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The Proliferative Capacity of Osteoblasts During Fracture Repair and a Comparison of DNA Synthesis in Normal and Hypophysectomized Rats: An Autoradiographic Study

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THE PROLIFERATIVE CAPACITY OF OSTEOCLASTS DURING FRACTURE REPAIR AND A COMPARISON OF DNA SYNTHESIS IN NORMAL AND HYPOPHYSECTOMIZED RATS:
AN AUTORADIOPHIC STUDY

By

Joseph Thomas Nichols

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

June
1967
Joseph Thomas Nichols was born in Pittsfield, Massachusetts on July 15, 1933.

He was graduated from Herbert Hoover High School, San Diego, California, June 1953. He then spent a two year tour with the U.S. Army and was honorably discharged in January 1956. From 1956 to 1958 he attended San Diego City College and State College, majoring in Zoology. He graduated from St. Louis University School of Dentistry, June 1962, with the degree of Doctor of Dental Surgery. He was accepted for an Oral Surgery Internship, Metropolitan hospital, New York City, and following one year in that position, he began his graduate studies at Loyola University School of Dentistry, Chicago, Illinois. After one year in the graduate school, he was accepted to a two year residency at Cincinnati General hospital, Cincinnati, Ohio.

He returned to Loyola University School of Dentistry on a Teaching Fellowship in Oral Surgery July 1966. And, in June 1967, he completed his thesis and was awarded the degree of Master of Science in Oral Biology.
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CHAPTER I

INTRODUCTION

The identification of a cell type while the cell is preparing for or in mitosis and the transformation of a cell of one type into other cell types can be deduced if the precursor cells can be identified by a permanent tag that labels these cells during subsequent transformations. Tritium labeled thymidine specifically labels deoxyribonucleic acid during the period of chromosomal replication.

The principal aim of this study is to demonstrate autoradiographically, the proliferative capacity of the osteoblasts which develop following mechanical fracture of the rat fibula. Also, the baseline of frequency of labeled cells in the osteogenic layer of the periosteum following fracture in the normal rat is used to compare DNA synthesis in the hypophysectomized rats.

An attempt will be made to gather further information concerning the osteogenic potential of the outer layer of the periosteum.
CHAPTER II

REVIEW OF LITERATURE

Thymidine was tritiated at Brookhaven by Hughes in 1955 and independently in Belgium. It has since become an invaluable adjunct to the study of cell dynamics. The compound is diluted in physiological saline solution to a concentration suitable for injection.

McGrath (1965) and Hinrichs (1964) state that thymidine is a specific precursor of DNA and is incorporated during the chromosomal (early prophase) replication phase previous to mitosis. Quastler (1959) reports that the compound may be rapidly incorporated into cell nuclei within a few minutes after intraperitoneal injection, and saturation is reached in approximately twenty minutes.

Messier & Leblond (1960) confirmed the findings of Quastler. They report that after intravenous injection of tritiated thymidine into man, plasma radioactivity fell rapidly to a low level one hour later. And, after subcutaneous injections, plasma radioactivity reached a peak in 20-30 minutes and decreased rapidly thereafter. This rapid metabolism implies that tritiated thymidine is available to the tissues for only a brief period; and any autoradiographic reaction found even months later must be due to radioactivity incorporated during the first hour after injection. Steel (1965) found that all but a few percent of tritiated thymidine injected into a rat appears within one hour either
incorporated into DNA or degraded to tritiated water. He also offers a hypothesis for the reutilization of tritiated thymidine.

Leblond (1959) states that because of the low beta energy (0.018 MeV) of tritiated thymidine, the photographic grains produced are found within 2-3 micra, and most are found within one micron from the source as measured with NTB-3 emulsion.

Toto (1964) on the other hand places the maximum range at approximately seven micra, although the average lies between 1.5 to 3.0 microns, and results in excellent resolution. He states further that resolution depends on a number of factors, the most important being the grain size, thickness of the specimen, thickness of the emulsion, and the distance between the emulsion and specimen. Caro (1962) and Taylor (1963) are in agreement with Toto but elaborate that the size of the silver halide must be small.

Kisieleski (1961) in his study correlating grain counts with varying concentrations of tritiated thymidine showed that in a tissue section three micra thick, from 100 to 200 disintegrations per minute (dpm) are required to render developable one silver halide grain in the emulsion overlying the labeled locus.

That tritiated thymidine is attached during synthesis in the six position and in the 5-methyl group was shown by Evans (1965). The decomposition rates for thymidine-5-methyl T., and for thymidine-6T at comparable specific activities are respectively 0.5 - 1.0 percent and 1.0 - 2.0 percent per month. This rate increases when the solutions are stored below zero degrees centigrade.

Decisive proof was obtained by Amano (1959) to show that labeled thymi-
Dine was taken up into DNA by treating sections from tritiated thymidine animals with the enzyme deoxyribonuclease to remove the DNA present. No autoradiographic reaction at all could then be found. They concluded therefore, that the only radioactive substance present after tritiated thymidine injection was DNA.

Autoradiography is a method for detecting radioisotopes, based on their ability to affect the silver halide crystals of the photographic emulsion, primarily the bromides. Such crystals act as micro-detectors of radiation and are useful in visualizing locations of radioelements. The resultant autoradiographs consist of accumulations of black silver granules overlying those areas in the tissue section which contain the radioactive material.

It is to be assumed that the chemical behavior of the labeled substance in this study, tritiated thymidine, is identical with that of its stable counterpart. This will also be true biologically if the amount of the isotope administered is small enough to prevent a harmful radiation effect. Fry(1961) and Young(1962) are in agreement that the cellular damage is insignificant at dosages of one microcurie/gram body weight of mice and rats.

