INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48108-1346 USA
800-521-0600

UMI
NOTE TO USERS

Page(s) missing in number only; text follows. Microfilmed as received.

62

This reproduction is the best copy available.

UMI
THE RICE INSTITUTE

QUANTITATIVE RELATIONSHIP OF OSTEOCLASTS
TO PARATHYROID FUNCTION

by

Robert Jens Toft

A THESIS
SUBMITTED TO THE FACULTY
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Houston, Texas
June, 1960
ACKNOWLEDGEMENT

I wish to thank Dr. Roy V. Talmage for his support of this project. His guidance and patience were greatly appreciated.

For technical assistance in certain phases of this work I wish to thank the staff of Dr. Talmage's laboratory.

Finally I would like to express my appreciation to the Atomic Energy Commission for their continued support of this project under a grant to Dr. Talmage.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>The Parathyroids</td>
<td>3</td>
</tr>
<tr>
<td>Bone</td>
<td>8</td>
</tr>
<tr>
<td>Parathyroids and Bone</td>
<td>19</td>
</tr>
<tr>
<td>Feed-back Mechanism</td>
<td>31</td>
</tr>
<tr>
<td>Statement of the Problem</td>
<td>40</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>41</td>
</tr>
<tr>
<td>General</td>
<td>41</td>
</tr>
<tr>
<td>Nephrectomy</td>
<td>42</td>
</tr>
<tr>
<td>Lavage</td>
<td>43</td>
</tr>
<tr>
<td>Histology</td>
<td>45</td>
</tr>
<tr>
<td>Statistics</td>
<td>46</td>
</tr>
<tr>
<td>RESULTS</td>
<td>47</td>
</tr>
<tr>
<td>Osteoclasts as an Index of Parathyroid Activity</td>
<td>47</td>
</tr>
<tr>
<td>Parathyroid Activity in Nephrectomized Rats</td>
<td>50</td>
</tr>
<tr>
<td>Lavage Studies Utilizing Variable Calcium and Phosphate Levels</td>
<td>55</td>
</tr>
<tr>
<td>Calcium- and Phosphate-free Lavage</td>
<td>55</td>
</tr>
<tr>
<td>Calcium-free and High Phosphate Lavage</td>
<td>57</td>
</tr>
<tr>
<td>High Calcium and Phosphate-free Lavage</td>
<td>57</td>
</tr>
<tr>
<td>High Calcium and High Phosphate Lavage</td>
<td>58</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>60</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>69</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>71</td>
</tr>
</tbody>
</table>
INTRODUCTION

Over the last decade the tempo of research on the parathyroid gland and its relationship to metabolism has increased rapidly. As new advances in our knowledge of calcium and phosphorus metabolism are made, old ideas and theories must be re-evaluated and some modified, others discarded. New concepts of parathyroid function are being brought forth from the mass of literature which is accumulating.

A reversal of thought is taking place regarding both the primary site of action of the parathyroid hormone and the mechanism of control of its secretion. For years it was stated that the primary action of the hormone was to regulate the kidney threshold for phosphorus excretion. In keeping with this idea, it was also felt that the phosphate level of the serum was the direct controlling agent of parathyroid hormone secretion.

Enough evidence has now accumulated to show that the primary action of the parathyroid hormone is probably on bone rather than on the kidney. In addition, it is now widely accepted that the serum calcium level controls the secretion of the hormone. To date there has been a general lack of evidence for the exclusive control of hormone secretion by calcium. This is due, in large measure, to the lack of an adequate index of endogenous parathyroid activity. It has been heretofore difficult, if not impossible, to adequately assess the degree of parathyroid secretion under a number of physiological conditions.
It is hoped that the work presented herein will prove helpful in providing additional evidence in support of the latter proposition.
THE PARATHYROIDS

In mammals there are usually two pairs of parathyroid glands. They are derived from the third and fourth pharyngeal pouches and come to lie in the thyroid such that the pair from the fourth pharyngeal pouches is anterior to the other pair. In the rat only those derived from the third pair are present. These lie on the ventral surface of the thyroid and are readily seen by examination of the surface of the thyroid.

The glands are enclosed in a connective tissue capsule and are supplied with blood from the inferior and superior thyroid arteries. The only innervation appears to be nerve fibers ending on the blood vessels for vasomotor control (Turner, 1955).

The parenchymal cells of the normal parathyroid gland are of two general types. The larger of these two types tend to be polarized with a basal nucleus and apical golgi apparatus. The mitochondria are unoriented and the cytoplasm is moderately acidophilic (Davis, 1959).

Rosof (1934) and DeRobertis (1941) viewed the second cell type, which is smaller, more acidophilic, and with denser nuclei, as being a terminal stage of a secretory cycle. This opinion was held also by Benseley (1947). On the basis of abnormally vacuolated endoplasmic reticulum and swollen mitochondria present in electron micrographs, Davis (1959) suggested that these smaller cells are senescent or dying.

Lipid droplets are randomly distributed throughout the cell. Trier (1958), working with monkey parathyroids, and Davis (1959), working with rat glands, have reported the presence of colloid-like
granules in the cells. These are possibly secretory inclusions (Davis, 1959). Since the hormone of these glands has been characterized as a protein or polypeptide, it is conceivable that this colloid-like substance is either the hormone or its precursor. More work will be necessary to elucidate such a relationship.

Hypertrophy of the parathyroid glands is rare and can be produced only with long-term diet regulation in the normal animal. However, bilateral nephrectomy will cause marked hypertrophy of the parathyroids within 48 hours. Baker (1945) reported a 45-fold increase in mitoses, hypertrophied cells, and a 32% enlargement of the gland within 48 hours after bilateral nephrectomy in rats. Weymouth (1957) and Lever (1958) reported that these hypertrophied cells have increased cytoplasmic basophilia. Baker (1945) reported that the golgi apparatus was rounded up and in a juxtanuclear position as opposed to the apical position described above for the normal cell. In addition to this rounded configuration, Davis (1959) reported another golgi form with dispersed, highly branched filaments. It is reported by Davis (1959) that the appearance of the hypertrophied cells is, in general, a homogeneous one. Thus, in extreme hyperfunctional states, all cells of the parathyroid are alike.

Although the parathyroid glands were first described in 1880 by Sandstrom, they were not mentioned extensively until Gley (1891) rediscovered the external pair and Kohn (1895) again described the internal pair. Vassale and Generali (1900) proved the relationship of the parathyroids to tetany. The relationship of these glands to calcium metabolism was first established by MacCallum and Voegtlin
(1909). With the preparation of an extract of the gland (Hanson, 1924; Collip, 1925) it was possible to look for the role of the parathyroid in body metabolism.

It is well established that the parathyroids function through their hormone, to control the calcium metabolism of the body. There is still argument as to how this is accomplished. The two primary sites of action are the bones and kidneys. A summary of the effects of the parathyroids on bone will be given below. For work concerning the kidney action of the parathyroid hormone the reader is referred to the following works: Albright and Reifenstein, 1948; Handler et al, 1951; Davies and Gordon, 1953; Bartter, 1954; and Greep and Talmage, 1960.

Having established the primary sites of action for the hormone, the question remains as to the control of the secretion of the hormone by the gland. There have been three principal theories proposed concerning this control. The first relates the secretion of the hormone to nervous control. In his study of the parathyroids, Raybuck (1952) intimated that the nerve fibers entering the gland actually had endings on the individual cells and that these fibers controlled the secretion of the cells. However, several transplantation and tissue culture studies have shown that the gland can produce its hormone when completely removed from its nervous connections (Barnicot, 1948; Chang, 1951; Gaillard, 1955, 1959; and Davis and Talmage, 1960).

A second theory of control for the parathyroids was that advanced by Anselmino et al (1934) in which they proposed that the pituitary controlled the output of parathyroid hormone. Although it has been shown that there is mild hypertrophy after hypophysectomy (Baker, 1942;
Weymouth, 1957) Baker has suggested that this is an indirect effect since it does not parallel the dramatic changes produced in the thyroids, gonads, and adrenals. It is to be expected, however, that the hypophysis would have an indirect effect on the parathyroids through its influence on general metabolic rate as mediated through the thyroid (Carnes et al., 1943; Tornblom, 1949). This effect is probably related to changes in phosphate levels and will be discussed in a later section.

