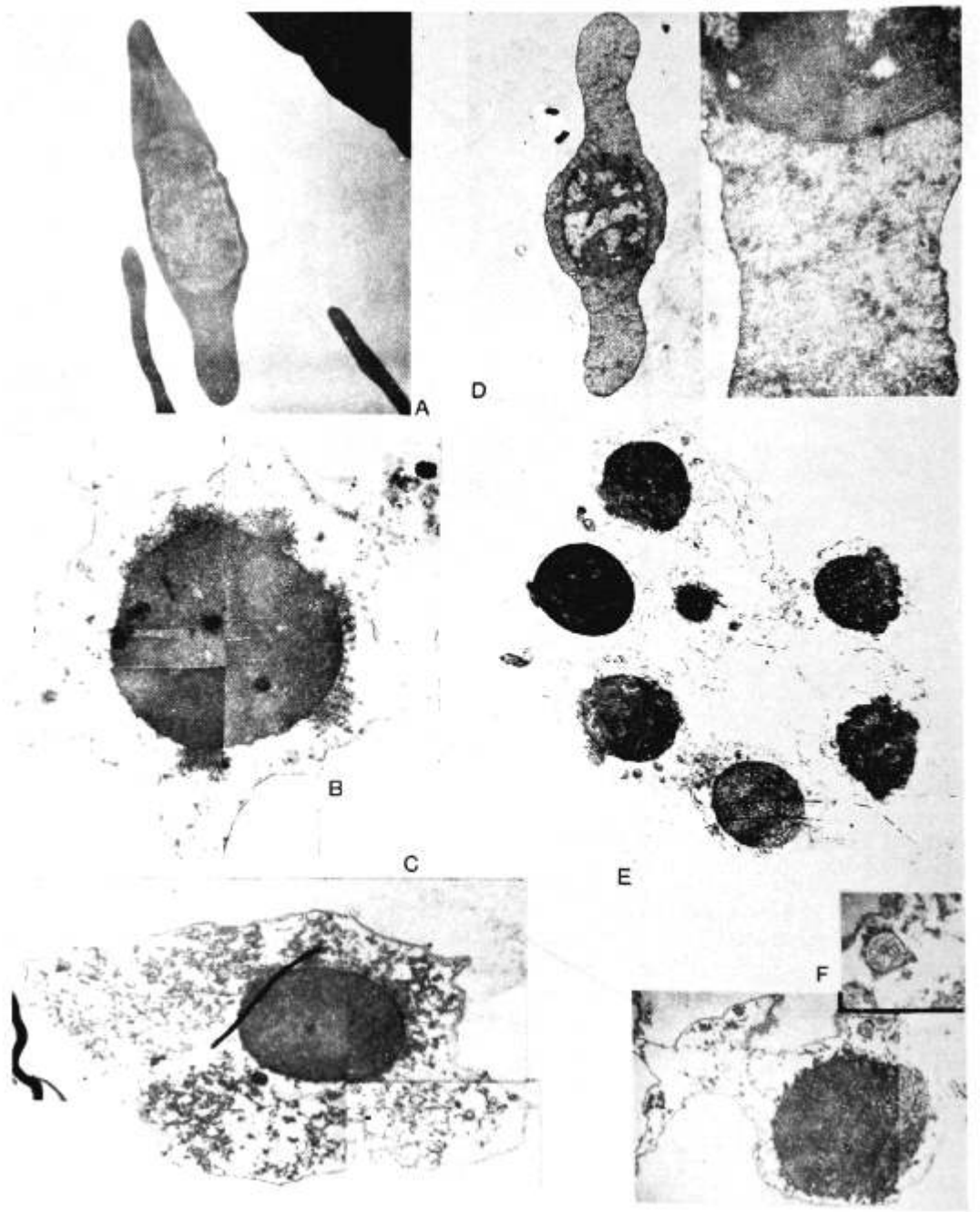


FIG. 17. Acridine orange stain of frog erythrocytes. *Upper left:* Smear of normal erythrocytes obtained by heart puncture. The yellow DNA fluorescence is visible in the nucleus, while the cytoplasm displays no orange RNA fluorescence. *Lower left:* Smear of frog erythrocytes from a 4 hour fracture hematoma. Several cells in the field demonstrate obvious orange fluorescence in the cytoplasm indicating an increase in the cytoplasmic RNA content. The altered cells have not changed shape due to clotting of the hematoma. Considerable fresh bleeding is unavoidable in such a preparation and many unaltered cells contaminate the smear. *Upper right:* Smear of frog erythrocytes removed from lucite chamber after exposure to effective levels of current. A mixture of cells in several stages may be seen. Initially there is a change from the yellow fluorescence in the nucleus to an intense red color indicating a marked increase in nuclear RNA content. This is followed by the appearancse of red-orange fluorescence in the cytoplasm, the nucleus retaining its red fluorescence or diminishing it slightly in intensity to a red-orange. The final stage is characterized by a retention of the cytoplasmic red-orange fluorescence and the reappearance of the yellow fluorescence in the nucleus. The final stage is typical of a highly active cell and should be contrasted with the normal erythrocyte similarly stained. *Lower right:* Smear of frog erythrocytes obtained after exposure of electrical current similar to the above but with pretreatment of the smear with ribonuclease before Acridine orange staining. The red-orange fluorescence is completely removed by this method, indicating that the original result was in fact due to an increase in RNA content.

(under light microscopy) appeared in the medium in the vicinity of the cellular aggregates. With serum sterilized by passage through 0.45 μ millipore serving as the culture medium, cells persisted as separate entities for 7 days and there was complete failure to form aggregates or extracellular material. Unfiltered, commercial fetal calf serum was also used as the culture media and the aggregation phenomena were observed, however, these experiments were not carried beyond 48 hours and no production of extracellular material was noted.

Some initial studies have been completed on the metabolic activities of these cells during and immediately following the exposure to electrical currents; these will be reported in detail at a later date. Of interest in the present context are the preliminary results of acridine orange staining for DNA and RNA and autoradiographic studies on the rate of protein metabolism. (Additional autoradiographic and cytochemic studies are ongoing and will be the subject of later reports).