Leblond et al(1959) recommends the use of tritiated thymidine as an investigatory tool and rejects the use of $^{35}$S and $^{14}$C on the basis of poor resolution. Also, the doses of $^{35}$S and $^{14}$C required to produce the desired effects may be high enough to produce radiation damage. They state that with tritiated thymidine the doses used and the radiation thereby produced causes no visible radiation damage even after months in the body.

Lajtha(1959) states that $P^{32}$ has a half life of only fourteen days, con-
sequently a problem of potency during storage arises. To the opposite end, $^{14}\text{C}$ has a half life of 5,600 years. More ideally placed between the above two is thymidine with a half life of 12.26 years with a disintegration rate of 0.016 percent per day.

Tonna (1960) in a study similar to the present thesis with a few exceptions, mechanically fractured the right femur of albino swiss mice of varying ages, and sacrificed them at 24 hours, one week, and two weeks post-fracture. One hour prior to sacrifice a subcutaneous injection of tritiated thymidine was given. The dosage was 0.5mc/gram body weight. A number of observations were made:

1. Osteogenic cells of the periosteum were most frequently labeled.

2. The number of labeled cells decreased with increasing age.

3. Twenty-four hours after fracture there appeared an increase in the population of labeled cells, even in mice 52 weeks old.

4. Labeled cells were most numerous when osteogenic or chondrogenic cells from the periosteum were actively converting the fracture callus into a cartilaginous mass.

He concluded that the potential for fracture repair resides essentially in the proliferative capacity of the cells of the periosteum, which diminishes with age. This reduced capacity accounts for the reduction in the rate of fracture repair in older animals.

In a more recent study Tonna & Cronkite (1962) using tritiated thymidine observed that the labeling index of the osteogenic cells of the periosteum in old mice respond, following trauma, in a similar manner to that in young cells.

According to Enoch (1962) the periosteum consists of an outer layer of
collagenous fibers in which most of the cells are fibroblasts, and an inner layer (cambium) or osteogenic stratum composed of single fusiform cells in the resting state. This latter becomes a multicellular layer of plump osteoblasts when trauma such as a fracture occurs. Bassett (1962) continues by saying "It is generally agreed that an active osteoblast is responsible for the manufacture of bone." He concludes however, that despite the conflicting views in the past, concerning the osteogenic potential of the fibroblast, there seems to be definite evidence that undifferentiated connective tissue cells have a latent capacity to specialize as osteoblasts under appropriate environmental conditions.

Pelc & Gahen (1959) claimed the frequency of labeled nuclei in the epithelium of the seminal vesicles of tritiated thymidine treated mice greatly exceeded that expected from the low mitotic index. They claimed that the ratio of the labeling index over the mitotic index was eighty-eight and felt that DNA turnover must have occurred outside the duplication stage.

Messier & Leblond (1960) in a similar study, showed the results of an average ratio of 6.7 percent of seminal vesicle epithelium in rats, and 6.5 in mice. Both of these figures are in the normal range found from other tissues. They concluded therefore, in total disagreement with Pelc and Gahen, that premitotic DNA synthesis must account for the labeling. Further, they concluded that the mitotic index is not altogether satisfactory to assess the rate of cell proliferation in a tissue. Similar difficulties were encountered when attempts were made to use the radioactive index soon after tritiated thymidine injection, as an indicator of the rate of cell proliferation.
theless, the radioactive index has two important advantages:

1. The cells tagged by tritiated thymidine can be traced long after mitosis has been completed.
2. Labeled cells are more readily identified than mitotic figures.

Using this method, one can identify sites and roughly estimate rates of cell proliferation, and follow the fate of the tagged cells.

Odartchenko (1964) in a study to evaluate the mitotic time in vivo using tritiated thymidine agrees that the use of the percentages of labeled versus nonlabeled cells to the exclusion of grain counts eliminates the uncertainties of quantitation of tritium activity in the autoradiograph.

Tonna & Cronkite (1961) in an attempt to determine the original cell types participating in fracture repair, and to find out whether a common progenitor cell exists, studied cell proliferation in the periosteum of intact and fractured femora of mice. One hour before sacrifice, tritiated thymidine was injected (flash labeling). This method gives one an estimate of the proliferative rate at that instance. He also observed that pre-osteoblasts are "flash" labeled more frequently than osteoblasts or fibroblasts. Osteocytes do not "flash" label. At twenty-four hours the fraction of cells labeled in the periosteum was greater throughout the entire shaft than at the fracture site.

One week after fracture, the labeled cells were most prevalent at the fracture site, but were much less than at twenty-four hours. From his studies he concluded that it is unlikely that fibroblasts produce osteogenic cells.

Tonna & Cronkite (1961) in a study to determine periosteal proliferative rates sacrificed fourteen rats of the Sprague-Dawley strain one hour after injection of tritiated thymidine (0.5mc/gram body weight). They reported that
there was no increase in proliferation from one to eight hours; however, be-
 tween eight and sixteen hours there was a significant increase in labeling in-
dicating that between this time cell proliferation had been stimulated by some
factor associated with the fracture(mid-diaphyseal). Thirty-two hours after
fracture, labeling of the periosteal cell population increased to a maximum of
25.4 percent. As was found in a previous study Tonna(1961), the labeled cells
were found throughout the entire diaphysis, and not limited to the fracture
site. Beyond thirty-two hours the labeling index began to decrease rapidly,
whereas a less rapid decrease in labeling index occurred in the area of the
callus. Even after fourteen days where cartilage was being replaced by bone,
intense labeling could be seen, and this picture continued until fracture re-
pair was complete.