The theory accepted by most workers is that which proposes the control of parathyroid secretion to be by the level of circulating metabolites. The two substances most suspected of controlling parathyroid secretion are the obvious ones, phosphate and calcium ions.

One of the earliest suggestions that serum calcium might be responsible for the control of parathyroid secretion is attributed to Ham and co-workers (1940). They concluded from diet studies that hypocalcemia and not hyperphosphatemia was the primary cause of physiological hypertrophy of the parathyroid glands. The work of Carnes et al (1942) and Stoerk and Carnes (1945) substantiated the work of Ham et al and again pointed to a low calcium level as being the stimulus for parathyroid hypertrophy in diet studies. Certainly the most direct evidence for calcium being the controlling factor was that given by Patt and Luckhardt (1942). They reported that perfusion, with decalcified blood, of the thyroid-parathyroid apparatus of dogs caused the elaboration of a substance into the perfusate which brought about an increase in plasma calcium when injected into another animal. In accord with this premise, Søngfeldt et al (1954), as a result of
their diet studies, suggested that increased phosphate levels were not the stimulus for increased parathyroid secretion. In opposition to this view is the work of Drake et al. (1937) and of Helfet (1940) who pointed to hyperphosphatemia as the stimulus for parathyroid secretion. As mentioned previously, the work of Baker (1945), Weymouth (1957), and Lever (1958) on the hypertrophy of the parathyroid glands might be taken as evidence for phosphate being the controlling factor. The studies of Tornblom (1949) and Crawford et al. (1950) also carried the suggestion that an increase in plasma phosphate was responsible for parathyroid stimulation. It is hoped that the data to be presented in this thesis will help clarify the relative positions of calcium and phosphate in controlling parathyroid secretion.
It has been mentioned previously that one of the primary sites of action of the parathyroid hormone is bone. That is to say, the hormone has an effect on a special kind of connective tissue which, in the words of Pritchard (1956),

"...is characterized by the presence of cells with long branching processes (osteocytes) which occupy cavities (lacunae) and fine canals (canaliculi) in a hard, dense matrix consisting of bundles of collagenous fibers in an amorphous ground substance (cement) impregnated with Calcium phosphate complexes."

While such a compact statement does not give an all-inclusive definition of bone, it does, however, point out the complexity of the tissue involved.

To attempt a discussion of each element of bone with some degree of accuracy and completeness is beyond the scope of this work. For the sake of brevity and clarity only those few elements intimately concerned with the problem of this study will be considered. For a more comprehensive view of bone as a tissue the reader is referred to McLean and Urist (1955), Bourne (1956), Neuman and Neuman (1958), as well as to standard histology texts. The three elements to be explored in the present discussion are the organic matrix, the mineral of bone, and certain cellular components.

**Organic Matrix.** The organic matrix of bone is composed primarily of collagen, a fibrous protein common to all types of connective tissue. Collagen makes up about 95% of the dry fat-free weight of the organic material in bone. It occurs in long fibers about 800 Angstroms wide which exhibit cross-banding at intervals of about 640 Angstroms (McLean, 1958). In adult bone the fibers are oriented
parallel to the long axis of the bone, but in embryonic tissues they
tend to be unoriented (Glimcher, 1959). From electron microscope
observations it is believed that bone collagen is secreted in soluble
form by osteoblasts as molecular units, with the capability of self-
aggregation (McLean, 1958).

The remainder of the organic matrix of bone is called the
ground substance. It constitutes less than 5% of the matrix, and is
found occupying the spaces between the collagen fibers and the apatite
crystals. It is generally spoken of as being amorphous. Ground
substance occurs in a continuum between the well-structured basement
membrane and the interstitial fluid. This extension of the form of
the ground substance allows for the continuous transfer of ions and
other substances from the blood. Present insight into the composition
of connective tissue ground substance is meager. Ground substance
from connective tissue has not yet been isolated and analyzed by chem-
ical means. Its constituents are inferred from histochemical tests
and from data regarding the composition of ground substance from other
tissues.

There appears to be one or more acid polysaccharides present in
the organic matrix, possibly in a high-molecular state as either hyal-
uronic acid or chondroitin sulfate (Sylven, 1956). Carbohydrate
components such as glucose for nutritional purposes, blood group
polysaccharides, and serum mucoproteins are also present (Sylven, 1956).

Extraction of several connective tissue ground substance
components has yielded polysaccharides mostly obtained as protein
complexes, often together with large amounts of other water-soluble
proteins of unknown origin. The proteins of the ground substance have been, as yet, poorly characterized. More will be said about them in a later section.

Among the other constituents of the ground substance are the electrolytes and water. The amount and kind of electrolytes are variable. Since the ground substance is in contact both with the extracellular fluids and with the bone mineral itself, it would be expected that the ionic composition of this substance would reflect that of both bone and blood. This is the case as reported by Sylven (1956). The presence of sodium, chloride, potassium, calcium, and magnesium has been demonstrated by many workers. In addition, the large amount of chondroitin sulfate present may offer partly ionized sulfate and carboxyl groups and may account for the cationic binding capacity of this substance (Neuman and Neuman, 1958). Much of the water found in the ground substance is considered "bound" to protein and to polysaccharides forming an hydration phase (McMaster and Parsons, 1950). There are, in addition to the above-mentioned substances, various metabolites passing either way between the cells and the vascular system. It should be pointed out that, taken as a whole, the ground substance is a very dynamic material, taking part in many of the physiological activities controlled both by the adrenal and the parathyroid glands.

**Bone Mineral.** Analytically, on a dry-weight basis, bone consists of 65 to 70% of the inorganic crystals of the calcium-phosphate salt, apatite. DeJong in 1926 demonstrated by x-ray diffraction that the crystal structure of bone mineral was similar to that of the apatites,
and more specifically hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ (Glimcher, 1959). The exact nature of the apatite in bone is still unknown due to difficulties arising from deviation of the Ca/P ratio from the theoretical ratio for hydroxyapatite. The Ca/P molar ratio of all apatites should be 1.67. Unfortunately, the composition of most apatite preparations has a Ca/P ratio varying from 1.3 to more than 2.0. These all exhibit the x-ray diffraction pattern of apatite, however (Neuman and Neuman, 1958).

There have been three main mechanisms proposed by which the composition of the bone mineral might vary without markedly affecting the apparent structure. McConnel and co-workers (1952) feel that substitution of one ion or group for another within the lattice (especially $\text{H}_3\text{O}^+$ for Ca) accounts for the deviation. Posner and associates (1954) propose that the presence of unsubstituted defects in the internal lattice are responsible for the varying Ca/P ratios. Finally, Hendricks and Hill (1942, 1950) favor the idea that surface substitution, exchange, or adsorption might account for the observed differences in Ca/P ratios. Neuman and Neuman (1958) feel that all three views are, in part, correct and that all may contribute to the observed picture.

The actual structure of the mineral as it occurs in bone has not been determined. One group of workers holds to the view that the bone mineral is essentially hydrated calcium triphosphate. Dallemagne (1956) terms this alpha tricalcium phosphate and gives it the formula $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{H}_2\text{O}{\text{H}}_2$. He says that the mineral of living bone is in an unstable form; that it is kept in this unstable form
by its close association with the organic matrix; that the characteristics responsible for its instability are also responsible for its reactivity in the living organism; and that on removal from the bone, except under very special precautions, it is transformed into the stable hydroxyapatite, which is then identical with preparations of this mineral prepared synthetically.

A description of the ultrastructure of the reactive crystals and their surroundings may lead to a better understanding of the phenomena of ion transfer or ion exchange between the crystals of bone mineral and circulating fluids of the body. For descriptive purposes the unit of bone mineral will be the crystal complex made up of a microcrystal of colloidal dimensions with an enormous surface to mass ratio. These microcrystals are a few hundred Angstroms long and only 20-50 Angstroms in thickness (McLean, 1958). Each ion which is held on the surface of the crystal is surrounded by oppositely charged ions on one side only; the effect being a residual charge on the crystal surface. Because of their small size, these crystals can attain greater stability by chemisorption.

