RICE UNIVERSITY

HORMONAL AND RADIATION EFFECTS ON OSTEOCLASTIC PROLIFERATION

by

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ABSTRACT

The past century of research on bone physiology has established that the osteoclast is involved in the resorption of bone. The presence of parathyroid hormone in the circulating body fluids causes the calcium concentration in these fluids to increase and there is an accompanying increase in osteoclastic activity in the bone tissue. The metabolic processes, which result in crystal removal from bone, are mediated by parathyroid activity and are necessary for maintenance of specific calcium levels in all land-dwelling vertebrates.

This study shows various hormonal and radiation effects on osteoclastic proliferation and the relationship between the activity of these cells and the maintenance of calcium concentrations in the rat. The proliferation of the osteoclasts was produced by the technique of continuous peritoneal lavage and/or nephrectomy. The experimental work consisted of: (1) altering the circulating calcium concentration by acidosis, peritoneal administration of NaF, or supplying exogenous calcium; (2) studying the effects of hypophysectomy, adrenalectomy, or estrogen administration to male rats; and (3) producing changes in bone by internal radiation with Ca$^{45}$ or Pu$^{239}$.

The results show that although wide variations are found in the osteoclast numbers produced by the various experimental conditions, the calcium levels are uniformly maintained. This would indicate either a constantly changing level of parathyroid activity, a change in activity of the osteoclasts present, or that the calcium concentration in the body fluids can be maintained without the necessity of osteoclastic proliferation.
ACKNOWLEDGEMENTS

This work was made possible because of the contributions made by Professor Roy V. Talmage and his laboratory staff:

To Dr. Talmage for originally stimulating my interest in this problem and for his constructive criticism along the way.

To the technicians, Mrs. Dorothy McRitchie and Mrs. Lynne Bishop, for their valuable assistance.

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And to Mrs. Eileen Moran for her care in typing this manuscript.
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INTRODUCTION

The modulating action of certain hormones on well-defined cell populations has always proven to be an interesting topic for study. However, when this relationship between hormones and cells is studied in vivo, specific cell populations cannot be completely isolated to observe the hormonal effects. For example, the primary effect produced by parathyroid hormone, in all the higher vertebrates, is the maintenance of a constant calcium concentration in the extracellular fluids. Parathyroid hormone is known to work by influencing the kidney (calcium and phosphate excretion), the intestine (calcium and phosphate absorption), and bone (calcium and phosphate deposition or resorption). It is not beneficial to study the hormonal influence on the cells of one of these tissues without considering the hormonal influence on the other two.

Chemical and radioisotope techniques have proven that calcium can be mobilized from bone in a matter of minutes, when parathyroid hormone is present. However, observable changes in the bone cell population are not evident until several hours after the calcium has already been removed from the bone tissue and added to the extracellular fluids. The most obvious change in morphology of bone, subjected to parathyroid hormone, is the increased numbers of osteoclastic cells present. These cells are responsible for the remodelling of bone, and normally are found in greatest numbers in the more metabolically active regions. The relationship between numbers of osteoclasts, parathyroid activity, and calcium metabolism will be discussed in this thesis.
STATEMENT OF THE PROBLEM

It has been shown previously (Toft and Talmage, 1960) that a quantitative determination of the osteoclast numbers in the distal metaphyseal region of the femur could be used as an index of parathyroid activity in the rat. This hypothesis was retested in the present work. Following this, a series of experiments were designed to alter the calcium metabolism and see how this affected the osteoclasts. Also, experiments were designed to study certain hormonal and radiation effects on bone cells and calcium metabolism. The primary purpose of these combined experiments was to determine whether or not maintenance of calcium levels was dependent upon osteoclastic activity.
A. The Osteoclast

The osteoclast is one of the largest cells in the body. It may have as many as 100 nuclei and in some cases a volume approximating 200,000 cubic microns (Hancox, 1956). When viewed in a seven micron section, generally only six to ten nuclei and in some cases only a mononucleated piece of osteoclast cytoplasm, may be seen. The osteoclast is very mobile (Hancox, 1946), and assumes many varied shapes in a fixed preparation. It may be flattened out close to the surface of trabecular bone, or may be more spherically shaped and at a distance from the trabeculae; or it may assume any shape and position between these two extremes.

The nucleus of this cell averages 3.0 to 3.5 microns in diameter, is usually spherical, and contains one or two nucleoli. There is no evidence that these cells undergo mitotic or amitotic division. Pyknotic nuclei are taken to be an indication of cell degeneration, but this is not an easy criterion to judge.

The cytoplasm shows various staining reactions, probably representing different stages of cellular activity. Generally, most of these cells in a particular section have basophilic cytoplasm with an occasional tendency towards oxyphilia. The degenerating cells have an eosinophilic cytoplasm which is usually well vacuolated (Hancox, 1956). In the degenerated cells, only an amorphous mass of heavily vacuolated, somewhat granular material, may be seen (Fig. 1). Sometimes the cytoplasm of the osteoclast will also
contain erythrocytes (Fig. 2), liberated osteocytes (Fig. 3), and PAS-positive stained material (Fig. 4).

An outstanding characteristic of the active osteoclast is a ruffled border, which is located on that surface of the cell adjacent to the bone (Fig. 5). This border consists of numerous folds of cytoplasm, which sweep across the bone surface. In the light microscope, this region often has a striated appearance due to the bone matrix fibrils that project into the invaginated cytoplasm of the ruffled border. The PAS reaction and tests for alkaline phosphatase are negative on this border (Kroon, 1954).

The electron microscope has greatly aided in elucidating the relationship between the ruffled border and the associated surface of bone. In all of the studies thus far: Scott and Pease, (1956); Robinson and Cameron, (1956); Gonzales and Karnovsky, (1961); Hancox and Boothroyd, (1961); and Takuma, (1962), hydroxyapatite crystals that have been associated with the bone matrix have been found far up into the folds of the osteoclast's border. Bone salts have sometimes been observed in pinocytotic vacuoles within the cell's cytoplasm. In one study (Takuma, 1962), it was shown that the structure and function of chondroclasts were very similar to those of osteoclasts and that salts and collagen were within the cisternae of this cell. However, due to the vast infolding of the ruffled border, these intracisternal contents may actually have been extracellular.

Both the electron microscope (Takuma, 1962) and high resolution x-rays (Greulich, 1961) show a band in the matrix about one micron thick which is of high density and located just beneath the ruffled
border of the osteoclast. Greulich believes this band is caused by an accumulation of those enzymes which are responsible for bone resorption. These radio-opaque bands are found only beneath areas where osteoclasts are in contact with bone.