No evidence was obtained in this study to confirm a significant role of
the subcortical endosteum in internal callus formation. Osteogenic cells with-
in the intertrabecular spaces and the osteoblasts lining the trabeculae were
heavily labeled. It is the impression that these cells form the internal cal-
lus.

Tonna(1961) in another study concerning normal bone growth, observed
that the pre-osteoblasts of the periosteum were frequently seen labeled, in-
dicating DNA synthesis. Occassionally, osteoblasts were seen labeled. In a
previous study a high percentage of osteoblastic labeling was observed after
fracture(Tonna & Cronkite 1961).

In appraising the success of hypophysectomized rats, Hall(1961) recom-
mends the operation be judged by testicular atrophy. Later, one sees"that
the metabolism of the ground substance seems to have been interfered with in such a way that its normal maturation with age has been arrested.

In order to maintain hypophysectomized rats or mice in a state of homeostasis, El Bolkainy (1963) suggest a simple protocol. The animals can be maintained well for short periods (two weeks) on Purina laboratory chow, and given 5% dextrose and water in 1/4% normal saline rather than plain drinking water. Survival is better if the room temperature is kept between 80 - 83 degrees F.

If longer periods of survival are required, hormonal replacement would probably be required.

Belanger (1960) reported an increase in mitotic activity in the gastric epithelium of hypophysectomized rats. This is a most unreasonable observation since after pituitary excision, general body growth ceases and all other cell types which have been studied to date divide more slowly.

In a similar study Clark (1963) in his observations could not confirm the previous results. His observations were in line with what is generally expected, (i.e.) general body growth ceases and cell types divide more slowly.

Leblond & Carriere (1955) in studying the effects of thyroxine on the mitotic rate of hypophysectomized rats reports that mitotic activity is significantly lowered in the intact animal. However, growth hormone was shown to accelerate mitosis.

Bois et al (1963) in a similar study to that of Leblond and Carriere with the exception that the former studied the epiphyseal cartilage and the latter the intestinal mucosa, agree with the finding of Clark and summarized; Hypophysectomized rats had a smaller cartilage plate and a lower mitotic index,
and that growth hormone significantly stimulated mitosis.

Reichlin (1964) has obtained similar results as Bois without performing hypophysectomy on the experimental animals. His method is to administer thyroxine which depresses the thyroid and simulates glandular inactivity.

Finally, Johnston & Doiss (1965) offer the hypothesis that endochondral and intramembranous bone respond differently to hypophysectomy. Male rats (85-95 grams) which were hypophysectomized, received a total of 225 units of parathyroid extract and were sacrificed. One-half of the calvarium was incubated with $^{14}$C-proline and the other half with $^{14}$C-glucose. The specific activity of hydroxyproline and hexosamine was determined after incubation. In a similar study using metaphysial bone, the specific activity of hydroxyproline and hexosamine of cartilage was reduced. In the former study, the specific activity was increased in the calvaria. They concluded that cartilaginous and membranous bone respond differently to hypophysectomy. And, they suggested that membranous bone of the calvarium grows primarily by cell division, consequently, a measured quantity of bone from either hypophysectomized or control animals could contain an equal number of cells and synthesize an equal quantity of hydroxyproline.
CHAPTER III

METHODS AND MATERIALS

Twenty-four mixed set young albino rats of the Sprague-Dawley strain were used. Their weights ranged between 80-90 grams each. Twelve of the animals were normal and were used as control specimens; while the other twelve were hypophysectomized by Hormone Assay Laboratories, and were used as the experimental animals. The rats were initially separated into two groups: (1) normal and (2) hypophysectomized. The animals were littermates and housed in wire bottom cages. The diet of the normal rats was Purina Lab chow and drinking water ad libitum; whereas the hypophysectomized animals were maintained according to El Bolkainy(1963) and the survival periods lasted throughout the entire study which required approximately two weeks.

The right fibula of each rat was mechanically fractured with digital pressure under inhalation ether anesthesia and then returned to their respective cages. The animals were sacrificed at six, twelve, eighteen, twenty-four, thirty, thirty-six, forty-two, forty-eight, fifty-four, sixty, sixty-six, and seventy-two hours after fracture (consult Table I for Protocol). One hour prior to sacrifice, each animal was injected intraperitoneally with 50μc of tritiated thymidine (sp. act. 1.9c/μM) at the rate of approximately 0.5μc/gram body wt. This process is known as "flash" labeling. Sacrificing the animals was done by the technic of ether suffocation in a Bell jar and immediately
following expiration, the right leg was severed at the proximal joint of the femur, the skin and gross muscle were removed, and the specimen was then placed into 10% buffered formalin and fixed for twenty-four hours. At this time all fractures were confirmed by x-ray (Figure 1). The specimen was then washed for twenty-four hours under running tap water and the bones were decalcified in a 10% solution of disodium versenate and the tissue samples were embedded in paraffin and sectioned at three to six micron, mounted on glass slides, dipped in MTB-3 liquid emulsion and allowed to air dry. They were then exposed for twenty days in a sealed container at four degrees centigrade, developed, washed, fixed and stained with hematoxylin and eosin. Staining was deferred until after the development of the autoradiographs in order to eliminate leaching out of stain from the tissue sections. This method of preparing autoradiograms is essentially according to Joffes (1959).