While this leads to a highly variable surface composition, it also results in increased reactivity of the crystal complex (Neuman and Neuman, 1958). Around the crystal and partly bound to its surface there is also a layer of hydrated ions and a shell of water which may exceed the dimensions of the crystal itself. McLean (1958) reports that synthetic apatite crystals may bind 0.8 grams of water per gram of crystal. This represents a volume of water greater than that of the crystal due to difference in density. Neuman and Neuman
(1957) have viewed each crystal complex as having, where adequate hydration is possible, a series of layers. Ions from the surrounding fluids can diffuse into the outer layers of the hydration shell. At the crystal surface, they state, there is a bound layer of hydrated calcium, phosphate, and hydroxyl ions, constantly interchanging with similar ions in the surface layer of the lattice. Since there are vacancies in the lattice itself there is a constant, though slower, ion interchange within the crystal itself. According to Neuman and Neuman (1957) potassium and chloride ions can penetrate the hydration layer only. Sodium, uranium, carbonate, and citrate ions can penetrate the surface-bound layer of the lattice by displacing calcium, phosphate, or hydroxyl ions. Other ions such as calcium, strontium, radium, phosphate, and fluoride can penetrate still further; that is, into the crystal interior. It may be assumed that all of these possible transfers take place constantly whenever the body fluids are in close contact with the crystal complex of reactive mineral.

In contrast to the picture just presented is the one representing "old" crystals. As a crystal ages and as it grows, it gradually displaces some of the water and thus becomes less reactive. McLean (1958) reports that such unreactive bone makes up more than 99% of the compact bone and that this bone can be made available to the organism as a whole only through cellular action, resulting in resorption. Throughout the body the bone exhibits this tendency toward excessive mineralization which would lead to a condition of stability for all bone that would be
incompatible with the life of the organism. This points up the necessity for constant remodelling of bone to be discussed later.

Cellular components. The bone cells or osteocytes occupy elliptical spaces or lacunae situated within or between layers of the Haversian systems. They have dark-staining nuclei and cytoplasm which varies from acidophilic to weakly basophilic. Since these cells are entrapped in a solid matrix with only slow diffusion of materials in or out, they must depend, for survival, on fine protoplasmic processes which extend out from the cell body along delicate canaliculi which interconnect the neighboring lacunae. This in contrast to the cartilage cells which have no processes but are embedded in a matrix which is quite permeable, by diffusion, to nutrients. The probable origin and fate of these cells will be discussed below.

A second cell component of bone is the osteoblast. Being associated with bone formation, these cells are not conspicuous elements of mature bone, but, during growth, they cover all the surfaces upon which osseous matrix is being deposited. They vary in shape. Some are cuboidal, others columnar, while many are pyramidal with either their broad base or their narrow apex resting on the underlying bone. They remain attached to one another by fine protoplasmic processes. As osteoblasts differentiate into osteocytes, these processes become more prominent, and it is the deposition around them of matrix that leads to the formation of the canaliculi. The osteoblast nucleus is round, eccentrically placed in the cell, and usually exhibits a single prominent nucleolus. The basophilia of the cytoplasm is attributed to the
ribose nucleoprotein. The basophilia varies with the activity of the cells and is probably related to the synthesis of protein components of the matrix. (Fawcett, 1954).

Heller-Steinberg (1951) has shown granules in the osteoblasts which, on the basis of staining reaction, are believed to be glycoprotein in nature. Since the ground substance of newly deposited bone has similar staining properties, she regards these osteoblasts as containing precursors of bone matrix. This hypothesis is supported by the observation that such granules are abundant in the osteoblasts and young osteocytes in the zone of growth at either end of a long bone, but they are inconspicuous or lacking in the relatively inactive cells at the middle of the shaft.

Osteoblasts appear to play an important part in calcification of bone. They are rich in alkaline phosphatase, the activity of which varies with the degree of osteogenic activity, and it is now felt that calcification results from a local increase in phosphate ions split off from organic phosphorus compounds by the action of the alkaline phosphatase of the osteoblasts as well as from phosphate from other sources (Follis and Berthong, 1949; Greep, 1948). This view has been challenged, not in principle, but in mechanism by Neuman and Neuman (1958), who proposed that the action of the phosphatase is important at the mineralization sites not by locally increasing phosphate, but rather by removing a crystal poison from the immediate fluid environment. This is based on the earlier observations of many workers that the presence of unhydrolyzed esters of phosphate prevents the formation of new bone crystals.
(Neuman and Neuman, 1958).

The third cell component of bone which is important to this discussion is the osteoclast. First discovered in 1849 by Robin, these multinucleate cells have been the subject of much controversy since Koelliker, in 1873, suggested that such cells were the universal agents of bone resorption and christened them "ostoklasts". The shape of osteoclasts varies greatly. They may be seen as round or oval masses a little distance from bone, or as thin sheets closely applied to the surface of bone. Osteoclasts are often found occupying indentations in the bone called Howship's lacunae which are probably formed by dissolution of the bone substance. Barnicot (1947), in his supravital studies on mouse osteoclasts, observed adjacent small cells to be pear-shaped with the stalks pointing toward one another as if they had been joined together. In tissue culture osteoclasts have been observed to form lobes which move apart and then flow together.

The nuclei are rounded or oval in shape with one or two nucleoli. There is considerable variation in nuclear shape. The cytoplasm of osteoclasts is weakly basophilic, finely granular, and often vacuolated. One of the most outstanding features of the osteoclast is its "striated", "brush", or "ruffled" border. It has been called by these three names by various workers since the early days of Koelliker (1873). Since electron microscope work has revealed that this area is not a striated or brush border the term "ruffled", used by Scott and Fease (1956) will be applied in future discussions. It is intimated by many workers that this ruffled
border aids in the dissolution of bone. This theory will be
discussed at a later time. It is sufficient to say here that the
evidence to date tends to support rather than reject such an
hypothesis. The histochemical localization of acid phosphatase
in the osteoclasts has added further evidence of such bone-destroying
action (Schajowicz and Cabrini, 1958).

Both the origin and fate of the osteoclast have been widely
discussed without a great deal of agreement. That they arise by
mitotic division of existing osteoclasts is doubtful, for very few
reports of mitoses have been made. Various precursors for the
osteoclasts have been proposed. Among the most common are the
osteoblasts, osteocytes, and chondrocytes. Heller et al (1950)
state that they have found areas in bone where there is clear
evidence of fusion of cuboidal or spindle-shaped osteoblasts into
multinucleate osteoclasts. This is in agreement with Arey (1916).
They go on to say that there are indications that osteoclasts break
down into individual cells which take on a spindle-shaped form.
They point out that, though there are large numbers of osteoclasts
present, no dead ones are ever seen when the numbers are diminished.
These workers have also proposed that osteocytes may form
osteoclasts. Benoit and Clavert (1950) have also suggested that
more than one source is involved in the formation of osteoclasts.
The work of Gaillard (1955, 1959) using tissue culture techniques
on the parietal bone of mouse embryos showed that osteoclasts appear
very rapidly, remain for several days, and disappear with no
evidence of dead or dying osteoclastic material. It has been noted
by Toft and Talmage (1960) that the nuclear to cytoplasmic ratio in osteoclasts remains constant even though great variations in the number of osteoclasts take place, thus supporting the idea of coalescence of discreet mononuclear units such as osteocytes and osteoblasts.
PARATHYROIDS AND BONE

Up to this point the parathyroid glands and bone have been spoken of independently and as separately functioning entities. While it is true that each maintains a certain degree of autonomy, it is incorrect to say that, in the organism as a whole, they are separate unrelated units. The parathyroids and bone interact to a great degree and because of this interaction an environment is created in which the other physiological processes can function properly.

The first difficulty encountered in assessing the effects of the parathyroids on bone was that of convincing certain groups of workers that the observed phenomena were not the result of kidney response to parathyroid hormone. Albright and Reifenstein (1946) have held to the idea that the primary action of the parathyroids is on the kidney threshold for phosphorus. They stated that the hormone caused the kidney to release more phosphate into the urine, thus lowering the serum phosphate level. This, in turn, caused calcium to be removed from bone because of a shift in the equilibrium of the blood. They considered the blood to be saturated with calcium and phosphorus at an ion product of approximately 40.

Tweedy et al (1936), in their study of the effects of complete renal insufficiency and parathyroid activity, stated that the mobilization of the calcium stores of the body into the blood by parathyroid hormone is dependent on kidney function. However, Stoerk (1943) showed that 100 USP units of parathyroid extract
would elevate the serum calcium of bilaterally nephrectomized-parathyroidectomized rats as much as 4 mg/100 ml serum. Monahan and Freeman (1944) were able to show that nephrectomized dogs could maintain normal serum calcium levels independent of a wide range of phosphate concentrations. As a further corollary to this work, Stewart and Bowen (1951) recorded increased serum calcium in nephrectomized dogs with intravenous injection of 500 units of parathyroid extract within 8 hours. They also noted the increase in serum calcium in nephrectomized-parathyroidectomized dogs with parathyroid extract.