Normal nucleated erythrocytes suspended in amphibian Ringer's containing 1.0 μc/ml of tritiated mixed amino acids (New England nuclear), were exposed to the usual current density and morphologic alterations produced. Subsequent autoradiographs demonstrated marked absorption of the amino



acids from the solution and concentration in the region of the nucleus (Fig. 16). Control cells under identical circumstances, except for lack of electrical current exposure, demonstrated no cellular accumulation of the amino acids. We conclude from this preliminary data that the electrically altered cells demonstrate a marked increase in metabolic activity compared to the normal unaltered cells in which metabolism is practically nonexistant.

Acridine orange staining was done by the method described by Gurr.[60] Normal erythrocytes suspended in amphibian Ringer's solution and exposed to effective levels of electrical current demonstrated 3 main stages

FIG. 18. Electron micrographs of frog erythrocytes exposed to electrical currents in vitro.

a. Normal cells obtained by heart puncture, diluted in amphibian Ringer's solution and treated similarly to the experimental cells except not exposed to electrical currents.

b. A cell that has reached the rounded clear stage demonstrating the appearance of small electron dense particles in the nucleus in numbers large enough to obscure normal nuclear detail. In several areas these particles appear to be extruding from the nucleus and the nuclear membrane is indistinct. The cytoplasm is relatively clear. The particles are presently interpreted to be ribosomes in process of being produced in the nucleus and extruded into the cytoplasm.

c. Cell in a stage slightly later than b. Ribosome production is continuing in the nucleus, but considerable numbers of polyribosomes have accumulated in the cytoplasm. The rate of extrusion from the nucleus is diminishing and areas of nuclear membrane are becoming visible.

d. A still more mature cell with a dense granular cytoplasm resolvable into apparent polyribosomes and with an active nucleus.

e. Cluster of several altered cells obtained from lucite chamber and subsequently maintained in all culture mediums for 48 hours. The typical expanded nucleus and branching pattern of the cytoplasms with multiple areas of close contact with other cells is evident. Several cytoplasmic organelles are visible.