Upon examination of the autoradiograms, those slides or areas which were obviously high in background grain counts were either discarded or disregarded. Background grains are due to tritium being metabolized and incorporated into other compounds, to cosmic rays and to processing artefacts. All observations were made at one-thousand magnification using oil immersion lens and significantly labeled representative cells of the periossteum were recorded by means of photomicrographs. The number of labeled osteoblasts as well as non-labeled osteoblasts along the periossteum of the diaphysis of the fractured fibula were counted from each section made. The labeling index was determined by counting the number of labeled cells of the osteogenic layer of the perios-
trum per one-thousand periosteal cells. A table of the labeling indices and the percentage of cells labeled demonstrates the range of proliferative capacity (Table II). A distribution curve illustrates graphically, the spread and comparison of synthesizing DNA (Figure 2).
CHAPTER IV

RESULTS

The extent of radiolabeling of normal (control) and hypophysectomized rats each receiving 50μc of tritiated thymidine intraperitoneally one hour before sacrificing is well illustrated in the photomicrographs (Figures 3-22). The results of cell proliferation in the normal animals coincide very closely to the results in a similar study by Tonna and Cronkite (1961). The "flash" labeling technic was used to prevent cells undergoing mitosis from dividing and therefore the proliferative rates reported gives a good estimate of DNA synthesis at time zero.

The six and twelve hour animals exhibited the same reaction to trauma as did the remaining animals (i.e.) a thickening of the osteogenic layer of the periosteum along the entire diaphysis (Figure 3). Labeled cells were almost absent with the exception of the finding of one labeled cell in each the six and twelve hour specimens (Figure 4).

Somewhere between 12 - 18 hours cell proliferation (DNA synthesis) has been stimulated by some factor(s) associated with the fracture, because the labeling index has increased to 0.250 from that of the twelve hour specimen of 0.001. Noted also at this time interval is definite evidence of a fibrous callus developing. Labeled cells are found in the osteogenic layer of the periosteum along the entire diaphysis (Figure 5).
In the 18 hour hypophysectomized animals the results are similar to the control with one exception. The proliferative capacity is reduced significantly and this can be observed by comparing Figures (5 & 6). Clark & Bois (1963) in separate studies report that general body growth ceases and cell types divide more slowly in hypophysectomized animals than in normal animals. The general results concerning DNA synthesis seem to corroborate their findings. In (Figure 6A) the osteogenic layer of the periosteum is greatly thickened. The cells are noted to be round and plump and highly labeled by tritiated thymidine (Figure 6B). Skeletal muscle can be observed to be void of label as are also the majority of the fibroblasts of the outer layer of the periosteum.

Peak labeling occurred at 24 hours in the control animal. In (Figure 7B) numerous labeled periosteal cells are observed and also among the osteoblasts are noted groups of macrophages. The fracture segments (Figure 7A) are in good apposition.

Although all mechanical fractures of the experimental animals were confirmed post-sacrifice by x-rays (Figure 1), sectioning the specimens was not always easily executed by the technician in order to expose the fracture site. When this occurred, thickening of the osteogenic layer of the periosteum was used as the criteria for the site of injury and radiolabel counts were made in these regions. This was the case in the 24 hour hypophysectomized rat (Figure 8A). Many labeled periosteal cells and unlabeled macrophages can be seen in (Figure 8B). The fibrous layer of the periosteum is well defined with many spindle-shaped and fusiform, unlabeled fibroblasts.
In the 30 hour control animal (Figure 9A) a displaced fracture with a considerable amount of interfragment space is noted. In various regions between the fragments there are some indications of osteoclastic resorption. Again numerous labeled cells can be seen in the osteogenic layer of the periosteum. For the first time labeled fibroblasts are seen, although they are few in number (Figure 9B).

There appears to be no great difference between the 30 hour normal and the 30 hour hypophysectomized animals; again however, the proliferative rate in the hypophysectomized animal is decreased by about five percent. In (Figure 10A) there is a suggestion of a Howship's lacunae containing osteoclasts. In (Figure 10B) scattered labeled osteoblasts are easily seen.

The photomicrograph (Figure 11B) of a 36 hour control animal shows an extensive amount of labeling. Labeled cells of the inner layer of the periosteum are found and plump suggesting that the cell is synthesizing DNA. Fibroblasts in the fibrous layer are noted to be labeled and their shapes to be characteristic.

Again the only apparent difference between the control animal and the 36 hour hypophysectomized rat is the proliferative capacity. In the latter it is decreased by the same value as in the previous example (5 percent). Labeling is heavy and diffuse (Figure 12B).

In the 42 hour control animals the fracture is seen to be oblique and in good apposition (Figure 13A). Numerous labeled cells of the osteogenic layer are observed. Fusiform and spindle-shaped fibroblasts are also noted to be
radiolabeled; they are however, again few in number. What appears to be labeled osteocytes are on closer observation several osteocytes within their respective lacunae overladden by grains due to radiation.

The cambium layer in the 42 hour hypophysectomized rat is greatly thickened; also the fibrous layer of the periosteum is increased in width, this latter is probably indicative of the developing fibrous callus (Figure 14A). Typically labeled osteoblasts are illustrated in (Figure 14B). It should be mentioned that from the peak labeled animal, the proliferative rate has steadily fallen off and in the 42 hour hypophysectomized animal the labeling index is 0.142 or 14.2 percent labeling.

Fractures are noted to be in excellent apposition in the 48 hour normal and hypophysectomized animals (Figures 15A & 16A). Labeled cells of the cambium layer are definitely decreasing in number if compared with the previous micrographs (Figure 15B & 16B).

Figure (17A) exhibits a characteristic fracture with good alignment of the fragments and to be noted is a very thick periosteum both inner and outer layers (compare with Figure 3). Labeling of the 54 hour control animal is excellent, again, however, there is a decrease in the number of labeled cells. A point of interest is a well-developed lacunae with anastomosing canaliculi (Figure 17B).

Large plump and infrequently labeled osteoblasts of the cambium layer are demonstrated in the 54 hour hypophysectomized animal in (Figure 18B). Also noted was a greatly increased fibrous layer of the periosteum.