As already noted, the final effect of the parathyroid hormone is to raise the serum calcium level. This is done by dissolution of mineral from bone. Bone resorption has been noted since the time of Koelliker (1873). The evidence for such resorption has been based on chemical, histological, and radioactive studies. Since bone resorption includes the disappearance of both the mineral and the organic portions of the bone, the question of which is removed first has long been debated. Many attempts have been made to show a mechanism for the primary removal of one or the other elements. It was noted by early workers (Jaffe and Bodansky, 1930; Selye, 1932) that long-term injection of parathyroid extract caused removal of bone mineral and an increase in fibrous connective tissue similar to the syndrome of osteitis fibrosa. This, however, must be considered as the long-term effect that it is. In terms of the connective tissue components of bone, it has been shown by Engel and Catchpole (1953) that with administration of parathyroid extract there was a dissolution of bone matrix with an increase in
plasma and urine mucoproteins. It has been shown that there is a
general depolymerization of the ground substance in response to
parathyroid hormone and that this may affect the calcium-binding
capacity of the ground substance, causing the calcium to be more
readily available to the circulation (Heller-Steinberg, 1951). For
further work on the connective tissue effects of the parathyroid
hormone, the reader is referred to Heller-Steinberg (1951), Shetlar
et al (1956), Bronner (1957), McLean (1957), Bernstein (1958) and
Greep and Talmage (1960). More will be said concerning the
dissolution of the matrix in a later section.

Aside from calcium, the ion which has caused the greatest
interest in bone metabolism in recent years has been citrate.
Since Dickens (1941) first showed that bone contains relatively
high amounts of citrate, there has been continued investigation of
this ion and its possible role in calcium metabolism. It has been
shown that parathyroid extract injection increases serum citrate
(L'Heureux and Roth, 1953) and that parathyroidectomy lowers serum
citrate (Elliott and Freeman, 1956, 1956a; Freeman and Elliott,
1956; Neuman and Neuman, 1958). It has also been shown by Freeman
and co-workers that the majority of citric acid oxidation takes
place in the kidney (1950). Later papers demonstrated that
nephrectomy causes a transient increase in citric acid coupled with
hypercalcemia (Freeman and Chang, 1950). A companion paper (Freeman
and Chang, 1950a) and three recent papers (Elliott and Freeman, 1956,
1956a; Freeman and Elliott, 1956) have appeared which indicate
quite conclusively that serum calcium, serum citrate, and parathyroid function are interrelated, since parathyroidectomy prior to nephrectomy prevents the hypercitricemia and hypercalcemia. Parathyroid extract restored the calcium and citric acid response of the parathyroidectomized rat to nephrectomy.

In view of this and other evidence, Neuman and Neuman, (1958) have proposed an hypothesis whereby citric acid produced in the bone causes the dissolution of calcium and its subsequent release into the body fluids.

The basic problem to be solved in determining the method of dissolution of bone mineral is how blood, which is already supersaturated with respect to bone, can pick up more calcium and phosphorus from bone as it passes over the mineral. Neuman and Neuman (1958) have proposed three mechanisms by which this could be accomplished. They are, briefly, a) the presence of a local, high concentration of a solubilizing ion such as magnesium, carbonate, or citrate, which could increase the apparent solubility of the hydroxyapatite; b) the production of a localized high concentration of a chelating agent for calcium, thus tying up the calcium and allowing for dissolution; or c) the localized production of acid which would lower the pH sufficiently to allow for dissolution of bone. Citrate would answer all three possibilities. Mordin (1957) suggested that the secretion of citrate by bone cells would be a means of maintaining serum calcium levels. It has been pointed out that citrate would be ideal for the proposed mechanism of bone
dissolution since it forms a highly associated complex with calcium, as an acid it has three carboxyl groups all ionized at physiological pH, and it is readily and rapidly oxidized by the kidneys (Neuman and Neuman, 1958). Neuman et al (1956) presented the citric acid theory with a brief description of an experiment to substantiate it. A portion of that report is quoted below.

"It was hypothesized that cellular elements of bone normally secrete citrate (or citric acid) in response to parathyroid activity. This citrate carries complexed calcium to the serum where extraskelatal tissues (the kidney primarily) oxidize much of the citrate leaving an excess of calcium ion in solution. If citrate were secreted as the acid, a local pH gradient would also contribute to the transport of ionized calcium to serum. It was technically very difficult to test the hypothesis directly. However, by simply drilling a small hole in the spongiosa of the femur of an intact dog it was possible to collect blood directly from the spongiosal circulation for comparison with simultaneous arterial samples. While this sample was only 'contaminated' with venous flow from the bone cells, the output of citrate from the bone was so great, clearcut analytical differences were easily shown. Furthermore, dramatic increases in citrate output from the bone were observed almost immediately following injection of parathyroid extract."

The mechanism of this accumulation of citric acid by the bone is unknown. However, Neuman and Neuman (1958) have proposed that perhaps the parathyroid hormone works by inhibiting the action of coenzyme II. Such an inhibition would cause the accumulation of pyruvic, lactic, oxalacetic, citric, isocitric, and aconitic acids. On the basis of such an inhibition there would be a depletion of glycogen stores. Heller-Steinberg (1951) reported an absence of glycoprotein granules at the site of rapid bone resorption. It has been shown by Dixon and Perkins (1952) that the enzymes necessary for citrate synthesis are present in bone and cartilage. On the
other hand, they showed that the enzyme necessary for citrate utilization (isocitric dehydrogenase) was unmeasurable in bone and very low in cartilage. This would suggest that the citrate cycle does not function significantly in oxidation in bone cells. The combination of these two phenomena might well lead to the accumulation of citrate in bone.

The question as to whether the mineral or matrix is first affected by the action of parathyroid hormone remains largely unsettled. Ham and Gordon (1952) reported the loss of bone mineral with the residual matrix being seen as fibrils projecting from the eroded surfaces of the bone. Heller-Steinberg (1951) noted that in the normal animal there is what she termed "reactive" bone mineral around the osteocytes and their canaliculi. After injection of parathyroid extract, the reactive area enlarged, thus indicating to her the mobilization of mineral as the first noticeable histological change.

Among the various elements of bone affected by the parathyroid hormone, the osteoclasts stand out as being the most controversial. Koelliker (1873) first described bone resorption from a histological standpoint and implicated the osteoclasts as the universal agents of bone resorption. Since that time many attempts have been made to relate the osteoclasts both to parathyroid function and to bone resorption directly.

One of the earliest reports of the effect of parathyroid extract on osteoclasts was that of Selye (1932) in which he suggested that the parathyroid hormone exerts a direct effect on the osteoclasts, thereby causing mobilization of calcium from osseous
tissue. Pugsley and Selye (1933) followed the histological changes in bone after the administration of parathyroid extract. They state that the first reaction of the bone to the parathyroid extract is the formation of osteoclasts. This increase in osteoclasts was pronounced after two days and these cells were numerous at four days as in osteitis fibrosa. They said that calcium excretion was high during the period when there were many osteoclasts. By 9 - 12 days the number of osteoclasts had diminished and the calcium excretion was back to normal. A conflicting opinion regarding the cause and effect relationship of the osteoclasts to bone resorption was given by Burrows (1938), who gave small doses (0.05 to 0.5 cc. of Collip's extract daily) of parathyroid extract to growing rats and noted changes in osteoclasts. With the lowest dose he found only a few osteoclasts present with a maximum at 4 days. With the largest dose he found some osteoclasts at 12 hours and more at 24 hours with the largest number at 2 days. He believed that the osteoclast increase was the result, and not the cause, of decalcification.