f. A cell obtained from the same culture as e, demonstrating the same nucleus and cytoplasmic appearance with a well resolved organelle [see insert] which appears to be a vacuole filled sack. Similar organelles are seen frequently in these preparations—occasionally in close proximity to the nucleus. Organelles with the same appearance are visible but unidentified in Figures 17, 19 and 20 of limb blastema cells in Watson's study.[68]

of change by this technic (Fig. 17). First, loss of hemoglobin and expansion of the nucleus accompanied by intense red-orange fluorescence of the nucleus with minimal orange color to the cytoplasm. This stage was succeeded by a stage in which the red-orange fluorescence of the nucleus remained intense but the cytoplasm acquired almost equally intense red-orange color. The final stage demonstrated the return of yellow fluorescence to the nucleus with one or more orange nucleoli and prominent orange fluorescence in the cytoplasm. Similar stains of smears of each of these stages first exposed to ribonuclease[2] demonstrated complete lack of the red-orange fluorescence in any stage. Controls consisting of normal erythrocytes suspended in amphibian Ringer's solution for the same length of time demonstrated green-yellow fluorescence of the nucleus only. Presumably a small amount of RNA is normally present in the cytoplasm but any fluorescence is quenched by the hemoglobin.

We interpret these changes in the experimental cells as a rapid, massive production of RNA in the nucleus with subsequent migration into the cytoplasm and final establishment of an active cell with large amounts of RNA in the cytoplasm and a nucleus containing nucleoli.

Electron microscopy was done on cells immediately after the production of morphologic alterations by exposure to electrical currents and after 72 hours in culture media (fetal calf serum). The procedure utilized was removal of the cells from the chambers or culture dishes by pipette, suspension in gluteraldehyde fixative and centrifugation in a Pasteur pipette. The sedimented cell layer was subsequently stained, dehydrated and imbedded in the usual manner. Sections were cut at 600–700Å thickness. The resulting electron micrographs were both interesting and unusual and are reproduced here with only preliminary interpretation which understandably may be subject to change (Fig. 18).

Because of the considerable technical difficulties inherent in this method, only a few of the morphologic stages have been studied and a complete report will be made at a later date. In brief, the major changes appear to be in sequence; clumping and expulsion of the hemoglobin, expansion and ap-

pearance of activity in the nucleus, with massive ribosome production in the nucleus and subsequent migration of these units into the now empty cytoplasm. Intracytoplasmic organelles of as yet undetermined types appear in the cytoplasm by the end of 2 days in culture. (We have not yet evaluated the EM appearance of cells cultured for longer periods of time).

The changes that have been observed are similar to those described for the early blastema cells in amphibian limb regeneration[45] and closely resemble the cells observed in the early fracture hematoma (Fig. 2). The intranuclear ribosomal production and subsequent migration through the nuclear membrane into the cytoplasm are in accord with the observations reported by Mephan.[44] It should now be noted that autoradiography, cytochemistry and electron microscopy have all yielded compatible results when applied to these cells, interpretable as massive production of ribosomes initially in the nucleus with subsequent cytoplasmic migration.

We have made some additional observations on certain cyclic variations in this species of amphibian which have a bearing on healing processes and cellular responses to electrical factors (see appendix).

In summary, except for animals in the winter state, the minimal electrical parameters necessary to produce the described morphologic alterations in normal reactive amphibian erythrocytes in vitro appear to be: a nonuniform field pattern, a voltage drop across a single cell of 0.3 mv, and a current density of approximately 1 $\mu\mu A/mm^2$. The current should be steady direct current and the foregoing values are calculated on that basis.

The failure to produce the morphologic alterations in the uniform field chambers at any current level and in the nonuniform field chambers at total current levels in excess of 1,000 $\mu\mu A$, plus the production of cellular alterations at both electrodes were taken to indicate that the effect is not produced by ionic migration or gradients, or to other secondary chemical effects associated with the current. Other phenomena associated with metallic electrodes may be ruled out by the experiments using chambers with no electrode contact with the amphibian Ringer's solution.