There is good histochemical evidence that osteoclasts contain many enzymes which can produce local changes in the environment of the bone surface with which it is associated. It has large quantities of acid phosphatase, beta-glucuronidase, succinic dehydrogenase, and cytochrome oxidase (Cabrini, 1961; Burstone, 1960a, 1960b), and the active osteoclast shows strong aminopeptidase reaction in the cytoplasm (Lipp, 1959). It has been reported that the Krebs cycle is altered in the osteoclast so that lactate and citrate are produced using glutamate as the substrate (Walker, 1961). Walker also found that parathyroid extract did not change the histochemical reaction of the cell types in bone, but only increased the numbers of osteoclasts.

Neuman, (1958) has concluded that solubilization of the apatite crystals must occur before the bone matrix can be resorbed by proteolytic enzymes. Whether the osteoclast does both of these things is unknown at this time, but the evidence shows that these cells have the necessary potential. A localized lowering of pH by osteoclasts has been measured with micro-technique by Cretin, (1951). Neuman, et al., (1960) has found that yttrium, which is ionic and diffusible only at a low pH, is attracted to absorption lacunae. The osteoclast has numerous mitochondria (Ch'uan, 1931), can produce acids, and shows proteolytic activity (Cameron, 1963; Hancox, 1946).
The origin of the osteoclast is an unsolved problem at the present time. A survey of the literature shows that the cells most likely to give rise to osteoclasts are: (1) undifferentiated cells, (2) wandering histiocytes, (3) osteoblasts and (4) monocytes or mononucleated leukocytes. Although the fusion of osteoblasts has been the most widely accepted theory of the origin of osteoclasts (Jordan, 1921; Heller, et al., 1950; Toft and Talmage, 1960; and Tonna and Cronkite, 1961), it is difficult to see how the union of several cells of the same type could confer upon the resulting giant cell a completely different metabolism and the property of great mobility. There are others (McLean and Bloom, 1941) who believe that the osteoclast may be a phagocytic cell, arising from wandering histiocytes, and responsible for "cleaning up" bone debris after bone resorption has occurred. Young, (1963) is one of the present proponents of the idea that osteoclasts arise from undifferentiated osteoprogenitor cells. Hancox, (1946) was probably the earliest advocate of osteoclasts arising from monocytes, but Fischman and Hay, (1962) have since proposed the same theory.

B. Hormonal Effects on Bone

There is no doubt that the presence of parathyroid hormone in the blood certainly causes changes in the cell population of bone tissue (Heller, et al., 1950; Tonna and Cronkite, 1961; and Young, 1963). It has been suggested that the initial effect of this hormone is to decrease osteoblastic activity, and increase osteoclast numbers (Pugsley and Selye, 1933; Burrows, 1937). Many workers in this field
agree that parathyroid hormone causes an increase in osteoclast numbers in the metaphyseal region of long bones, although they disagree about the function of these cells. Toft and Talmage, (1960) have shown that increased osteoclasts can be quantitated in this region of bone and this can be used as an index of parathyroid activity in the rat. However, if excess hormone is present over long periods of time, as in cases of hyperparathyroidism, the osteoclasts eventually disappear in this region and increased fibrous tissue or abnormal bone is produced (Burrows, 1937; McLean, 1956).

It has been suggested that bone resorption may result from localized pH changes between bone cells and bone surface, or by a general lowering of pH as suggested by Nordin, (1961). Elliott and Talmage, (1958) showed that citric acid and parathyroid hormone resulted in radiocalcium removal from the same areas of bone. There is evidence that parathyroid extract increases CO₂ production (Cohn and Forscher, 1962) and decreases oxygen consumption (Lekan, et al., 1960) in bones treated with this hormone. The pentose shunt plays a very minor part in the formation of the total metabolic products in bone tissue (Walker, 1961; Cohn and Forscher, 1962). In vitro studies (Borle, et al., 1960b; Vaes and Nichols, 1961, 1962) have indicated that parathyroid extract may act by decreasing the oxidation of citrate, thereby increasing the citric acid accumulation in bone. However, these same studies show much more lactate produced than citrate in the presence of parathyroid hormone. Kenney, (1961, 1959) has shown that lactate accumulation in bone results in a pH change, but the citrate accumulation is accompanied by the most bone resorption.
The adrenal corticoid hormones seem to have no effect on calcium levels if used in physiological quantities, but they may influence the serum phosphate levels (Schartum and Nichols, 1961; Lobeck and Steinkraus, 1960). Extensive hormonal therapy in the rat results in increased density of the metaphyseal bone, probably due to interference of resorption of bone beneath the epiphyseal plate (Follis, 1951).

Estrogens have effects on bone and bone cells which are antagonistic to parathyroid hormone. Radioactive estrogens are seen distributed along the endosteum and metaphyseal trabeculae, but have not been found in cartilage (Budy, 1962). The specific activity of serum calcium is higher in estrogen-treated rats (Linguist, et al., 1960), yet Ca\textsuperscript{45} after incorporation into the matrix, is not as readily exchangeable after estrogen treatment. The protein bound fraction of serum calcium, after administering estrogen, has not been seen to increase in mammals as it does in birds (Urist, et al., 1960). These steroids inhibit growth of the long bones so that the normal amount of bone is packed into a smaller volume. Estrogen-treated rats are smaller in size than normal, have shorter femurs and tibias, and the bone marrow can be completely displaced by trabecular bone (Urist, et al., 1950). Estrogens inhibit the normal resorption in the long bones (Linguist, et al., 1960), but testosterone can overcome this inhibition (Barker and Crossley, 1962). Although Linguist, (1960) feels that estrogen does not change the accretion rate in bones treated with estrogen, Ranney, (1959) says the accretion rate is increased.
Some other effects of estrogen on bone are: (1) Citrate production and oxidation is increased in bone (Vaes and Nichols, 1961) and more plasma citrate is incorporated into bone matrix (Ranney, 1960); (2) Synthesis of mucopolysaccharides is inhibited in cartilage, according to Priest and Koplitz, (1962), but stimulated in bone according to Suzuki, (1959); (3) Simmons, (1962) observed increased numbers of osteoblasts in bone treated with estrogen.

C. Radiation Effects on Bone Cells

The reviews on radiation effects on bone (Heller, 1948; Vaughn, 1962; and Daugherty, 1962) are mainly concerned with gross changes resulting from radiation damage. Heller, (1948) mentions that there was a hint of increased osteoclasts after x-ray irradiation of bone; however, this effect has never been quantitated.

Arnold and Jee, (1957) have studied the uptake of plutonium by osteoclasts. They noted that Pu$^{239}$ was deposited on the trabecular surfaces within two hours after injection, and four days later practically every osteoclast had ingested some plutonium. Vaughn, (1962) noticed that osteoclasts were more resistant to radiation than the other bone cells, and Jee and Arnold, (1961) found osteoclasts that had received up to 280 rep/day and were still resorbing bone. The osteoclast's resistance to radiation may be linked to the fact that these cells do not undergo mitosis and a dividing cell is much more susceptible to radiation than a resting one. Kember, (1962) has shown that the mitotic activity in the metaphysis can be decreased or entirely stopped by giving various amounts of P$^{32}$. He states
that after giving the larger doses, when mitosis finally does return to a normal rate, the cells produced are spindle cells rather than normal osteoblasts or osteocytes.
MATERIALS AND METHODS

A. Standard Lavage Procedure

Male rats of the Holtzman strain weighing between 180-200 grams were used in these experiments. There were exceptions to these weights which will be noted under the particular experiment in which they were used. All animals were maintained on calcium-free diet and deionized water for three days prior to use. The rats gained very little weight while on this regimen.