McLean and Bloom (1941) proposed that the mechanism of action of the osteoclasts on bone resorption as controlled by the parathyroids was the simultaneous dissolution of both the bone salts and organic matrix by local cellular action. Heller et al (1950) reported extensive cellular transformations in mammalian bones induced by parathyroid extract. They demonstrated coalescence of osteoblasts into osteoclasts and also noted that as osteocytes were liberated from their lacunae by erosion of the surrounding bone they were incorporated into the osteoclasts.
Beginning in 1946 with the work of Hancox there have been a
number of studies done using either tissue transplant or tissue
culture methods to study the effects of parathyroid extract on bone
and on osteoclasts. Hancox reported that there were certain cells
in his bone tissue cultures which resembled osteoclasts. He reported
that they were very motile and moved around through the culture. They
had a relatively short life and usually disappeared or dedifferen-
tiated within two days. Barnicot in 1948 made intracerebral grafts
of parathyroid tissue and noted the extensive erosion of bone
adjacent to the graft. He noted many osteoclasts associated with the
area of resorption. The studies of Chang (1951) were done with
similar grafts of parathyroid and other tissues to bone. She noted
that the parathyroid tissue caused local bone resorption of the
calvarium while other tissue similarly transplanted did not cause
resorption.

Perhaps the most impressive tissue or organ culture studies
to date have been those of Gaillard. Working with fragments of
parietal bone from 15 day old mouse embryos in tissue culture
Gaillard has shown some very striking effects of parathyroids on
bone. In one series of experiments (1955) he incubated in one vessel
parathyroid tissue from chick embryos, human embryos, or parathyroid
adenoma, and in another vessel the parietal fragments. In one case
the parathyroid fragments were then placed on top of the bone
fragments in the same vessel and incubated together for several
days. Upon histological examination there was a great deal of bone
resorption of the explants with parathyroid tissues. Control
cultures did not show resorption. In another set of experiments only the fluid taken from the cultivated parathyroid tissue was introduced into the bone cultures and in this case there was also a great deal of resorption in experimental cultures. He was also able to observe resorption by adding very small amounts of parathyroid extract to the cultures.

In a recent paper Gaillard described a method for assessing changes in embryonic mouse radii under the influence of parathyroid culture fluid, parathyroid extract, of the much more purified hormone substance prepared by Rasmussen (1960). By establishing a number of criteria concerning changes in the bone cartilage, and connective tissue, Gaillard was able to evaluate the effect of parathyroid hormone by the percentage of positive reactions obtained. He found a linear relationship between the percentage of positive reactions and the dosages applied (2 - 0.01 USP units/cc of culture medium). This work, which was reported at the recent Symposium of Parathyroid Research Trends, represents the only in vitro quantitative assessment of the relationship of bone changes to parathyroid function.

With regard to the osteoclasts, Gaillard noted another interesting phenomenon; namely, the sudden disappearance, in the presence of parathyroid hormone, of the osteoclasts as soon as the available bone matrix had vanished. From this he hypothesized that parathyroid hormone is only capable of "creating the conditions favoring the survival, the formation and the functioning of osteoclasts" if bone matrix is present and obviously conditions
its surroundings (Gaillard, 1959). This represents the only
description in the literature of such a relationship between the
matrix and the osteoclasts. In most other references it is concluded
that the osteoclasts affect the matrix, but little mention has been
made of a reverse situation.

Of great interest to morphologists is the ruffled border of
the osteoclasts. This feature has been variously described and its
significance interpreted in different ways. Where the surface of
the osteoclast is in contact with the mineralized matrix, and where
the matrix shows definite signs of resorption, the plasma membrane
presents an area of intricate infolding which is the "brush" border
first described by Koelliker and the "ruffled" border of Scott and
Pease (1956). Using light microscopy Ham and Gordon (1952) concluded
that the ruffled border was actually part of the bone and represented
the fibers of the matrix left after the mineral had been dissolved
away. A study of these same cells by Kroon (1954) revealed more of
their true nature as elaborations of the cellular membrane. Although
there have been several recent papers concerning electron microsc-
ropy of bone, none has dealt with the osteoclast in as complete a
fashion as did Scott and Pease in 1956.

Close scrutiny of the ruffled border shows that it lacks the
orderliness of arrangement noted in renal tubular cells and it seems
to be more like the folded membrane of macrophages as described by
Palade (1955). There are deep invaginations of the plasma membrane
which produce channels into the cell. These have associated with
them large vacuoles in the cytoplasm. Communication between the
channels and the vacuoles has been shown. Loose crystals have been noted in the channels and also in the vacuoles. In the immediate area of the ruffled border there is a loosening of the osseous matrix, giving an appearance similar to that seen through light microscopy by Ham and Gordon (1952). This material appears fringed and is composed of dense elongate columns separated by clear irregular spaces which often contain numerous crystalline aggregates. The appearance of the fringe material strongly suggests that some of the collagen fibers, which originally bound the osseous matrix together, had undergone solution. The dense particles are assumed to be bone crystals freed by resorption of the organic matrix (Scott and Pease, 1956).

Though it has long been recognized that the osteoclast is associated with resorbing bone, conflicting views have been expressed by different groups of investigators as to whether it functions as a primary or secondary agent of resorption. As stated previously, Koelliker felt that the osteoclasts were the primary agent of bone resorption and associated this with the brush border he described. Jordan (1920, 1921), Dodds (1932), Kroon (1954) and others were of the same opinion.

The opposite view is represented principally by Ham and Gordon (1952), who thought that the brush border was due to the bone surface, not the osteoclast surface. This group feels that osteoclasts are more likely the result of resorption than the cause.

Scott and Pease (1956) state that the osteoclasts probably
function by secreting a collagen-dissolving substance which
dissolves the matrix and frees the inorganic material which is
then trapped in vacuoles and dissolved.

Whatever the ultimate function of the osteoclast is dis-
covered to be, it will probably be a considerable while before
the question is settled. The problem has remained unresolved
for 37 years and may well round out a century of controversy.
FEED-BACK MECHANISM

Having shown in the previous section that the parathyroids exert a direct effect on bone for the release of calcium to the serum, it is now fitting to discuss the method of control of this system. The controlled release of calcium to the serum would be as disastrous to the animal as would be the lack of its release. It is well known that a certain titer of calcium is essential for the heart beat and for coagulation of the blood. The part played in nerve transmission by calcium is readily seen in the tetany and death which result from parathyroidectomy with the consequent lowering of the serum calcium level. Perhaps less understood, yet equally important, is the function of calcium in the constitution and maintenance of the cell membrane structure.

Since the level of circulating serum ionized calcium seems to be closely controlled, the mechanism for the control of this level has long been the subject of discussion and dispute. Out of the mass of data which has accumulated has come the idea of a feed-back mechanism for this control. This was first proposed by McLean and Urist (1955) and has been extended by Talmage et al (1960).

Before a detailed discussion of this mechanism can be attempted, the criteria necessary for the operation of such a system must be established and supported by experimental evidence. The first point to be considered is the physico-chemical equilibrium which exists between the calcium and phosphate of body
fluids and that of bone. It has been shown by many workers that plasma is supersaturated with respect to tertiary calcium phosphate (Holt et al, 1925; Logan, 1940; Strates et al, 1957). This is interesting in view of previous statements in which it was reported that bone mineral might be essentially a tertiary calcium phosphate complex. In addition, Shear et al (1929) showed that serum was undersaturated with calcium and phosphate with respect to secondary calcium phosphate. They emphasized the importance of considering secondary calcium phosphate in relation to the solubility of calcium and phosphate in the serum. They supported their idea with work which showed that when calcium and phosphate precipitate from a saturated solution, the precipitate has a Ca:P ratio which approximates that of secondary calcium phosphate, and only later does the Ca:P ratio of the precipitate approach that of tertiary calcium phosphate. This idea has also been supported by Greenwald (1942).

Levinskas (1953) explains this undersaturation with respect to secondary calcium phosphate by stating that the solubility of these ions is limited by some factor (probably the surface of the solid phase) before reaching the solubility of secondary phosphates. Strates et al (1957) were able to show that above pH 6.9, secondary calcium phosphate is not stable and therefore the hydroxyapatite governs the solubility equilibrium when the solid phase is present.

A fundamental feature of the feed-back mechanism is the existence of a basic level of equilibration between the body fluids and bone. This level is maintained constant because of the interaction of the body fluids with bone. As mentioned in the preceding
section, normal serum is supersaturated with respect to bone. Thus when the blood passes over the solid bone phase, the calcium and phosphate in excess of the equilibration level are precipitated on the bone. If, on the other hand, the serum was lower in calcium and phosphate than the equilibration level, then these ions would be added to the serum from the bone.