Therefore, we postulated that the action of the electrical factors (field pattern and current density) was directly upon the cells themselves. The apparent requirement for a nonuniform field suggest that the dielectrophoretic effect may be involved in some fashion.[47] However, since cellular migration was not observed, the effect, if acting at all, must be upon subcellular units of some type. We have been unable to disassociate the effects of field pattern and density from current flow and the latter may be necessary for production of the phenomena. Since ionic movement does not appear to be a factor, one must consider the possibility that hydrated electrons are involved.[27]

Regardless of the physical mode of action of the electrical factors, a cellular mechanism of some sort must be activated. Initially, it was felt that the rapidity with which the morphologic change could be completed under optimal circumstances precluded its being the result of activation of nuclear DNA. It is generally considered that specific patterns of cellular proteins are governed by the DNA-RNA system being coded for the necessary pattern,[68] the specific code in action being referred to as a genetic operon. Therefore, to produce any major morphologic alteration such as dedifferentiation, a complex sequence of events would be required consisting of the repression of the currently functioning operon, the de-repression of a previously repressed operon, manufacture of newly coded transfer RNA (tRNA) and messenger RNA (mRNA) and the production of a new protein pattern by protein synthesis. However, Scott and Malt[56] have reported the presence of stable messenger RNA in nucleated erythocytes and their consequent ability to synthesize small amounts of new protein without the preceding DNA stages. Therefore, we postulated that stable messenger RNA, suitably

coded to initiate the dedifferentiation process, was present in these cells in the inactive state and that the action of the electrical current was to convert this from the inactive to the active form. Since the inactive form of this molecule may be considered to be folded or coiled and held in this configuration by hydrogen bonds while the active form is extended,[68] it is conceivable that a non-uniform field pattern accompanied by the availability of hydrated electrons could produce such a conversion. This hypothesis was available for testing to a certain extent through the use of puromycin and actinomycin D. The former selectively blocks protein synthesis by the messenger RNA, while the latter has a widespread inhibiting effect including blocking RNA synthesis by DNA.[23]

The addition of 4 μg of puromycin per milligram of the Ringer's solution in a chamber containing competent erythocytes completely prevented the appearance of the morphologic change despite the administration of effective levels of current. However, if the current is turned off and the puromycin diluted by several changes of the amphibian Ringer's solution, the cells will then begin to change and follow the normal sequence of morphologic alterations even in the absence of the current (however, the time required to complete the sequence is slightly increased, due probably to some residual puromycin). Apparently, the action of the current is in the nature of a switch (activation of inactive mRNA?) which may be turned on even in the presence of the metabolic blocker. If the blocking agent is subsequently removed, the action called forth by the switch closure will then take place.

Actinomycin D (18.25 μg/ml of amphibian Ringer's solution) also prevented the morphologic alterations but its action could not be reversed by subsequent washings. This result is to be expected since it has been reported that this agent is strongly bound to cellular elements.[23]

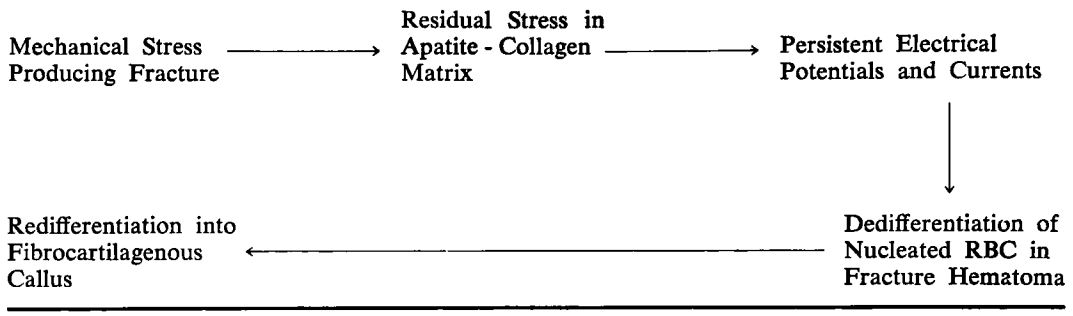
In view of the reported production of dedifferentiation (blastoid transformation) in mature mammalian lymphocytes by phytohemagglutinin (PHA),[52] it seemed of interest to determine the effect of this agent on amphibian nucleated erythrocytes. Utilizing the same technic, we found that PHA did produce a sequence of morphologic alterations in these cells similar to that produced by appropriate levels of electrical current. However, the change required approximately twice as long using PHA and only about 50 per cent of the cells altered completely.