On the day before the lavage, the animals were weighed, numbered, and a "plug" was inserted into their peritoneum. This "plug" was the stainless steel plug designed by Kloff and Page, (1954). Animals which were nephrectomized had their kidneys removed through a mid-ventral incision and then the plug was sewn into the same incision. The rats were under ether anesthesia during all operating procedures.

When animals were parathyroidectomized, it was done about eight hours prior to use of the rats. The operation consists of anesthetizing the animal with ether, making an incision above the thyroid gland, locating this gland, then locating the parathyroids by the use of a binocular dissecting scope, and removing the parathyroid tissue with jewelers forceps. Sham parathyroidectomies were done to rule out any effects due to surgical trauma.

The peritoneal lavage procedure consisted of introducing fluid into the peritoneum via the peritoneal plug, allowing it to remain for one hour, then draining it out and replacing it with new lavage fluid. Unless stated otherwise, the fluid consisted of a buffered
rinse*, calcium-free at pH 7.35 that was heated to body temperature before introducing it into the peritoneum.

The samples that were collected for each hour from each rat were stored in vials at 10 - 15 degrees centigrade. Calcium measurements were made on aliquots of these samples by the use of a Beckman Model D U Spectrophotometer. All calcium values are expressed in terms of milligram per cent.

Unless there is a note to the contrary, all calcium values in the following results refer to those values obtained in the lavage fluid. Serum calcium values were much higher than the values considered throughout this work, nevertheless the same relative picture was true in both fluids.

In all cases, both femurs were removed for histological study; in some cases, tibias and mandibles were also removed. For longitudinal sections, the whole femur was removed, but for cross sections, the femur was separated at the distal epiphysis and then sawed in half so as to include the distal metaphysis and one-half inch of the shaft.

B. Special Procedures

I. Exogenous Calcium: The animals, prior to use, were maintained on 2.4% calcium lactate in the drinking water for five days. Twenty-four hours before the lavage each rat was given a total of 15 mg of calcium by stomach tube in three doses over a twelve-hour period. The lavage fluid used was the normal buffered fluid but also contained

* Buffered lavage solution contained NaCl (6.75 gms/liter), 2% glucose, KCl (0.1225 gms/liter), NaHCO₃ (2.19 gms/liter), and lactic acid (0.67 cc/liter).
10 mg % calcium as calcium lactate. It is estimated that during each hour of the lavage, each animal received about 2.5 - 3.0 mg of calcium.

II. Sodium Chloride Acidosis: Acidosis was produced by introducing a lavage fluid containing unbuffered 0.84% sodium chloride at pH 7.4.

III. Fluoride Studies: The animals in these studies were about ten to twenty grams lighter than those used in the above experiments. The primary modification in this procedure consisted of adding sodium fluoride to the buffered lavage fluid at a concentration of 0.072 mg/ml.

IV. Hypophysectomized Animals: These rats were obtained from the Hormone Assay Laboratories in Chicago, Illinois. The animals were hypophysectomized on Saturday, allowed to recuperate over Sunday, and received in our laboratory on Monday. The animals used for the lavage procedure were maintained on cortisone (1 mg/day).

V. Adrenalectomized Animals: The rats were treated in the manner outlined under General Lavage Procedure except that they were adrenalectomized two hours prior to lavage. These animals were given either cortisone (1 mg/day) or epinephrine (0.1 mg/hour of lavage).

VI. Estrogen Studies: Immature male Holtzman rats, weighing 50 grams, were used in these experiments. Each animal received two mg of Delestrec* injected subcutaneously every two weeks for a four week

* Delestrec is an experimental product from Squibb Laboratories. Because of the ten-carbon side chain in the 17-position, this estrone derivative is slowly absorbed.
period. All rats were maintained on Holtzman diet and tap water during this period.

VII. Radiation Experiment Using Ca\(^{45}\): At the beginning of this experiment the animals weighed about 200 grams. Half of the rats received fifty microcuries of Ca\(^{45}\) apiece and the others were injected with one millicurie of radioactivity each. The injections were divided in half and given eight hours apart. Two lavages which lasted three hours each were done on days five and ten after the injections. On day 21, after the injections, the animals were lavaged for eight hours and then killed.

VIII. Radiation Experiment Using Pu\(^{239}\): This project was carried out at the Argonne National Laboratory in Chicago, Illinois. The rats were of the Sprague-Dawley strain and weighed about 200 grams. One group was injected five days prior to lavage with one microcurie of Pu\(^{239}\) per rat, and the second group was injected 24 hours before the lavage. The lavage rinse itself consisted of the normal buffered solution.

C. Histological Procedures

In the beginning of this study, the bone tissue was fixed overnight in the refrigerator in 90% Zenkers fluid and 10% formol (Kelly's), and then decalcified for three days in 5% formic acid. In later studies, Bouin-Hollande fixative was used for three to four days at room temperature and decalcification was completed with 18.5% EDTA at pH 7.0 for three days. This latter technique did not change the results.
significantly but did seem to create fewer artifacts in the sections. That is to say, the bone cells were not so frequently removed from the matrix, and the various cellular components were more easily distinguished when "abnormal" bone was studied. Also, it was convenient in some of the studies to have a fixative that was stable for longer than one day. The mercury salts in Zenker's fixative also caused problems in the staining of slides and interfered with autoradiographs when emulsions were used.

After the bone tissue had been fixed and decalcified according to one of the two procedures discussed above, it was washed in tap water and then dehydrated through a series of alcohols. The double embedding technique was used whereby the bones were impregnated with celloidin and paraffin (m.p. 54-56°C.). After mounting the tissues on wooden blocks, they were sectioned at five to seven microns with the Spencer 820 Rotary Microtome. The resulting sections were fixed on chemically clean glass slides and stained with Hematoxylin, Fast Green and a mixture of Biebrich Scarlet and Orange II.

The osteoclast counting technique was made possible by a Harvard grid (0.26 mm²) placed in one ocular of the binocular microscope. At a magnification of 430X, this grid was moved in a successive series of steps so as to cover the whole cross section of the femur under study. The average osteoclast count was obtained by determining the total number of osteoclast cells in the whole cross section and dividing by the number of times the grid fits on this piece of tissue. Mononucleated osteoclasts were usually not counted. In all cases, the quantitative count was made on a sample from the distal metaphysis.
of the femur, immediately beneath the epiphyseal plate.