The microscopic finding of the 60 hour control animals are similar to
the findings in the 54 hour animals with the exception that the proliferative rate is decreasing more as illustrated in the distribution curve in (Figure 2).

The first demonstration of a well developed fibrous callus is observed in the 60 hour hypophysectomized animal(Figure 20A). It bridges the opposing fragments of the fracture and is twice the thickness of the fracture segment. In a low power field the callus can be seen to extend a considerable length of the diaphysis. Where a green stick type fracture is present, the callus can be noted to bridge only one side of the diaphysis, the other side appearing normal in reference to thickness of the two layers of the periosteum.

Labeling of osteoblasts of the cambium layer in 66 hour normal rats is excellent(Figure 20B). Also identified are numerous macrophages and fusiform fibroblasts, all of which are observed to be void of label. In (Figure 21A) labeling is less extensive than in the previous photomicrographs and is demonstrated by comparison with (Figures 3-20) to have a much decreased proliferative capacity. The labeling index being 0.074 or 7.4% is compared with that for the 24 hour hypophysectomized animal which was 0.255 or 25.5%.

In the 72 hour normal animal(Figure 21B) a well developed fibrous callus is seen bridging the fracture site and as before under low power it is seen to extend well above and below the fracture. Noted in most sections of this study was the finding that labeled osteoblasts were quite rare in the vicinity of the fracture and extensive along the remainder of the diaphysis.

The fracture in (Figure 22A) is slightly impacted and a fibrous callus is developing. Labeling in the 72 hour hypophysectomized animal (Figure 22B) is noted to be sparse. The most constant feature of all the specimens was
the visible reaction to injury (i.e.) all fractures demonstrated a highly thickened cambium layer of the periosteum and later the fibrous layer.

The proliferative capacity in control and hypophysectomized rats appears to be inversely proportional to the duration of time. As time increases post-fracture, the proliferative capacity decreases. Of course this rule of thumb is not without exception and that being the time interval between 0 and 24 hours. This relationship is well illustrated in the distribution curve (Figure 2). A very rapid peaking at 24 hours is seen in both the control and hypophysectomized animals. From this point the proliferative capacity gradually falls off to a low of 8.1% (control) and 6.7% (experimental). The latter figures are listed in table form in (Table II). In (Figure 2) the proliferative rate is plotted as a function of time post-fracture in hours and of labeling index (number of labeled osteoblasts per 1000 unlabeled osteoblasts).
The relative ease with which tritiated thymidine may be incorporated into cells and its specificity for deoxyribonucleic acid is demonstrated by the extremely high intensity of label observed within the tissues of the experimental animals (e.g.) cells of the cambium layer of the periosteum and marrow.

Watson and Crick (1959) demonstrated that thymine is one of the four bases making up deoxyribonucleic acid. The specificity of tritiated thymidine is exclusively the number six position (predominantly) and in the 5-methyl group (Evans 1965).

Since 1955 the use of tritiated thymidine has been most useful for studying various aspects of nucleic acid and protein metabolism at the intracellular level, and as tags for study of cell proliferation and cell turnover in various tissues. The percentage of cells incorporating label is a measure of the percentage of the cycle occupied by DNA synthesis (Taylor 1963). Thymidine enters the cell nuclei during interphase and may later be identified by microautoradiography.

Thymidine is either incorporated into deoxyribonucleic acid or promptly degraded (Hughes & Rubini 1960). Following the incorporation of tritiated thymidine into DNA, it is diluted only by successive cell divisions.

The amount of tritium incorporated into DNA of labeled cells has no de-
monstrable effect on growth and survival. For laboratory studies it is usually desirable to limit the amount of radioactivity to such an extent that the biologic effects are minimal or avoided entirely. This implies their use in 'tracer' amounts (Behrens 1964).

According to McLean & Budy (1962) an autoradiograph is made by placing an object (tissue section) containing a radioactive material in contact with photographic emulsion and upon development an image is produced localizing the radioactivity which appear microscopically as blackened grains. The product of this technic is referred to as an autoradiogram.

In this study osteogenic cells of the periosteum were labeled by injecting tritiated thymidine intraperitoneally into normal (control) and hypophysectomized rats to determine the proliferative rate and to compare the two. Comparisons were made by calculating the labeling index of both animals. This index represents the fraction of cells synthesizing deoxyribonucleic acid at time zero (Mendelsohn 1960).

Tonna (1960) in a study similar to the present one concluded that the potential for fracture repair resides essentially in the proliferative capacity of the cells of the periosteum, which diminished with age. The reduced capacity accounts for the reduction in the rate of fracture repair in older animals. He was interested in the proliferative capacity as it was related to ageing. Whereas, the present study was interested in the difference in the proliferative capacity between control and hypophysectomized animals. The results of this study in reference to the normal animals closely duplicated the
Bassett (1962) reports that although it is generally agreed that an active osteoblast is responsible for the manufacture of bone, there seems to be definite evidence that undifferentiated connective tissue cells have a latent capacity to specialize as osteoblasts under appropriate environmental conditions. However, he fails to give any evidence confirming this thesis.

Much has been written concerning the above hypothesis (i.e.) that bone is derived from mesenchyme. Because of the very sparse radiolabeling of the fibrous layer of the periosteum in comparison to the great abundance in the osteogenic layer, the results of this study are in agreement with (Church 1963) who states that "the outer layer of the periosteum lacks evidence of osteogenic potential."

Another theory of bone formation and repair is advocated by Trueta (1962). He presents evidence that endothelial cells of the walls of the advancing vessels divide and lay down a progeny of either osteoblasts or their near predecessors. This theory is supported by the work of Rigal (1961) who found that following fracture, many of the endothelial cells of vessel walls were preparing to divide; this was detected by radiolabeling with tritiated thymidine.