The observed cellular alterations appear to be the result of activation of a naturally occurring mechanism and not due to toxic or deleterious effects of the electrical factors. The weight of the evidence presented (morphologic alteration, cytoplasmic basophilia, histo-chemical evidence of RNA production, increase in mitrochondria and ribosomes, protein synthesis as observed by autoradiography, blocking action of puromycin, the survival and behavior in cell culture, etc.) indicates that the exposure to the electrical factors results in the transformation of an almost metabolically inert, mature, well differentiated cell into a highly active, more primitive cell with a markedly altered function.

We conclude from these observations that the action of the electrical factors is directly upon the cells and consists of the activation of some unit, possibly a stable form of messenger RNA. This activation process results in synthesis of a new sequence of proteins and the morphologic process of dedifferentiation. It is of interest to consider this proposal in the light of Commoner's[25] criticism of the Watson-Crick hypothesis,[69] particularly in view of the extensive morphologic alterations in the nucleus that occur following the primary cytoplasmic changes.

GENERAL DISCUSSION

These observations on the relationship between the electrogenic properties of bone and the cellular phenomena basic to fracture healing appear to constitute a closed loop control system similar to that previously described for Wolff's law.[14] In perhaps over simplified forms, this is as follows:



Several aspects of this schematic diagram require additional discussion. First, as mentioned previously, our observations of the cellular mechanism for fracture healing in this vertebrate class vary with those reported by Pritchard.[49] However, in the original description of amphibian fracture healing, Wurmbach[71] discussed the role of the hematoma, describing some morphologic alterations in the erythrocytes within it and expressed concern for his inability to accurately relate their fate. Viewed as a regenerative process, our observations of a dedifferentiation mechanism as the source of the blastema do not vary with current, well-founded concepts.[34, 53] In addition, recent evidences have been presented indicating the ability of mature mammalian circulating monocytes to dedifferentiate into fibroblasts in wounds,[32] and a large body of data has been accumulated on the "blastoid" change produced in the small mature lymphocyte by exposure to phytohemagglutinin.[52]

One might speculate that immature cells of the erythroid series in mammalian hematopoietic tissue may be similarly capable of dedifferentiation provided they had not matured to the extent of losing their nuclei. If this is so, one would expect that fractures of bones having active hematopoietic marrow would heal at a more rapid rate than similar bones with inactive fatty marrow. Clinically, of course, long bone fractures in infancy have a rapid rate of healing as do rib and sternal fractures in adults. The differences between our data and that of Pritchard,[20, 48, 49] who reported healing of amphibian fractures by periosteal stem cell mitotic activity, may be resolved by considering the differences in experimental conditions. Pritchard maintained his animals at an abnormally high environmental temperature, while ours were kept at a temperature approximating that of the normal amphibian environment. However, it is interesting to speculate on whether 2 cellular mechanisms might be available, dependent upon the organism body temperature.

It should be noted that the electrical phenomena so far have been shown to be responsible only for the dedifferentiation process. The nature of the signal producing redifferentiation into fibrocartilagenous cells is presently not known.

We consider it inappropriate to discuss at any length in this paper the origins of the electrical potentials at the fracture site. A considerable body of literature has been presented in the last few years dealing with the origin of stress generated electrical potentials from bone and connective tissue.[1, 3, 11, 14, 15, 16, 31, 57, 58, 59]

In addition, there are several reports indicating a polarity-dependent bone growth response in vivo in response to the administration of low levels of DC electrical current.[4, 30, 39a, 46] While the exact source of the stress generated potentials in bone is evidently not clear, we interpret the results reported in this paper as mitigating against their being derived solely from the piezoelectric effect of collagen. The periosteal potentials seem to be clearly related to the innervation of the periosteum and it is our thesis that they represent a response to trauma on the part of the previously de-

scribed neural direct current control system.[9, 10, 12] Since the postulated control system signal is electrical in nature, one may relegate to the periosteal, neural related potentials a secondary regulatory role. Even without the nerves entering the hematoma, there would be, on purely physical grounds, an interaction between the periosteal electrical field and the field derived from the stressed bone matrix. Furthermore, the secondary increase in the periosteal potential noted some days after the fracture in amphibia—and possibly the source of the skin potentials observed by Friedenberg in mammals[29]—may have a further regulatory effect, although one cannot at this time rule out the possibility that it is a second order phenomenon resulting from the metabolic activities at the fracture site. Friedenberg's failure to observe a positive potential at the fracture surface is expected since all his measurements were made from the skin surface and reflect the periosteal potentials only.