In some of the autoradiographic work where Kodak emulsion (NTB3) was used, the tetrachrome stain technique was not desirable to use since the emulsion took up so much of the coloration. Therefore, a PAS stain was used on the tissue before the slides were dipped in emulsion, then after developing the autographs, the slides could be counterstained with Hematoxylin. This procedure allowed reasonable definition of the cell types.

During this work it was discovered that cross sections could be taken of the metaphyseal region of the calcified femur. These calcified sections were useful in some of the radioautography work, but in general did not produce the same quality results as bone that had been decalcified with EDTA. Cross sections of these bones near the shaft and longitudinal sections of the whole calcified femur were impossible to obtain by this procedure.
RESULTS

A. STANDARD LAVAGE

Table I

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Parathyroid intact -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Non-lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td>10</td>
<td>3.61 ± .03</td>
</tr>
<tr>
<td>b. Non-nephx.</td>
<td>12</td>
<td>2.29 ± .10</td>
</tr>
<tr>
<td>II. 8-hour Lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td>21</td>
<td>7.07 ± .11</td>
</tr>
<tr>
<td>b. Non-nephx.</td>
<td>14</td>
<td>4.39 ± .20</td>
</tr>
<tr>
<td>III. 12-hour Lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td>3</td>
<td>7.01 ± .47</td>
</tr>
<tr>
<td>b. Non-nephx.</td>
<td>4</td>
<td>5.71 ± .37</td>
</tr>
<tr>
<td>B. Parathyroidectomized -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. 8-hour Lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td>19</td>
<td>3.50 ± .01</td>
</tr>
<tr>
<td>b. Non-nephx.</td>
<td>15</td>
<td>3.26 ± .16</td>
</tr>
<tr>
<td>II. 12-hour Lavage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td>4</td>
<td>4.16 ± .28</td>
</tr>
<tr>
<td>b. Non-nephx.</td>
<td>5</td>
<td>4.20 ± .32</td>
</tr>
</tbody>
</table>

The standard lavage procedure, which involved eight hours of continuous lavage with a buffered rinse changed hourly, produced an increase in osteoclast count, with the degree of stimulation depending upon the presence or absence of the parathyroid gland and of the kidneys. No effect of lavage is seen in the parathyroidectomized, nephrectomized
rats. If the kidneys are functional, minor stimulation of osteoclasts can be produced in the absence of the parathyroids. The increase in osteoclasts is doubled, however, if the parathyroid glands are functional. Under these standard conditions, maximal response to the eight-hour lavage was obtained in nephrectomized rats with functional parathyroids.

If the lavage was extended to twelve hours, the only group showing an increased osteoclast stimulation over the eight-hour values was that group composed of rats with both intact kidneys and parathyroid glands.

In these experiments, the calcium values normally measured in the lavage fluid was 5.5 to 6.0 mg% for all parathyroid-intact animals, and 4.0 to 4.5 mg% for parathyroidectomized animals.

Short Discussion

Although the counting technique used in this study was slightly different from that used by Toft (he counted mononucleated osteoclasts), his results have been repeated. The results presented here and by Toft show that lavage and/or nephrectomy results in quantitative increased in osteoclast numbers (Toft, 1960). Only in the parathyroid-intact animal is the lavage-produced increase significant. When probability values are not given, a significant difference between two means implies that they are ±3 standard errors apart in value. The lavage stimulates secretion of parathyroid hormone because of the continuous removal of calcium by this procedure. The increased parathyroid secretion is accompanied by increase in osteoclast numbers, rate of bone resorption,
and liberation of calcium ions into the extracellular fluids. Nephrec-
tomy also stimulates parathyroid secretion (Talmage and Toft, 1961).
Removal of the kidneys results in high serum phosphate values and the
elevated $\text{Ca} \times \text{PO}_4^-$ product increases the rate of deposition of these
ions in bone, thereby lowering serum calcium levels. Increased para-
thyroid secretion results from the fall in blood calcium with the
subsequent stimulation in osteoclast activity.

It is interesting to note that the lavage raised slightly the
osteoclast count in the animal without parathyroids. This shows that
some parathyroid-like activity must still be present, which might be
explained by the presence of aberrant parathyroid tissue, or some
change in osteoclast formation not yet understood.
B. EXOGENOUS CALCIUM

Table II

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
<th>Lavage Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Non-nephrectomized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Parathyroid intact</td>
<td>6</td>
<td>3.44 ± .22</td>
<td>7.2 ± .0</td>
</tr>
<tr>
<td>b. Parathyroidectomized</td>
<td>4</td>
<td>3.35 ± .10</td>
<td>6.5 ± .1</td>
</tr>
</tbody>
</table>

The addition of calcium to the buffered lavage fluid, plus the pre-treatment of these animals with calcium lactate, prevented the increase in osteoclasts which normally occurs when a lavaged animal has intact parathyroids.

The calcium levels were extremely high under these conditions. It is interesting to note, however, that the calcium in the animal without parathyroids could not be increased to the same value as that of the parathyroid-intact group.

Short Discussion

The addition of exogenous calcium to these animals completely negated the stimulation of the parathyroids as measured by the osteoclast numbers. Because these results show a low osteoclast count in the presence of a high calcium level, it is reasonable to conclude that the demand on the animal's calcium supply determines osteoclastic activity and not the lavage procedure per se.
C. ACIDOSIS

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
<th>Lavage Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Parathyroid intact -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Nephx.</td>
<td>11</td>
<td>6.60 ± .08</td>
<td>6.5 ± .2</td>
</tr>
<tr>
<td>a. NaCl lavage</td>
<td>10</td>
<td>7.17 ± .11</td>
<td>5.9 ± .3</td>
</tr>
<tr>
<td>b. Buffer lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Parathyroidectomized -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Nephx.</td>
<td>5</td>
<td>2.92 ± .01</td>
<td>5.5 ± .2</td>
</tr>
<tr>
<td>a. NaCl lavage</td>
<td>10</td>
<td>3.52 ± .28</td>
<td>4.5 ± .1</td>
</tr>
<tr>
<td>b. Buffer lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The pH of the lavage fluid, when removed from the animals in this experiment, was checked hourly. In those animals that received the buffered wash, the pH of the rinse was 7.50 throughout the eight hour period. The pH of the rinse from the sodium chloride-treated animals, decreased from 7.40 to 7.00 during the same eight hour period.

This decrease in pH was accompanied by a significant increase in the calcium levels of the parathyroidectomized animals. A slight increase was also seen in the calcium level of the parathyroid-intact group, but this was not highly significant (P < .05).

Concerning the osteoclasts, the condition of acidosis resulted in a significant decrease (P < .001) of their numbers in the parathyroid-intact animals compared to those animals on buffered lavage fluid.
Short Discussion

When the parathyroid glands are removed, the calcium concentration in the extracellular fluid immediately decreases to a new value which is known as the "basic" level. The basic calcium level is maintained primarily by the physical and chemical processes that regulate calcium exchange between bone and the surrounding fluids (Talmage, 1962).