In 1959, Wimer reported on some studies in which normal serum was placed in a vessel with a piece of fresh rat bone and shaken at 37°C for 2 hours with a proper atmosphere of oxygen and carbon dioxide to maintain the pH at 7.4. In each case, the calcium level dropped from 11 to 6 mg/100 ml. of serum. In another series of experiments normal serum was shaken with tertiary calcium phosphate, and the serum calcium level dropped to about 2 mg/100 ml. serum. This depleted serum was placed in a vessel with fresh rat bone and again shaken for 2 hours. In this case the serum calcium level was raised, but only to a level of about 5 - 6 mg/100 ml. serum (figure 1). In addition to this in vitro work, Talmage and associates have done a great deal of in vivo work utilizing the technique of continuous peritoneal lavage. Talmage and co-workers have found that, following parathyroidectomy, rats subjected to continuous peritoneal lavage are able to supply large amounts of calcium to the extracellular fluids for long periods of time (Talmage et al, 1957). Copp (1957), using continuous versus injection in dogs has also demonstrated the ability of the bones to supply large amounts of calcium to the extracellular fluids for long periods of time.
FIGURE I

EFFECT OF INCUBATION OF LIVING FEMURS IN PLASMA ON THE CALCIUM AND PHOSPHATE LEVEL OF THE PLASMA

Notes:

1) All incubations were for two hours.

2) pH was maintained at 7.3 - 7.4 with atmosphere of CO₂ and O₂

3) Serum was depleted of calcium and phosphate by shaking with tertiary calcium phosphate

4) Data taken from Wimer (1959)
An interesting facet of the problem of the equilibration between bone and blood is found in tracer studies using radiocalcium and radiophosphorus. Talmage and Elliott (1958) found that, when the animals were parathyroidectomized during continuous peritoneal lavage, there was a marked decrease in lavage calcium levels within half an hour. However, the amount of tagged calcium or strontium removed from bone was not decreased unless the radioactivity had been injected several weeks before the experiment. They interpreted these data as indicating that the parathyroids were removing calcium from a different area of bone than that available for exchange on a physico-chemical basis. In a later paper (Talmage et al., 1959) Talmage and associates studied the removal of both radioactive phosphorus and radiostrontium by the method of peritoneal lavage. In one series of experiments the radioactive isotopes were both given 18 hours prior to lavage. In the parathyroidectomized animals the amounts of radioactive phosphorus and strontium were essentially the same as in the controls. In another series of experiments the isotopes were given 3 weeks prior to lavage. In the parathyroidectomized animals the removal of both radiophosphorus and radiostrontium was much lower than the amount in the control. Parathyroidectomy caused a 25% drop in radiostrontium removal and an 18% decrease in radiophosphorus removal. This again points up the idea that a different area of bone is being affected by the parathyroid hormone and by the equilibrium with serum in the absence of the parathyroid hormone.

Accepting then that there is a basic equilibrium between bone
and extracellular fluid which is independent of the parathyroid hormone, the next task is to see how the parathyroid gland maintains the serum calcium above this basic level. The data just presented from the studies of Talmage and co-workers point out that the parathyroids are causing the release from bone of calcium and phosphorus from an area which differs from that in close contact with circulating fluids.

The concept of Neuman and Neuman (1958) regarding parathyroid control of citric acid production in bone has already been discussed and will be mentioned again only with respect to the supposed area of this reaction in bone. They feel that this phenomenon takes place only in "reactive" bone which constitutes the newly laid down tissue. McLean (1960) has taken issue with the Neumans in respect to the area affected. McLean believes that the osteoclasts must be taken into account and has advanced the view that, by virtue of osteoclastic resorption, under the control of the parathyroid glands, the organism has access to the non-exchangeable or stable fraction of the bone mineral. He states that his view requires at least provisional acceptance of the often advanced, but never proved, hypothesis that osteoclasts do, in fact, resorb bone, and that their activity in this respect is regulated by the parathyroid hormone. McLean further states that, while the parathyroids may control the osteoclasts, there is osteoclastic resorption even in the absence of the parathyroids.

A reconciliation of the views of the Neumans and McLean might come from the earlier work (1950) of Heller-Steinberg. She noted
that parathyroid extract caused changes in the staining characteristics of the bone around the lacunae occupied by osteocytes and around their canaliculi. She interprets these changes as indicating resorption around the lacunae. These areas are far removed from the bone surfaces or "reactive" areas. If her interpretation is correct, then it must be concluded that the osteocytes, as well as osteoclasts, participate in parathyroid-induced resorption, and this would possibly furnish the link between the theories of Neuman and McLean. Because of the evidence which exists suggesting the formation of osteoclasts from osteocytes and osteoblasts, it would not seem unreasonable that these cells might all be able to react to a common stimulus under the right circumstances.

Having assembled in one place the essential components of a feed-back mechanism for calcium, a fabrication of the over-all mechanism itself now seems in order. To begin with, calcium is usually in demand, since much of the calcium which is ingested is in a form which is unavailable to the body. Therefore, it must be conserved. Phosphate, on the other hand, is not only plentiful in the diet but is also released by various metabolic processes in the body and as such is constantly being poured into the blood stream.

Under normal circumstances the amount of circulating serum calcium and phosphorus is high enough that, as the blood passes over the solid phase of bone, calcium and phosphate are precipitated onto the crystal. This causes a lowering of the serum calcium level. As this "depleted" blood flows through the parathyroid
glands the lowered ionized calcium level furnishes the stimulus for the gland to secrete its hormone. Neuman hypothesizes that the hormone then reaches the bone via the blood and causes the local increase in citric acid which dissolves bone by a pH change, or by chelation of calcium. This calcium citrate complex leaves the bones and enters the general circulation. When it reaches the kidneys the citrate is rapidly metabolized, releasing the calcium into the serum, thus re-establishing the normal serum calcium level. However, accompanying the calcium from bone is phosphate which is also released as a result of the breakdown of the crystal. This would lead to an accumulation of phosphate were it not for the kidney effects of the parathyroid hormone. These kidney effects have been left largely unmentioned since they do not fit into the picture of the direct action of the parathyroids on bone. However, in the normal homeostatic functioning of the body they play an important part with regard to calcium and phosphate.

The presence of parathyroid hormone in increased quantities has two immediate effects on the kidneys. First, the threshold for calcium excretion is raised. That is, there is either greater resorption of calcium or less secretion of calcium in the renal tubules. The net result is a conservation of calcium. At the same time there is a lowering of the threshold for phosphate and a consequent increase in excretion of phosphate. This prevents the accumulation of excess phosphate in the serum which would cause faster precipitation of the calcium on bone. The combination of
the two actions of the hormone allow for the greatest change in the shortest time with the least amount of calcium being involved.

It can be seen that under such a system there would be continuous production of hormone at a low level to offset the continual deposition of calcium and phosphate on bone. It is hoped that the evidence presented in the work to follow will substantiate this hypothesis.

Although the basic idea of a feed-back mechanism for the control of serum calcium came from McLean and Urist (1955), the elaboration and refinement of the theory is the work of Talmage. The bulk of the operational plan of the feed-back mechanism as presented above was taken from the report of Talmage and Toft (1960) given at a recent symposium on parathyroid research trends.

The significance of such a system to the animal has been pointed out by Talmage et al (1960). The proposed system would allow for the control of calcium within very narrow limits, the necessity of which has already been discussed. Further, since the phosphate level in animals varies with age, metabolic rate, etc. it could not as easily be controlled. There are two primary advantages to the animal of such a feed-back mechanism. First, due to the constant precipitation of calcium and phosphate on bone, the stimulus and means are provided for new bone formation. In support of this idea is the fact that the serum phosphate level of children is twice that of the adult. By the system proposed, calcium would be precipitated faster in bone because of the higher (Ca x(P))
product, thus aiding bone growth. The second obvious advantage is the stimulus for bone remodelling due to the constant resorption of bone to supply the calcium needed by the depleted serum. Through such a mechanism one can see why such a hard and rock-like substance as bone is regarded not as a static, but as a dynamic system in the body.
STATEMENT OF THE PROBLEM

The problem of this thesis was twofold. The first aim was to establish, if possible, a quantitative relationship between the amount of osteoclastic material in a specific area of the long bones of rats and the activity of the parathyroid glands. Such a relationship might be used as an index of endogenous parathyroid secretion.