In any event, the observations are consistent with the exchange of information between the periosteum and the fracture hematoma via interaction between their respective electrical fields. This obviates, at least in part, the paradox that while Singer has shown a very definite requirement for the presence of neural elements in regenerating tissues,[61] bone is basically aneural.[26, 60] If one may assign to the periosteal potentials a supraregulatory function over the local cellular electrical process of fracture healing then the observations of "over abundant" or unregulated callus formation in paraplegic patients[51] and the abnormal response to minor fracture in the denervated digits of patients with leprosy[21] may be somewhat understandable.

The production of cellular dedifferentiation in vitro by exposure to exceedingly small currents accompanying low-voltage, direct current, nonuniform electrical fields appears to have significance beyond that related to the healing of fractures. It must be emphasized that only the nucleated erythrocyte has been found to be capable of reacting in this fashion. Circulating amphibian leucocytes and mature epithelial cells have not been observed to display morphologic alterations under similar circumstances while other cell types remain to be investigated. In view of its role in fracture healing and observed in vitro reactions, the nucleated erythrocyte appears to function as a "target cell" especially susceptible to such electrical factors. Considering its general distribution and availability at sites of trauma, it may play a role in the healing of other wounds by responding to the current of injury associated with tissue trauma. It is interesting that a hematogenic origin of limb blastema in amphibians has been previously proposed, first by Colluci in 1884[24] and most recently by Ide-Rozas in 1936.[37] While the work of Butler and O'Brien[22] in 1942 was considered at the time to negate this thesis, subsequent works of Rose on the influence of irradiation on regeneration have indicated that it remains a possibility (see review Rose[53]).

Should the nucleated erythrocyte be shown to play a role in blastema formation, it would then fulfill the criteria for "complete" dedifferentiation as opposed to partial dedifferentiation which is followed by redifferention only according to the cells original specific genetic determination.[55] Other cells as well may possess this facility, both in the vertebrate classes evaluated and in the mammalia where nucleated erythrocytes are not generally available. It would seem obvious that these target cells must possess certain specific characteristics enabling them to undergo this major morphologic transformation under these conditions. We propose that these cells are specifically designated by the organism to perform this function when triggered by appropriate electrical factors in the local environment resulting from trauma. It should be noted that from a physical point of view, the effect of the electrical factors must be primarily upon the cell membrane and associated structures, or both, and that enhancement of the cell sensitivity to electrical factors by hormone changes must also

be at the same locus (see appendix). While we have presented some evidence indicating that at least part of the mechanism bringing about the internal cellular reorganization may be a stored inactive mRNA, additional factors must be present to produce first, the major nuclear reorganization noted, and second, the subsequent assumption of fibroblastic properties.

To transfer the observations to mammalia, one need only postulate that similar electrical factors at the fracture site stimulate the target cells, in this case the periosteal and endosteal stem cells (as well as possibly the associated hematopoietic marrow). The requirements for blastema formation in this class are no different than in the other vertebrate classes, and similar stimulating factors should apply. Bassett, Pawluk, and Becker[4] have previously reported in vivo stimulation of endosteal mitotic activity and subsequent formation of a bony callus in mammals with administration of small continuous electrical currents in a nonuniform field pattern from implanted devices.

Similar results have recently been reported by Friedenberg[30] and O'Connor.[46] While these experiments were aimed at substantiation of our thesis regarding the control system governing Wolff's law, the results are directly transferable to the context of the present paper. Therefore, we propose that the fracture healing process in all vertebrates is governed by basically the same control system. The stimulus for the initial cellular response, be it dedifferentiation of nucleated RBC's in the hematoma or mitotic activity in periosteal or endosteal stem cells, is electrical in nature and derived from the electrogenic properties of the bone matrix and periosteum.