The result of making an animal acidotic (as evidenced by a decreased pH of the peritoneal wash) was an increased exchange of calcium from bone to extracellular fluids due to increased solubility of the bone salt (Talmage and Kraintz, 1961). Since the basic calcium level was increased (see Table III), less activity of the parathyroids was needed to maintain the normal calcium removal from bone. Associated with these factors was a lower osteoclast count in the acidotic animal.
D. FLUORIDE STUDIES

Table IV

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
<th>Lavage Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Parathyroid intact</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Buffer lavage</td>
<td>7</td>
<td>6.50 ± .21</td>
<td>5.9 ± .1</td>
</tr>
<tr>
<td>b. NaF lavage</td>
<td>7</td>
<td>7.74 ± .23</td>
<td>5.2 ± .2</td>
</tr>
<tr>
<td>II. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. NaF lavage</td>
<td>6</td>
<td>7.25 ± .33</td>
<td>5.4 ± .1</td>
</tr>
<tr>
<td><strong>B. Parathyroidectomized</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Buffer lavage</td>
<td>7</td>
<td>3.85 ± .14</td>
<td>4.0 ± .1</td>
</tr>
<tr>
<td>b. NaF lavage</td>
<td>7</td>
<td>3.81 ± .05</td>
<td>2.8 ± .2</td>
</tr>
<tr>
<td>II. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. NaF lavage</td>
<td>8</td>
<td>4.01 ± .23</td>
<td>3.2 ± .1</td>
</tr>
</tbody>
</table>

In the nephrectomized animals, the fluoride in the lavage fluid caused a highly significant drop (P < .005) in the calcium level in the parathyroidectomized animal. This decreased calcium level was reflected by the sixth hour in the decreased level in the parathyroid-intact rat. In the non-nephrectomized rat, similar decreases occurred.

The numbers of osteoclasts in the parathyroidectomized animals are essentially the same for fluoride and non-fluoride treated groups. However, the rats with intact parathyroid glands and lavaged with fluoride showed a markedly significant increase (P < .005) of osteoclasts over the non-fluoride treated animals. In fact, this is the only experiment
where the osteoclast count in the parathyroid-intact, non-nephrectomized, lavaged animal was as high as the nephrectomized, lavaged animal.

Short Discussion

The results with fluoride have shown that when NaF was added to the lavage fluid, the basic calcium value decreased. The Warburg studies indicate that this quantity of fluoride is not acting to block the metabolic processes in bone. Data from radioactive fluoride studies (Applegren, et al., 1961; Carlson, et al., 1960; Parkinson, et al., 1955; and Weidman, et al., 1959) indicates that the only tissues which significantly concentrate fluoride are the kidney and the metaphyseal regions of bone. F\textsuperscript{18} is out of the blood and concentrated in these tissues within minutes after the fluoride injection. Neuman (1958) has shown that fluoride decreases the solubility of hydroxyapatite crystals. This decreased reactivity may be due to the changes in crystallinity of the apatite crystal (Menczel, et al., 1962; Zipkin, et al., 1962). All of these factors would suggest then, that the observed decrease in the basic calcium level was due to the decreased solubility of the bone salts. The parathyroid-intact, fluoride-treated animal had to maintain normal calcium levels in spite of the decreased reactivity of the bone crystal; consequently, the parathyroids were stimulated more than in non-fluoride treated animals. This increased parathyroid secretion was accompanied by a large increase in osteoclast numbers.
E. HYPOPHYSECTOMY

Table V

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Hypophysectomized parathyroid intact)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Lavaged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) + cortisone</td>
<td>4</td>
<td>6.49 ± .13</td>
</tr>
<tr>
<td>II. Non-lavaged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) + cortisone</td>
<td>3</td>
<td>3.69 ± .21</td>
</tr>
<tr>
<td>(2) - cortisone</td>
<td>4</td>
<td>4.33 ± .48</td>
</tr>
<tr>
<td>b. Non-nephx.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) + cortisone</td>
<td>6</td>
<td>3.61 ± .37</td>
</tr>
<tr>
<td>(2) - cortisone</td>
<td>6</td>
<td>3.22 ± .41</td>
</tr>
</tbody>
</table>

The hypophysectomized animals that were lavaged but received no cortisone died before completion of the experiment. The rats on cortisone did survive the lavage and maintained normal calcium levels (5.8 ± .2). However, these animals had an osteoclast count which was lower than the non-hypophysectomized animal under the same conditions (see Table I).

In the non-lavaged, hypophysectomized group of animals, the nephrectomized rats, with and without cortisone, had osteoclast counts which were not significantly different from the non-hypophysectomy values (see Table I). In the non-nephrectomized, non-lavaged, hypophysectomized group, only those animals that received cortisone showed significantly different osteoclast numbers than the count usually obtained from non-nephrectomized, non-lavaged animals.
Short Discussion

In these short-term studies, it was shown that cortisone was adequate therapy to allow the maintenance of normal calcium levels in hypophysectomized rats. Since the calcium level was normal and there was an obvious increase in osteoclasts due to the lavage, the parathyroids must be actively secreting in hypophysectomized animals. It would seem from this rather limited data that parathyroid hormone production is not dependent \textit{per se} on the pituitary.
F. ADRENALECTOMY

Table VI

<table>
<thead>
<tr>
<th>Condition (Parathyroid intact)</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
<th>Lavage Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Lavaged</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) cortisone</td>
<td>6</td>
<td>3.93 ± .22</td>
<td>5.3 ± .2</td>
</tr>
<tr>
<td>(2) epinephrine</td>
<td>11</td>
<td>3.98 ± .25</td>
<td>5.1 ± .2</td>
</tr>
</tbody>
</table>

No results are noted here for adrenalectomized animals that were not given replacement therapy. These animals could not withstand the stress of the lavage and died before completion of the experiment.

The lavaged, adrenalectomized animals given cortisone or epinephrine maintained calcium and osteoclast values within the normal range (see Table I).

Short Discussion

In adrenalectomized rats with intact pituitaries, there was essentially a normal calcium and osteoclast response to the lavage. This would indicate that the adrenal secretions are not dominating factors in the parathyroid control of calcium metabolism.
Table VII

<table>
<thead>
<tr>
<th>Condition</th>
<th>Avg. Osteoclasts</th>
<th>Lavage Calcium</th>
<th>Hydroxyproline</th>
<th>Specific Activity $^{45}$Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Lavaged)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Parathyroid intact</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Estrogen treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx. (9)</td>
<td>6.99 ± .15</td>
<td>6.1 ± .2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Non-nephx. (13)</td>
<td>4.82 ± .26</td>
<td>5.7 ± .1</td>
<td>0.020 ± .002</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>II. Non-estrogen treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Non-nephx. (10)</td>
<td>4.39 ± .20</td>
<td>5.7 ± .1</td>
<td>0.036 ± .001</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>B. Parathyroidectomized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Estrogen treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Nephx. (5)</td>
<td>3.68 ± .23</td>
<td>4.0 ± .3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Non-nephx. (9)</td>
<td>3.05 ± .15</td>
<td>5.0 ± .1</td>
<td>0.019 ± .002</td>
<td>78 ± 6</td>
</tr>
<tr>
<td>II. Non-estrogen treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Non-nephx. (5)</td>
<td>3.26 ± .16</td>
<td>3.8 ± .1</td>
<td>0.026 ± .002</td>
<td>60 ± 2</td>
</tr>
</tbody>
</table>

Number of animals given in parentheses.