More recently Urist (1965) reported that wandering histiocytes are stimulated by degradation products of dead matrix to grow in and repopulate the area of decalcified bone. This process is followed immediately by new-bone formation by autoinduction in which both the inductor cells and induced cells are derived from ingrowing cells of the host bed. The inductor cell is a de-
scendent of a wandering histiocyte; and the induced cell is a fixed histiocyte or perivascular young connective tissue cell. Differentiation of the osteo-progenitor cell is elicited by local alteration in cell metabolic cycles that are as yet uncharacterized. This evidence was offered in favor of the theory of induction.

Many theories of bone formation have been presented. From a logical outlook, it is more rational to accept a theory for which one may offer evidence. Of the theories presented to repeat: undifferentiated connective tissue cells, transition of endothelial cells into osteoblasts and more recently the theory of autoinduction; all with the exception of the first seem to lack definite evidence to confirm the respective thesis. However, evidence at the present as to the source of osteoclasts points to the osteogenic layer of the periosteum. From the results and the photomicrographs, the distribution of labeled cells of the inner and outer layer of the periosteum implies positive evidence to the cambium layer of the periosteum as the source of osteogenic cells.

Catabolism of labeled cells with the resultant liberation of the radiolabel can be used by tissue cells preparing for duplication. This phenomena is known as the process of reutilization (Figure 23).

According to Robinson (1963) labeling many continue for some time after the initial administration of tritiated thymidine. The substance transferred is the thymidine itself. This thesis is supported by the work of Goljan (1964).

Reutilization is mentioned only to compare this method with the technic.
used in the present study which was the "flash" technic. The former method facilitates the availability of the radiolabel for long periods of time and consequently many generations of cells very possibly will become labeled. In the latter or "flash" technic, the isotope is available to duplicating cells for a period of one hour (maximum), after which the animal was sacrificed; therefore second generation cells were not labeled. This would explain the absence of labeled osteocytes in respective lacunae, and also the absence of diluted radiolabeling.

Tonna and Cronkite (1962) in a study on ageing found that old mice respond following trauma, in a similar manner to the proliferative capacity normally found in young mice. He was referring to the labeling index of the osteogenic cells of the periosteum. The greatest increase in the proliferative rate in his study was at time 24 hours — where the index was approximately 0.300. The results of the present study agree very closely with his results. However, his study did not attempt to compare the proliferative rates between normal and hypophysectomized rats which was done in the present study. The results were about what one would expect for a hypophysectomized animal, and LeBlond (1955) describes it well "mitotic activity is significantly lowered after thyroidectomy or hypophysectomy in the intact animal." He found that growth hormone corrected the deficiency and accelerated mitosis.

This study was interested in what the injured cells were doing at the time zero (i.e.) immediately following sacrifice after "flash" labeling with tritiated thymidine.
The results of this study appear to support the hypothesis that the proliferative capacity of hypophysectomized animals is markedly decreased compared to the normal animal. Differentiation of the osteoprogenitor cells was constant in both animals -- the change occurring in the proliferative rates as illustrated in the distribution curve (Figure 2). It is the feeling of the author that the purpose of this study was completed successfully in demonstrating autoradiographically, the proliferative rates of the osteogenic layer of the periosteum forming, following mechanical fracture of the rat fibulas. Also DNA synthesis was able to be significantly compared between the control and experimental animals (Table III).

Statistical significance was calculated by the method illustrated in (Figure 2A). The individual figures for (T) were plotted with values ranging from 0.95 to 5.8. The factor(s) involved and the probable cause of the diffuse spread of values is believed to be the various sacrificial times. The time of sacrifice varies from early morning to as late as eight o'clock in the evening. Nevertheless, the values for (T) on the whole indicate the significance of the differences in the proliferative capacities from 18 hours to 72 hours. Therefore, the spread of the (T) factor in calculating the scientific significance can be considered a diurnal physiologic inconsistency.
CHAPTER VI

SUMMARY AND CONCLUSIONS

This investigation was conducted in order to demonstrate autoradiographically, the proliferative capacity of the periosteum developing following fracture of the rat fibula. Also, the baseline of labeled cells in the control animal was used to compare DNA synthesis in the hypophysectomized rats.

In the periosteum, preosteoblasts were more frequently labeled than osteoblasts or fibroblasts. Labeled osteocytes were not seen. Between twelve and eighteen hours cell proliferation of the osteogenic layer of the periosteum was stimulated. During this time DNA synthesis as manifested by cell proliferation greatly increased to a maximum of 30.0% (control) and 25.5% (hypophysectomized) at 24 hours.

The labeled cells were found throughout the entire diaphysis, indicating that proliferation had been stimulated throughout the diaphyseal periosteum and was not limited exclusively to the fracture site. The periosteum was missing for some distance from the fracture site and necrosis of osteocytes in areas close to the fracture site was observed.

A number of conclusions are in order: First, the proliferative capacity in the hypophysectomized rats is significantly lower than that of the normal animals. The difference in DNA synthesis varied from 1.5 to 5.2 percent.

Second, no further evidence was obtained to confirm studies favoring the
osteogenic potential of the outer layer of the periosteum. Rather, findings of this study are in almost total agreement with similar studies by Tonna (1960), Tonna and Cronkite (1961) and Tonna & Cronkite (1962). Their studies however, were concerned only with normal animals and they were more interested in the ageing process as it affected the proliferative rates.

Third, cells of the cambium layer of the periosteum, as a result of the trauma from the fracture, became a multicellular layer of plump osteoblasts. And, the thickness of the osteogenic layer and fibrous layer multiplied by many fold.