Having established such a relationship, the second aim was to use this index to study the effect of exogenous calcium and phosphate on animals subjected to nephrectomy or to continuous peritoneal lavage in an attempt to define the substance which controls parathyroid secretion.
MATERIALS AND METHODS

General. In these series of experiments approximately 400 male rats were used. They were obtained from the Holtzman colony and weighed between 225 and 250 grams. In a study of this type involving calcium metabolism, it was desirable to maintain a close control over the level of exogenous calcium which could be absorbed from the gut. To aid in the standardization of this factor, the animals for each experiment were locally procured at approximately the same time of day because of their feeding habits. The Holtzman Company attested to the constancy of the diet mixture used in feeding their colony. When the animals were received, they were placed on a diet consisting of sucrose, dextrose, Wesson oil, corn starch, and Alphacel (non-nutritive cellulose) with distilled water ad lib. They were maintained on this diet for 24 hours prior to each experiment. In cases where the experiment continued for several days, as in the nephrectomy study, the animals remained on this modified diet. It was noted, however, that following nephrectomy there was little tendency for the animals to eat. This seemingly rigorous diet accomplished a two-fold purpose. First, it emptied the gut of much of its former contents, including calcium, by displacement. Secondly, it kept the nephrectomized animals in better physiological condition. The lack of salt and proteins in the diet slowed the accumulation of toxic metabolites in the blood.

After each experiment blood was taken by heart puncture and analysed for calcium and phosphate. Phosphate analysis was done
according to the method described by Allport and Keyser (1957) using a Spectronic "20" spectrophotometer to quantitate the color reaction. The calcium was precipitated with ammonium oxalate, washed with a mixture of ether, 95% ethanol, 2% ammonium hydroxide, and water. After drying, the precipitate was redissolved in perchloric acid, diluted with 10% isopropyl alcohol, and read on a Beckman M del DU Spectrophotometer at wavelength 623 μm.

In the lavage studies, samples of the lavage fluid removed during each period and samples of urine were analysed in a like manner.

As part of the initial control series of experiments, normal animals were sacrificed upon arrival from the supplier. Others were placed on the modified diet for 24, 48, or 72 hours and then sacrificed. In another series, parathyroid extract (Lilly & Co.) was given intraperitoneally in one or more doses and the animals sacrificed 18 hours after the initial injection.

In all experiments where parathyroidectomy was necessary it was performed under ether anesthesia. After location with a binocular dissecting microscope, the parathyroids were individually removed from the animal with fine-tipped jewelers' forceps. Sham operations were performed to check any effect of operational trauma.

Nephrectomy. Nephrectomies were performed under ether anesthesia through a midventral incision. In one series of experiments progressive changes after nephrectomy were studied and, therefore, the animals were sacrificed at varying intervals up to
48 hours post-nephrectomy. Parathyroidectomy, as previously described, was performed just prior to nephrectomy in those experiments where both operations were necessary.

The effects of calcium administration were also studied, both in the nephrectomy series and in the lavage series. In each case, a calcium chloride solution of varying concentration was given in a volume of 0.5 ml or smaller by stomach tube. The dose was repeated every four hours except where otherwise noted. In the lavage studies additional calcium was given in the lavage fluid and will be described below.

**Lavage.** The technique of continuous peritoneal lavage used in these experiments is a modification by Talmage and associates (1957) of the original technique reported by Kolff and Page (1954). A stainless steel indwelling tube with a large flange on its inner aspect was sewn into the abdominal cavity in the midventral line about one inch anterior to the penis. This was done 24 hours before the start of the lavage to allow time for initial healing processes to seal any small openings around the plug which might subsequently cause a loss of fluid from the peritoneal cavity during the lavage. No antibiotics were used nor was any special attempt made to maintain aseptic conditions. The short duration of the experiment and the high resistance of the rat to infection made more elaborate precautions unnecessary.

For the basic lavage studies the lavage fluid which was introduced into the peritoneal cavity consisted only of 0.8% NaCl
and 0.5% glucose in distilled water. The pH was adjusted to 7.2 to 7.4 with dilute HCl and NaOH. No buffer solution was used in the lavage fluid. In later studies it was necessary to add either calcium or phosphate, or both, to the lavage fluid. The calcium was added as calcium chloride and the phosphate as Na₃HPO₄. The individual concentrations of these ions are given with the data from each experiment. In some experiments it was necessary to augment the lavage calcium with injections by stomach tube of a calcium chloride solution as mentioned previously.

For the duration of the lavage the animals were placed in individual metabolism cages in order that urine collections could be made during the lavage. The urine flow was above normal as a result of the slight hypotonicity of the lavage fluid. A constant temperature water bath containing coils of tubing filled with the various lavage solutions insured delivery of these solutions at body temperature. Each piece of tubing was connected at one end to a 30 ml syringe body, which could be filled from a reservoir, and at the other end to a hollow metal tapered tip which fitted the opening in the stainless steel plug. A pinch-clamp controlled the movement of fluid from the gravity-flow apparatus.

Each animal was filled with 30 ml of lavage fluid and placed in its metabolism cage. After one hour the fluid was removed by inserting a catheter into the indwelling tube and collecting the fluid in a graduate cylinder. The animal was then immediately refilled with another 30 ml and left for another hour. Urine collections were made
every four hours. The volumes of lavage fluid and urine were recorded for future consideration.

The duration of continuous lavage varied from 8 to 39 hours. Intermittent lavage was continued for periods up to 72 hours with rest periods of 4 hours out of every 12.

**Histology.** At the termination of each experiment the femur was exposed and the distal epiphysis snapped off. The femur was then sawed in half about 1/4 inch from the distal metaphysis. This piece of bone was then fixed in Helly's fluid for 12-18 hours. After fixation, the bone was washed in running tap water for an equal time. Decalcification was accomplished by using two changes of 5% formic acid per day for three days. Dehydration was followed by infiltration with 1% celloidin in methylbenzocate:ether:alcohol solution for 4 days. The bone was then embedded in paraffin (60-63°C) and mounted for sectioning. Sectioning inward from the metaphysis the bone was cut down to the area just below the deepest penetration of the cartilage cells into the spongiosa. It was felt that this represented, in all animals, the area of most uniform growth. Sections seven microns thick were taken from this area for histological examination and for quantitative determination of osteoclastic material. The sections were mounted on slides and stained with Erlich's hematoxylin, a mixture of Biebrick scarlet and Orange II, followed by Fast Green PCF. This tetrachrome stain aided greatly in identification of the osteoclasts. The bone and cartilage were green, erythrocytes were orange, and osteoblasts and osteoclasts were magenta.
The tissues were examined with a Spencer binocular microscope with 43x objective and matched 10x, wide-field oculars. A Harvard grid was placed in one ocular and represented the area (0.26mm²) taken as the unit for all quantitative counts of osteoclasts. This area is referred to in all subsequent data as the field. Successive fields of each cross section were scanned and the osteoclasts were counted. The results were expressed as osteoclasts per field. It must be stated at this point that, since the osteoclast, as a cell, is too large to be contained within a 7 micron section, only portions of osteoclasts were actually counted. Therefore the concentration of these parts of osteoclasts gave a measure of the amount of osteoclast material which was present in the given area. This is, in fact, a better index of parathyroid activity than would be obtained by determining the total number of cells, since osteoclasts vary greatly in size (Toft and Talmage, 1960).

Statistics. The only statistical treatment of the data was the determination of the standard error according to the following formula:

\[ S.E. = \sqrt{\frac{\sum X^2 - \bar{X}^2}{n-1}} \]

In a few instances a Student's T test for the significance of differences between means was run on two groups. Those are noted where this test was used. In all other statements concerning the significance of a difference between two means, it may be assumed that if the difference between the two means was larger than three times the larger of the two standard errors, the difference was significant.
RESULTS

Section I. Osteoclasts as an Index of Parathyroid Activity.