Conclusions

Viewing the fracture healing process in the frog as part of the spectrum of regenerative processes demonstrates its relationship to the same process in the human. In both, a recognizable blastema is formed which leads to an initial soft callus and subsequent ossification. In both, electrical events occur simultaneously with the fracture and subsequent cellular healing and in both, the process serves a useful function for the organism. Furthermore, the frog should not be viewed as an organism having a generally higher level of regenerative competence than man; in fact, the frog represents a highly specialized amphibian, much in the same way as man represents a highly specialized mammal. The regenerative abilities of the adult frog closely parallel those of man being generally limited to tissue regeneration and completely lacking any ability of limb regeneration under usual circumstances.

The observations reported in this paper appear to have some relevance for regenerative processes in general. The demonstration of an electrical control system initiating fracture healing lends support to the previously reported thesis that limb regeneration is also regulated by an electronic system.[6] It is particularly interesting that Smith[62] has recently reported the stimulation of limb regeneration in the frog by the implantation of small electrogenic bimetallic couplings in the amputation stump. Regeneration occurred only if the orientation of the coupling was such as to enhance the distal negativity and was lacking or much decreased with all other orientations. These were relatively crude devices and could do no more than enhance or diminish the electrical potentials at the amputation site. The process of limb regeneration is complex and undoubtedly requires sophisticated information and control systems throughout its course.[53] However, the data reported in this paper, plus Smith's observations, would seem to indicate that the initial or primary stimulus for this process may be a relatively simple electrical field requiring only a minimal current level acting upon a population of susceptible cells. Its function would be to produce the cellular responses necessary for blastema formation and possibly to activate the control system governing the subsequent events. The subsequent control over the regenerate may be related to the DC potential system of the central nervous system, e.g., the possible

regulatory function of the periosteal potentials over fracture healing.

The entire regenerative process would, therefore, require 2 control systems, an initial simple stimulus signal derived from the direct effects of the trauma and a second complex control system, neurally based, relating the regenerate to the host organism.

If this view is correct, which system is missing or inadequate in those organisms lacking limb regenerative ability?

Singer's demonstration of a minimal mathematical ratio between nerve and total tissue mass necessary for regeneration[61] need not indicate that the second control system is at fault. Certainly Smith's results indicate that in frogs, it is the initial simple stimulus that is inadequate, and once this is restored an adequate secondary system is furnished by the normal innervation of the limb. While the sectioning of the long bone attendant to amputation undoubtedly is productive of some electrical effect based upon the properties of the bone matrix, a major source of the amputation potential must be derived from the current of injury from the other tissues at the amputation site. In this light, the observations of Zhirmunskii[72] on a direct relationship between the current of injury and the innervation of the part appear to be most important. Therefore, Singer's data may be interpreted as indicating an inadequacy of the primary stimulus resulting from an increase in total tissue mass relative to nerve mass in the limb in the mammalia. One might, therefore, logically infer that restoration of limb regeneration to this vertebrate class may be accomplished by properly augmenting the local electrical phenomena secondary to the trauma and simultaneously enhancing the capability of the cells to respond to this factor by the proper manipulation of hormone levels.

SUMMARY

The authors report in this paper the identification of the factors inducing and regulating the cellular processes responsible for fracture healing in the amphibia. The cellular events in the fracture healing sequence in this animal were studied by several technics. It was concluded that the nucleated erythrocytes (the normal circulating cell in all vertebrates except the mammals) in the fracture hematoma undergo a sequence of morphologic alterations similar to dedifferentiation and that they subsequently redifferentiate as fibrocartilage cells responsible for the initial fracture callus.

The electrical effects produced by fracture of the long bones in this animal were studied on the bone itself and on the intact periosteum. The fracture was shown to produce measurable electrical potentials on both structures. The bone potentials approximated an opposed dipole in an idealized subperiosteal fracture, while the periosteal potentials were longer in duration and appeared related to both the underlying fracture and the periosteal innervation.

The current flow through the hematoma was calculated and application of similar current densities and field configurations to normal nucleated erythrocytes in vitro produced a similar sequence of morphologic alteration. Both the in vivo and in vitro cellular events were studied by a variety of technics and it was concluded that both processes represented a true cellular dedifferentiation produced by exposure to specific but low levels of electrical currents. The cellular mechanism effected by the electrical parameters is theorized and its relationship to regenerative processes in general is discussed.