Last, it is noted at 54 hours (distribution curve) that the respective curves are closely equated. At this time interval and beyond, proliferative capacity of the periosteum in both control and experimental animals has continued to decrease. It is suggested that cells are undergoing differentiation from preosteoblasts to osteoblasts.

The data presented has demonstrated again what has been the topic of much discussion for a number of years; and that is, that mitotic activity is significantly lowered following hypophysectomy in the intact animal.
Figure 1  Representative examples of mechanical fractures of rat fibula as evidenced by radiographic examination.
### TABLE I

<table>
<thead>
<tr>
<th>Rats \ H&amp;N</th>
<th>FF</th>
<th>ITT</th>
<th>SF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-hr</td>
<td>5 hr</td>
<td>6 hr</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>11 hr</td>
<td>12 hr</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>17 hr</td>
<td>18 hr</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>23 hr</td>
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<td>29 hr</td>
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<td>66 hr</td>
</tr>
<tr>
<td>12</td>
<td>&quot;</td>
<td>71 hr</td>
<td>72 hr</td>
</tr>
</tbody>
</table>

**PROTOCOL OF RAT TREATMENT**

Legend:  
- **N** = normal animal  
- **H** = hypophysectomized  
- **FF** = fractured fibula  
- **ITT** = injected tritiated thymidine  
- **SF** = sacrificed  
- **hr** = hour
Figure 2  The proliferative rate is plotted as a function of time post-fracture in hours and of labeling index (number of labeled osteoblasts per 1000 unlabeled osteoblasts).
### TABLE II

**Labeled Cellular Population of Osteogenic Layer of Rat Periosteum After Fibular Fracture**

<table>
<thead>
<tr>
<th>Post-fracture (hours)</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>42</th>
<th>48</th>
<th>54</th>
<th>60</th>
<th>66</th>
<th>72</th>
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<tbody>
<tr>
<td>Labeling index</td>
<td>-</td>
<td>-</td>
<td>.253</td>
<td>.302</td>
<td>.250</td>
<td>.210</td>
<td>.185</td>
<td>.164</td>
<td>.118</td>
<td>.106</td>
<td>.090</td>
<td>.081</td>
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<tr>
<td>Labeling %</td>
<td>-</td>
<td>-</td>
<td>25.3</td>
<td>30.2</td>
<td>25.0</td>
<td>21.0</td>
<td>18.5</td>
<td>16.4</td>
<td>11.8</td>
<td>10.6</td>
<td>9.0</td>
<td>8.1</td>
</tr>
</tbody>
</table>

# Cells counted per specimen (1000 cells)

#### CONTROL ANIMALS

<table>
<thead>
<tr>
<th>Post-fracture (hours)</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>42</th>
<th>48</th>
<th>54</th>
<th>60</th>
<th>66</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeling index</td>
<td>-</td>
<td>-</td>
<td>.200</td>
<td>.255</td>
<td>.204</td>
<td>.160</td>
<td>.142</td>
<td>.113</td>
<td>.095</td>
<td>.088</td>
<td>.074</td>
<td>.067</td>
</tr>
<tr>
<td>Labeling %</td>
<td>-</td>
<td>-</td>
<td>20.0</td>
<td>25.5</td>
<td>20.4</td>
<td>16.0</td>
<td>14.2</td>
<td>11.3</td>
<td>9.5</td>
<td>8.8</td>
<td>7.4</td>
<td>6.7</td>
</tr>
</tbody>
</table>

# Cells counted per specimen (1000 cells)

#### EXPERIMENTAL ANIMALS

Legend: Control = normal rats

Experimental = hypophysectomized rats

Post-fracture - in hours
Figure 2A  Statistical significance calculated as below (example)  

1. Standard Deviation  
   
   $0.256$  
   $0.237$  
   $0.267$  
   
   Average $= 0.253$  
   
   total divided by three equals average or mean labeling index.  

   Difference between respective indices and average is squared, totaled, divided by two and the square root determined.  

   $S.D. = \sqrt{\frac{\text{total of square of labeling index}}{2}}$  

2. Standard Error  

   $S.E. = \sqrt{\frac{\text{Stnd. Dev. Normal}}{3} + \frac{\text{Stnd. Dev. Hypophysectomized}}{3}}$  

3. Statistical Significance $= \frac{\text{Mean for normal} - \text{Mean for Hypophy}}{\text{Standard Error}}$
### TABLE III

#### STATISTICAL SIGNIFICANCE

<table>
<thead>
<tr>
<th>Animal Period</th>
<th>Standard Deviation</th>
<th>Standard Error</th>
<th>(T)</th>
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<tbody>
<tr>
<td>18 hour animals</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>0.015</td>
<td>0.009</td>
<td>5.8</td>
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<tr>
<td>Hypophysecto-</td>
<td></td>
<td></td>
<td></td>
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<tr>
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</tr>
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<td>0.018</td>
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<td>Normal</td>
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<td>42 hour animals</td>
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<td>Normal</td>
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<tr>
<td>54 hour animals</td>
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<tr>
<td>66 hour animals</td>
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<tr>
<td>72 hour animals</td>
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<td>0.95</td>
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<tr>
<td>Normal</td>
<td>0.0216</td>
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</tr>
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</table>

Legend: (T) = statistical significance
Figure 3  Representative example of normal periosteal thickness before traumatizing by digital fracture (A). Thickened periosteum of six hour animal after fracture (B). Note areas between arrows and compare. X750 H & E.
A. Representative examples of six and twelve hour labeling. (A) six hour normal rat (arrow) can see one labeled periosteal cell. (B) also see one labeled cell in twelve hour normal rat (arrow). X750 H & E.