The estrogen-treated animals that were nephrectomized prior to lavage showed the same calcium levels and increased osteoclast numbers as the non-estrogen-treated rats (see Table I). This was true for the parathyroid-ectomized and parathyroid-intact animal.

The results obtained from the non-nephrectomized animals in this group were more unique. The osteoclast numbers and the calcium level in those animals with intact parathyroids were the same as found in
non-estrogen-treated animals under the same conditions. However, in the parathyroidectomized rats, the calcium level was significantly higher than similar non-estrogen-treated animals, even though the osteoclast count was the same.

The pre-treatment of these rats with estrogen also caused a change in the specific activity of Ca\(^{45}\) (injected 24 hours prior to lavage) in the circulating fluids. The parathyroid-intact and parathyroidectomized estrogen-treated animals showed higher specific activity of their calcium values, compared to the non-estrogen animals.

Estrogen treatment resulted in significantly lower amounts of circulating hydroxyproline in both the parathyroidectomized and parathyroid-intact animals.

The body weight of these male animals was significantly affected by the estrogen administration. The rats injected with sesame oil gained an average of 42.5 grams/week, those given a total of 2 mg. Delestrec gained 30.8 grams/week, and those given 4 mg. Delestrec gained 30.0 grams/week.

**Histology of the Estrogen Bones:**

The morphology of the longitudinal sections of femurs from estrogen-treated rats showed that the trabeculae were not resorbed during growth of the young animal (Figs. 8 and 9). Evidence of excessive bone accretion due to estrogen administration was evident in longitudinal and cross sections of the femur. Osteoclasts were seen in abundance in the trabecular regions studied.

It was noted in the normal non-estrogen animal that the osteoclasts, using the tetrachrome stain, showed varying degrees of cytoplasmic
basophilia (Figs. 1-6). The non-estrogen-treated bones showed some osteoclasts with very few ameboid-like cytoplasmic extensions, while other cells had a highly vacuolated appearance with many extensions of its "frothy" cytoplasm.

In the estrogen-treated animals, the cytoplasm of the osteoclast tended toward acidophilia and vacuolation was often not seen. In many cases, the nuclei were polygonal and their outlines were indistinct. Osteoclasts in the same seven micron section often showed different degrees of cytoplasmic acidophilia (Fig. 7). On rare occasions, one could see the cytoplasm of the same osteoclast transcending from acidophilia to basophilia.

It is recognized that the stain technique used was not quantitative and determination of the acidophilia was open to subjectiveness. The assumption was made that the red blood cells in the tissue section would show about the same amount of acidophilia for each estrogen experiment. The erythrocyte's acidophilia was then used as a crude index to give an indication of the osteoclasts acidophilia. The results of this indicated that estrogen resulted in more acidophilia of the osteoclast's cytoplasm in the non-nephrectomized than in the nephrectomized animal.

Short Discussion

Because there was such a large weight difference between the animals that were estrogen-treated and those that were not, it was thought that the hydroxyproline and calcium differences might be due to differences in animal size rather than to the steroid. An identical lavage
was run using different size rats but not including any estrogen animals. The results of this showed that the large and small parathyroid-ectomized animals did not differ in maintaining identical calcium and hydroxyproline levels. The large and small parathyroid-intact rats also maintained identical results to each other. This confirmed the idea that the changes seen in the estrogen animal were due to the steroid and the changes it caused in bone, and not to some other factor.

As was pointed out earlier, there is abundant evidence in the literature that estrogens will inhibit the resorption of metaphyseal trabeculae. The results from this experiment support that same conclusion, as evidenced by inhibition of normal body weight gain, and the presence of more trabecular bone. Additional evidence obtained in non-nephrectomized animals showed: (1) reduced levels of circulating hydroxyproline; (2) decreased basophilia of osteoclastic cytoplasm; and (3) increased specific activity of the lavage calcium. However, with all these indications of decreased bone resorption, the lavage technique and nephrectomy still stimulated osteoclastic proliferation.

Although estrogens inhibit the resorptive process, there have been no studies to indicate if the inhibition can be overcome by stimulating parathyroid activity. The results from this study show that parathyroid activity still occurs in the estrogen-treated animal, but there is no evidence as to whether bone resorption by osteoclasts is still possible. In the non-nephrectomized, estrogen animals, the decreased basophilia of the osteoclast's cytoplasm would argue in favor of decreased activity of these cells. A complete analysis of this experiment is dependent upon determining whether the decreased basophilia of these cells is associated with decreased resorption, such as was found in the osteoclasts of the ia rat strain (Bhaskar, et al., 1960).
H. Ca\textsuperscript{45} RADIATION

Table VIII

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclast</th>
<th>Lavage Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Parathyroid intact)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Lavaged -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 50 μc.</td>
<td>2</td>
<td>3.79 &amp; 2.14</td>
<td>6.2 &amp; 4.7</td>
</tr>
<tr>
<td>b. 1 mc.</td>
<td>2</td>
<td>3.37 &amp; 2.26</td>
<td>5.9 &amp; 4.7</td>
</tr>
<tr>
<td>c. No - Ca\textsuperscript{45}</td>
<td>2</td>
<td>5.58 &amp; 4.41</td>
<td>5.9 &amp; 5.5</td>
</tr>
<tr>
<td>B. Non-lavaged -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 50 μc.</td>
<td>1</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td>b. 1 mc.</td>
<td>1</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td>c. No - Ca\textsuperscript{45}</td>
<td>2</td>
<td>3.40</td>
<td></td>
</tr>
</tbody>
</table>

The results of this project could not be fully appreciated because of the few number of animals that were used. Because of the expense involved in purchasing this much radiocalcium, it was not feasible to try this experiment on a larger group of animals.

The weight changes in these rats seemed to reflect the amount of radioactivity each one carried. For example, the non-treated controls had an average weight gain of 60 grams over the three week period, while the 50 μc. group averaged a gain of 23 grams and the 1 mc. group had an average loss of 10 grams.

In the lavaged animals, the osteoclast count in those treated with Ca\textsuperscript{45} was lower than the non-radioactive rats. The calcium levels in all three lavaged groups were essentially the same.
In the non-lavaged group, the radioactivity again seemed to lower the osteoclast count. In this group as well as the lavaged group of animals, the radiocalcium did such extensive damage to all the bone studied that the validity of these results is certainly questionable.