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APPENDIX

The frog, being a poikilothermic animal, normally enters a hibernating state during the winter months in the north temperate zone. While it is commonly assumed that frogs kept at normal temperatures in the laboratory during the winter months are physiologically equivalent to summer frogs, it is known that such animals respond differently in clinical pregnancy testing[36] and alterations in the central nervous and cardiovascular systems have been noted.[13] Therefore, it may well be that these animals, even though kept normothermic, are in an altered hormonal state during the time of their usual hibernation. However, they should not be assumed to be metabolically inert since the female engages in active oögenesis during the hibernating state. A further complication is the fact that generally the frog engages in one major episode of hematopoiesis during the year, this being purportedly at the time of emergence from hibernation.[28] Therefore, throughout the yearly cycle starting from the spring emergence, one encounters progressively older erythrocytes in the peripheral blood.

The healing of fractures and the responses of the normal erythrocytes to electrical currents in the winter frog reflect a similarly complicated situation. In general, the healthy frog during the period from mid-December to mid-March heals its fractures much more slowly than during the remainder of the year and his erythrocytes are correspondingly much less responsive to electrical factors in vitro. In the latter instance, changes are so slow as to be masked by bacterial overgrowth, anoxia and other artifacts. However, the winter frog kept in the laboratory under normothermic conditions is much more susceptible to infections and parasitic diseases and blood stream infestations with trypanosomes, and protozoa are common. These appear to be associated with some measure of active hematopoiesis and the resulting younger erythrocytes in the peripheral blood will respond to the electrical factors in vitro. Whether the unresponsiveness of the remainder of the red cells is related to physiologic alterations attendant to the "winter" state or to the age of these cells is not known at this time. However, a few individuals near the end of hibernation were noted to have erythrocytes that responded to high-electrical current levels. These animals did not appear to have engaged in hematopoiesis and therefore, the responding cells were considered to be "old" erythrocytes. On this tenuous basis, one can speculate that red cell age is related to a decreased sensitivity to electrical factors, and that the nonresponding red cells in hibernation are the result of factors other than age, possibly hormonal in nature.

A different situation is revealed by the female frog in the winter state. Apparently at certain stages of oögenesis, the sensitivity of all red cells in the peripheral blood to electrical factors is markedly increased, with a corresponding decrease in the value of the upper level of effective current. In preparations from these animals, we observed cellular alterations proceeding to completion in control chambers through which no externally generated electrical current was flowing. These alterations occurred in the area where the glass cover slip and the plexiglass chamber were in close approximation. We have determined that these changes were not induced by light, oxygen lack, mechanical disturbance or exposure to glass or plastic surfaces alone or to the vaseline used to seal the chambers. We theorized that these alterations were produced by a fixed electrical charge differential between the glass and plastic and performed the following experiment.

Three chambers were run simultaneously using diluted heart blood from the same animal, each exposed to a different current level from the same electrical supply. This was accomplished by arranging the chamber circuits in parallel each with a different value of resistance added to it. Under these circumstances, the total current in the entire circuit is divided into various amounts for each chamber according to Ohm's law (i.e., a total current of 99.9 $\mu\mu$A may be divided into 90 $\mu\mu$A in one chamber, 9 $\mu\mu$A in another, and 0.9 $\mu\mu$A in the third). Erythrocytes from females in this stage were found to demonstrate complete morphologic alterations throughout the entire 0.9$\mu\mu$A chamber in the usual time period, while those in the 9 $\mu\mu$A chamber altered somewhat but not completely, and those in the 90 $\mu\mu$A remained unchanged. The latter chamber in fact demonstrated less morphologic alterations than the control chamber with no external current. At this time we do not know whether this markedly increased sensitivity in females during oögenesis reflects some hormonal pattern or is the result of a wave of active hematopoiesis. When active hematopoiesis begins in the spring, both male and female frogs have smaller than normal RBC's, which are susceptible to a wide range of currents, centered in the 10–100 $\mu\mu$A range, the final clear cell being normal in appearance, only slightly smaller.