Figure 4
Figure 5  Representative examples of 18 hour normal rat fracture and labeling. (A) fracture is outlined by arrows and can also see increase in thickness of periosteum, some evidence of fibrous callus formation. X320 H & E. (B) extensive labeling is seen and also a part of the fracture. X750 H & E.
Figure 6. Representative examples of 18 hour hypophysectomized rat fracture and labeling. (A) periosteum can be seen to be greatly thickened (compare with figure 3). (B) plump osteoblasts can be seen labeled extensively. Arrow is pointing to skeletal muscle cells to show the lack of labeling. X750 H & E.
Figure 7  
Representative examples of 24 hour normal rat. (A) fracture can be seen to be slightly displaced. X320 H & E. (B) labeled cells are easily seen. Arrow points to group of macrophages. To the left of the periosteum, osteocytes are seen without label. This finding is expected when using the "flash" technic. X750 H & E.
Figure 8  Representative examples of 2½ hour hypophysectomized rats.  
(A) periosteum is greatly thickened and is bordered by bone on left and fibrous periosteum on the right.  X320 H & E.  
(B) thick periosteum, numerous labeled cells and several macrophages(arrows).  To the extreme right are fibroblasts, which did not take up radiolabel.  X750 H & E.
Figure 9  Representative examples of 30 hour normal rats. (A) displaced fracture with considerable gapping between fragments. X320 H & E. (B) thickened periosteum with numerous labeled cells. X750 H & E.
Figure 10  Representative examples of 30 hour hypophysectomized rats.  
(A) again is seen a displaced fracture with wide gapping,  
(arrow) points to osteoclasts in a lacunae.  X320 H & E.  
(B) thickened periosteum under higher magnification showing  
numerous labeled periosteal cells.  Arrow points to one of  
many labeled cells.  X750 H & E.
Figure 11. Representative examples of 36 hour normal rats. (A) fracture of fibula is slightly displaced. X320 H & E. (B) numerous labeled cells bordered on left by bone and on right by skeletal muscle which can be seen to be without label. The peripheral skeletal cells are easily noted. X750 H & E.
Figure 12  Representative examples of 36 hour hypophysectomized rats. (A) fracture is located between the two arrows, and also a comminuted fragment lies to the left. X320 H & E. (B) arrows indicate a few of the many labeled cells. The cells can be seen to be very plump in appearance. X750 H & E.
Figure 13  Representative examples of 42 hour normal rats. (A) oblique fracture located between two arrows. X320 H & E. (B) many labeled periosteal cells are seen. What appears to be labeled osteocytes to the left are osteocytes underlying background grains. X750 H & E.
Figure 14. Representative examples of 42 hour hypophysectomized rats. (A) the osteogenic layer of the periosteum is thick and outlined between the arrows. Lacunae with many osteocytes are noted to the left. X320 H & E. (B) periosteal cells of the osteogenic layer are well labeled. Bone with lacunae and osteocytes to the left are not labeled but are overlaid with background grains. X750 H & E.
Figure 15  Representative examples of 48 hour normal rats. (A) oblique fracture in center of photomicrograph, bordered on left by marrow and on the right by periosteum. X320 H & E. (B) Labeled cells are noted in center of micrograph. X750 H & E.
Figure 16
Representative examples of 48 hour hypophysectomized rats.
(A) an undisplaced fracture is noted. To right (arrow) are some indications of resorption and osteoclasts. X320 H & E.
(B) labeled cells are scattered throughout the periosteum X750 H & E.
Figure 17  Representative examples of 54 hour normal rats. (A) fracture is noted with good apposition and in areas there appear to be a lytic process. To the left is a thickened periosteum and skeletal muscle fibers. X320 H & E. (B) numerous labeled osteoblasts in center of photomicrograph, and to the left arrow indicates an empty lacunae with canaliculi. X750 H & E.
Figure 18  Representative examples of 54 hour hypophysectomized rats.  
(A) thickened periosteum and numerous unlabeled osteocytes within the bony lacunae.  X320 H & E.  (B) labeled enlarged osteoblasts.  To the left of the labeled cells is noted the fibrous layer of the periosteum.  X750 H & E.
Figure 19  Representative examples of 60 hour normal rats.  (A) the fracture is well visualized and to the right is noted a thickened periosteum.  X320 H & E.  (B) the number of labeled cells are becoming fewer as the duration post-fracture increases.  X750 H & E.
Figure 20 (A) fracture in 60 hour hypophysectomized rat -- can be seen in the center of the photomicrograph bordered by arrows. The horizontally placed arrows outline the fibrous callus which is greatly increased in width. X200 H & E. (B) 66 hour normal rats -- can see labeled osteoblasts. Upper two arrows exhibit macrophages and the lower arrow shows two fibroblasts lacking label. X750 H & E.
Figure 2.1 Fracture in center of photomicrograph of 66 hour hypophysectomized rat (A). To extreme left of fracture can see many labeled osteoblasts. X750 H & E. (B) 72 hour normal rat — large arrow points to fracture site and small arrows outline the fibrous callus. X200 H & E.
Figure 22 Representative examples of 72 hour hypophysectomized rats. (A) impacted-type fracture is easily seen. Also to left of fracture is an extensively developed fibrous callus. X200 H & E. (B) labeling is more sparse compared to previous sections. X750 H & E.
Figure 23  Factors involved in the incorporation of a labeled precursor in DNA. (after Quastler 1963).
BIBLIOGRAPHY


Lajtha, R.W., Oliver, R. The Application of Autoradiographs in the Study of Nucleic Acid Metabolism. Lab. Invest. 8:400- 1959.


APPROVAL SHEET

The thesis submitted by Dr. Joseph T. Nichols has been read and approved by three members of the department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given full approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.