The Ca$_{45}^+$ removed in each lavage showed no difference in the removal rate between those animals that received 50 μc. and those that received one millicurie.

Histology of the Ca$_{45}^+$ Radiated Bones:

The morphology of the bone tissue from the 50 μc. group was not noticeably different from the non-injected animals, except for the number of spindle shaped cells present in the Ca$_{45}^+$ group. The long bones showed in longitudinal section that there was fibrous tissue and spindle cells interspersed among the bone trabecular, but it was not found in wide areas of the metaphysis (Fig. 10).

The morphology of those bone tissues radiated with 1 mc. of Ca$_{45}^+$ was greatly different from that of the non-injected controls. The longitudinal sections through the metaphyseal region of the femurs of these animals showed that the normal cellular elements were displaced with fibroblasts and spindle cells (Figs. 11 and 12). Osteoclasts were still present in the peripheral regions adjacent to these areas of fibrous tissue. Myeloid elements and osteoblasts were definitely below their normal numbers. The osteons often appeared abnormal in that the osteocyte and the bone matrix were not always closely apposed and quite frequently the osteocyte was absent from the Haversian system (Fig. 13).
Short Discussion

In this experiment, although a minimum number of animals were used, an increase in osteoclasts was noticed. In fact, the group radiated with the higher dosage (1 mc.) had 25% more of an increase after stimulation than did the 50 microcurie radiation group. However, it is this 1 mc. group that had the lowest osteoclast quantities throughout the experiment.

The osteoblasts are thought to be the bone cells most susceptible to internal radiation (Vaughn, 1962); and in fact, in areas of obvious damage, these cells were absent. The osteocytes are also highly susceptible to radiation, and were frequently absent in the trabeculae of the radiated animal. In areas where only fibrous tissue predominated, the osteoclasts were not found, but on the edges of these areas, this cell was found in large numbers. It has generally been true in areas of active resorption that bone is replaced by fibrous tissue (Selye, et al., 1963). These fibrous areas as seen in longitudinal section correspond closely to the same region that is used when the quantitative osteoclast count is used. Therefore, by taking sections further down from the epiphyseal plate, a larger number of osteoclasts could be located.

This experiment shows that with the osteoclast functioning and the osteoblasts of the metaphysis possibly disabled, the radiated animal could still maintain almost normal calcium levels when placed under calcium stress. However, the exact significance of the osteoclastic activity cannot be determined because of the change in their
population and the unknown role that is played by the bone cells in other areas of the organism. Nevertheless, we can conclude that the osteoclast is much less susceptible to radiation than other bone cells, and after significant radiation damage to the metaphysis, the osteoclast still responds to parathyroid activity.
I. $^{239}$Pu RADIATION

Table IX

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of Animals</th>
<th>Average Osteoclasts</th>
<th>Lavage Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>(Parathyroid intact)</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. 8-hour Lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 5 day</td>
<td>4</td>
<td>5.74 ± .31</td>
<td>5.8 ± .1</td>
</tr>
<tr>
<td>b. 24 hour</td>
<td>4</td>
<td>4.06 ± .31</td>
<td>5.6 ± .1</td>
</tr>
<tr>
<td>c. No inj.</td>
<td>6</td>
<td>5.60 ± .36</td>
<td>6.1 ± .2</td>
</tr>
<tr>
<td>B. 4-hour Lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 5 day</td>
<td>4</td>
<td>4.04 ± .48</td>
<td>5.7 ± .1</td>
</tr>
<tr>
<td>b. 24 hour</td>
<td>4</td>
<td>3.37 ± .51</td>
<td>5.2 ± .0</td>
</tr>
<tr>
<td>C. Non-lavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III. Non-nephx.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. 5 day</td>
<td>4</td>
<td>4.47 ± .40</td>
<td></td>
</tr>
<tr>
<td>b. 24 hour</td>
<td>4</td>
<td>3.37 ± .22</td>
<td></td>
</tr>
<tr>
<td>c. No inj.</td>
<td>6</td>
<td>3.39 ± .08</td>
<td></td>
</tr>
</tbody>
</table>

These animals were all non-nephrectomized but were of a different strain and on a slightly different regimen while at Argonne Laboratory. Their osteoclast values are higher than the standard values obtained from the Holtzman rat (see Table I).

The osteoclast numbers are slightly lower in the animals injected 24 hours prior to 8-hour lavage, as compared to the non-injected ($P < .025$) and five day injected groups ($P < .025$). The 24-hour injected animals also have slightly lower calcium values; however, it is not highly significant ($P < .1$). All three groups show the same level of free hydroxyproline.
Comparing the non-lavaged rats to the lavaged ones, it is noticed that there is essentially no increase in osteoclast numbers in the 24-hour injected group. The increase in osteoclast numbers in the 5-day injected rats, lavaged vs. non-lavaged, is certainly significant. The increased numbers found in this 5-day injected group is seen to occur during the last four hours of lavage, which is typical for the normal response.

Although the two groups of calcium values from the 4-hour lavage are significantly different, the results from the first four hours of the 8-hour lavage show no differences. The small number of samples in the 4-hour lavage may have given erroneous results.

Histology of Pu\(^{239}\) Radiated Bones:

It was noticed that the 8-hour lavaged, 5-day injected animals had more Pu\(^{239}\) concentrated in each osteoclast (Figs. 15 and 16). In the other injected animals, the alpha tracks were spread throughout the matrix and cellular components of the bone (Fig. 14). It seems then that Jee's (Arnold and Jee, 1957) estimate of five days before Pu\(^{239}\) is concentrated in all osteoclasts is about right. Single or double tracks of an alpha-particle are seen in about 50% of all osteoclasts after twenty-four hours, but there is no heavy concentration of radioactivity in matrix or bone cells.

Short Discussion

Plutonium, like several other radioactive elements, will be immediately taken up by those areas of bone that are the most metabolically active. Unlike other bone-seeking elements, however, we know very little about the physiological actions of plutonium.
There is no doubt that Pu$^{239}$ can cause internal radiation damage to bone (Jee, et al., 1962). With the doses used in this experiment, it would have to be left for long periods of time (certainly longer than five days) before producing noticeable morphological changes in bone. Its destructive power is due to the high energy of the alpha particle which plutonium emits. In bone, alpha particles may have their initial effects on the osteoblasts and those cells undergoing mitosis (Jee and Arnold, 1961). In radiation studies with other alpha and beta emitting sources, the osteoblasts and osteocytes are affected before the osteoclast; whether that is the case in this experiment, we cannot say. It seems possible, however, that since the 24-hour injected animals showed less osteoclastic proliferation, the precursors of these cells are more affected by the irradiation than are the osteoclasts themselves. All of the groups, with and without Pu$^{239}$, showed the same level of free hydroxyproline, and almost the same calcium values. Using these parameters, it seems that bone resorption is probably not decreased by this dose of plutonium. However, this does not preclude the possibility that since osteoblasts are affected by radiation first, their bone accretion ability may be temporarily halted and more osteoclasts are not so necessary at this time. Also, it may be that in unusual conditions (e.g., radiation and estrogens) osteoclastic proliferation is not synonymous with increased osteoclastic bone-resorbing activity.
Combining the results of various experiments studied in this thesis, it was found that parathyroid activity was closely correlated with osteoclastic proliferation. These experiments included alterations of the bone crystal, bone matrix, and bone cells. In all cases, the parathyroid-intact animal maintained calcium concentrations that were within a normal range.

The results indicate that the parathyroids are completely regulated by the circulating calcium ions. The pituitary and adrenal hormones seem to have only a minor effect on parathyroid activity.

The question that was asked at the beginning of this work was whether maintenance of calcium levels was dependent upon osteoclastic activity. The arguments against their being necessary are:

1. In lavaged animals, the noticeable increase in osteoclast numbers occurs six hours after calcium levels have been maintained.

2. Selye (1963) can produce osteitis fibrosa in parathyroidectomy-ized animals, with associated increases in osteoclast numbers.

3. Some of the present studies (Table I, 12-hour vs. 8-hour lavage; Table II, acidosis vs. buffered) shows that the osteoclast numbers may vary irrespective of the calcium values even in the parathyroidectomized animals. However, these are not significant figures.

4. Some of the radiation experiments showed low osteoclast numbers, but normal calciuims.

Some of the arguments for the necessity of the osteoclastic proliferation are:
1. Early workers like Burrows (1937), and Pugsley and Selye (1933), showed that serum calcium and calcium excretion went to a lower value after the osteoclast numbers decreased.

2. In the fluoride-treated, non-nephrectomized animal (Table IV), the osteoclasts were stimulated very greatly as the calcium had a tendency to fall below normal.

3. When exogenous calcium was present, the osteoclasts were not stimulated at all.

In general, it appears that the stimulation for osteoclast proliferation may be the degree of calcium stress which the animal is under and not the parathyroid hormone itself. However, since this hormone is responsible for the maintenance of the calcium level, it is probably indirectly responsible for changes in osteoclast numbers. One way to separate this close association might be to parathyroidectomize an animal which had a large number of active osteoclasts to determine whether or not calcium levels fell as rapidly as in parathyroidectomized animals without the stimulated osteoclasts.

This problem could be further resolved by the aid of the electron microscope. As was discussed earlier in the text, some of the problems with osteoclasts could be resolved if the workers knew whether the cells were active at the time they were studied. This same problem was noticed in the interpretation of the estrogen experiment. Radioautography, in combination with electron microscopy, is still in the early stages of development, but might be useful in determining the ultrastructure of the osteoclast in various stages of activity. The variations in the literature on osteoclast ultrastructure may be due to variations in
activity of these cells when they were studied. This technique would also be beneficial in determining if labelled collagen and calcium were actually taken up by the osteoclast's cytoplasm.

From the evidence produced by Kenny (1959, 1961), it appears that the pH changes produced in bone are caused by lactate, but that actual bone resorption is dependent upon citrate accumulation. Histochemical studies combined with electron microscope observations might aid in clarifying this relationship. The new technique of using paper chromatography on tissue sections might help to isolate various chemical components in areas adjacent to the region used for ultrastructure studies.

In conclusion, this study has shown that the interrelationship between parathyroid activity and osteoclast numbers may be regulated via the circulating calcium levels. In conditions of very extreme calcium stress, it may be that osteoclasts increase even in the absence of parathyroid glands. In cases such as this, the osteoclast may be an alternate mechanism for maintenance of the calcium concentration; whereas, normal demands on bone tissue would not be dependent upon osteoclastic proliferation. A corollary to this is that calcium metabolism is maintained in spite of wide variations in osteoclast numbers. A final answer to the question must wait until a correlation is made between the activity of these cells and the proliferation of osteoclasts.
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Fig. 1

A degenerate osteoclast with oxyphilic cytoplasm.
Other osteoclasts present are basophilic.

(480X)

Fig. 2

A large osteoclast which contains an erythrocyte
(arrow).

(480X)
Fig. 3

Arrows indicate an osteoclast which extends into an osteocyte so that the two cells are indistinguishable. (200X)

Fig. 4

On the border, opposite the active surface of this osteoclast are granules (arrows) which contain PAS-positive stained material. (480X)

Fig. 5

The ruffled border (arrow) of this osteoclast is shown in association with an absorption lacunae. Also note the rouleaux of erythrocytes near the osteoclast which indicates the close relationship between these bone cells and the blood supply. (480X)
Fig. 6

An oxyphilic osteoclast with well-formed nuclei, but serial sections show no cytoplasmic extensions. The degree of activity of this cell is unknown.

(480X)

Fig. 7

This section is from an estrogen-treated animal and shows osteoclasts (arrows) with different degrees of basophilia.

(200X)
Fig. 8

The longitudinal section of the femur from a control rat. The top of the picture is located immediately beneath the epiphyseal plate, and some trabecular bone (tb) is shown scattered through the marrow.

(18x)

Fig. 9

The longitudinal section of the femur from an estrogen-treated rat. The top of the picture is located immediately beneath the epiphyseal plate and trabecular bone (tb) occupies much of the space previously filled with marrow.

(18x)
Fig. 10

A longitudinal section from the femur of a rat, three weeks after injecting with 50 microcuries of Ca$^{45}$. This view shows the abnormal bone (ab) produced by the radiation and contrasts it against the normal trabecular bone (tb). A portion of the epiphyseal cartilage (ec) can also be seen.

(50X)

Fig. 11

The longitudinal section from the femur of a rat, three weeks after injecting one millicurie of Ca$^{45}$. More abnormal bone (ab) is present beneath the epiphyseal cartilage (ec) than in the group given 50 microcuries of Ca$^{45}$. Trabecular bone (tb) has been extensively resorbed and replaced by fibrous tissue.

(50X)
Fig. 12

Higher magnification of the fibrous tissue and abnormal bone seen in Fig. 11. The cellular elements (arrows) are fibroblasts and spindle cells.

(200X)

Fig. 13

A cross section through some trabecular bone from the femur of the animals injected with 1 millicurie of Ca$^{45}$. This bone tissue shows unusual osteocytes (arrows) and perhaps some residual cartilage cells. The blood sinusoid (S) is lacking in erythrocytes and the myeloid elements (m) have degenerated.

(200X)
A cross section through the distal metaphysis of the femur from an animal injected five days before sacrifice with 1 microcurie of Pu$^{239}$. The alpha tracks, in general, are associated with the cellular elements of bone rather than the matrix. The highest concentration of plutonium is found in the osteoclasts.

(200X)

Higher magnification of an osteoclast from Fig. 14, showing the alpha tracks originating from the radioactive element within the cytoplasm.

(480X)