Problems concerning the role of osteoclasts in bone resorption have been investigated for many years. Numerous studies have attempted to clarify this basic relationship, particularly in reference to the part played by osteoclasts in parathyroid hormone effect on bone dissolution. These earlier studies have been reviewed by Hancox (1949, 1956). Of particular interest are reports of Selye (1932), which referred to increases in numbers of osteoclasts after parathyroid extract administration, and of Bloom et al (1941), which demonstrated qualitative increases in osteoclasts coupled with decreases in numbers of osteoclasts during the laying cycle of pigeons. More recently, Gaillard (1955) noted significant increases both in number and activity of osteoclasts in incubated foetal bone, under the influence of a substance elaborated from concurrently incubated parathyroid tissue, or from parathyroid extracted to the media. Talmage et al (1957) were able to show qualitative increases in numbers of osteoclasts in long bones of rats undergoing continuous peritoneal lavage with calcium- and phosphate-free lavage fluid. The only attempt to use a quantitative determination of osteoclast concentrations as an index of metabolic activity has been the recent work of Myers et al (1959) on the mandibular condyle. The purpose of this section of the study was to develop a method for quantitation of osteoclastic material which could be used as an index of bone dissolution and of parathyroid activity.
In Table I are summarized the results of a variety of experiments designed to show a quantitative relationship between parathyroid stimulation and osteoclast formation. The concentration of osteoclasts found in animals maintained on stock diet served as the norm. Since 2 or 3 days on a calcium- and phosphate-free diet did not significantly affect this norm, animals so treated were grouped with the controls. Parathyroidectomy for 7 days reduced significantly the osteoclast count. Treatment of such animals with 50 USP units of parathyroid extract returned the count to normal, as determined by bones removed 18 hours after hormone administration. Quantitative increases in osteoclasts count above normal were produced by treatment with 250 USP units of extract, by the technique of continuous peritoneal lavage, to be discussed below, and by nephrectomy. The degree of stimulation noted was maximum in bones taken from rats nephrectomized 24 hours or from those undergoing continuous peritoneal lavage. Both of these conditions will be dealt with in greater detail in the following sections. Parathyroid extract (250 USP units) produced a stimulation in bones, removed 18 hours after administration, similar to that found in bones removed after only 12 hours of continuous lavage.

A further study was made to determine if the ratio of cytoplasmic to nuclear material in these multimucleated cells varied with the degrees of parathyroid stimulation studied. This data is summarized in Table II. Counts of nuclei per osteoclast, however, remained constant even in bones showing a 100% variation in osteoclast count. The fact that the number of nuclei per osteoclast
TABLE I

EFFECTS OF VARIOUS STIMULI ON OSTEOCLAST CONCENTRATION
IN RAT FEMURS

<table>
<thead>
<tr>
<th>Group</th>
<th>No. Rats</th>
<th>Osteoclasts/Field$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Normal Control</td>
<td>32</td>
<td>3.55 ± .07</td>
</tr>
<tr>
<td>2 Normal 250 USP PTH$^2$</td>
<td>4</td>
<td>4.77 ± .44</td>
</tr>
<tr>
<td>3 PTX$^3$ 7 days</td>
<td>4</td>
<td>2.85 ± .18</td>
</tr>
<tr>
<td>4 PTX 7 days 50 USP PTH</td>
<td>4</td>
<td>3.42 ± .49</td>
</tr>
<tr>
<td>5 NEPX$^4$ 24 hrs</td>
<td>16</td>
<td>6.95 ± .32</td>
</tr>
<tr>
<td>6 LAV$^5$ 12 hrs</td>
<td>9</td>
<td>4.54 ± .48</td>
</tr>
<tr>
<td>7 LAV 32 hrs</td>
<td>5</td>
<td>6.09 ± .18</td>
</tr>
</tbody>
</table>

Notes: 1) Field size 0.26 mm$^2$
2) Parathyroid extract given 18 hrs before sacrifice
3) Parathyroidectomized
4) Nephrectomized
5) Continuous peritoneal lavage
6) Groups 2 and 3 are statistically different from group 1 (P = .001)
TABLE II

EFFECT OF STIMULATION OF OSTEOCLAST
INCREASE ON NUMBER OF NUCLEI
PER OSTEOCLAST

<table>
<thead>
<tr>
<th>group</th>
<th>0/F*</th>
<th>nuclei/osteoclast</th>
</tr>
</thead>
<tbody>
<tr>
<td>unstimulated</td>
<td>3.31 ± .03</td>
<td>3.12 ± .03</td>
</tr>
<tr>
<td>stimulated</td>
<td>8.24 ± .27</td>
<td>3.22 ± .30</td>
</tr>
</tbody>
</table>

Notes: 1. Unstimulated group composed of normal animals with a low number of osteoclasts.
2. Stimulated group composed of nephrectomized animals after 8 hours of peritoneal lavage.

* Osteoclasts per field.
remained constant, even though the concentration of osteoclasts changed greatly, lends support to the hypothesis that osteoclasts are formed by coalescence of other cell types; namely, osteoblasts and osteocytes.

As stated previously, the purpose of this section was to determine if changes in parathyroid activity could be detected by quantitative changes in the osteoclast concentration of a selected area of long bone. The data indicate that such is the case and also demonstrate that the procedure establishes a reliable index of changes in endogenous production and exogenous administration of parathyroid hormone.

The data are also of interest in regard to the normal functioning of the parathyroid gland. According to the work of McLean and Urist (1955) and the recent report of Talmage et al (1960), bone is under the continuous influence of hormone from the parathyroids due to the necessity of "feeding back" calcium into body fluids. This concept is further substantiated by the above data which indicate that the normal osteoclast concentration can be maintained only in parathyroid-intact animals, and that a significantly lower level appears following parathyroidectomy.
Section II. Parathyroid Activity in Nephrectomized Rats.

Having established an index of endogenous parathyroid activity, the next task was to show that changes in calcium and/or phosphate alone were able to influence this activity.

Because of the syndrome which follows nephrectomy it was felt that ablation of the kidneys might be a useful technique to use in attempting to elucidate the factor(s) controlling the parathyroids. It had been well established, as pointed out earlier, that parathyroid hormone was able to produce an effect in the nephrectomized animal, thereby indicating its locus of action in bone. It had been demonstrated that parathyroidectomy after nephrectomy, in all species studied, was followed by a drop in serum calcium levels which, later, could be restored toward normal by administration of parathyroid extract. It was also known that elevated calcium levels could be produced in the nephrectomized animal by hormone administration.

Equally well known was the fact that nephrectomy produced a rapid and major hypertrophy and hyperplasia of the parathyroid gland within 48 hours (Baker, 1965; Davis, 1959). Armed with these reports from the literature, a study was made of the progressive changes following nephrectomy.

For these experiments, the osteoclast count was taken at the indicated intervals after nephrectomy as an illustration of the intense activity of the parathyroids produced by nephrectomy. These data are summarized in Table III. An increased osteoclast count can be noted as early as six hours following the removal of the kidneys.
TABLE III

EFFECTS OF NEPHERECTOMY ON OSTEOCLAST CONCENTRATIONS AND
ON PLASMA CALCIUM AND PHOSPHATE VALUES

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rats</th>
<th>O/F*</th>
<th>mg Ca/100 ml</th>
<th>mg P/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>32</td>
<td>3.55±.07**</td>
<td>11.0±.2</td>
<td>7.3±.2</td>
</tr>
<tr>
<td>NEPX 6 hrs</td>
<td>6</td>
<td>4.67±.13</td>
<td>12.4±.4</td>
<td>12.5±.4</td>
</tr>
<tr>
<td>NEPX 12 hrs</td>
<td>7</td>
<td>5.36±.15</td>
<td>10.6±.1</td>
<td>13.5±.2</td>
</tr>
<tr>
<td>NEPX 18 hrs</td>
<td>6</td>
<td>5.42±.47</td>
<td>11.8±.4</td>
<td>13.4(2)</td>
</tr>
<tr>
<td>NEPX 24 hrs</td>
<td>16</td>
<td>6.95±.32</td>
<td>11.6±.4</td>
<td>13.7±.5</td>
</tr>
<tr>
<td>NEPX 32 hrs</td>
<td>6</td>
<td>5.93±.74</td>
<td>11.1±.4</td>
<td>17.3±.6</td>
</tr>
<tr>
<td>NEPX 48 hrs</td>
<td>20</td>
<td>5.91±.14</td>
<td>12.3±.1</td>
<td>16.6±.4</td>
</tr>
<tr>
<td>NEPX-PTX 24 hrs</td>
<td>5</td>
<td>3.53±.27</td>
<td>6.7±.3</td>
<td>15.8±1.0</td>
</tr>
<tr>
<td>NEPX-PTX 32 hrs</td>
<td>11</td>
<td>3.69±.45</td>
<td>7.4±.5</td>
<td>15.1±.9</td>
</tr>
</tbody>
</table>

* Osteoclasts per field (field size = 0.26 mm²)

** S.E. = ± \sqrt{\frac{\sum x^2}{n} - \bar{x}^2}
A maximum, but more variable, stimulation is seen 24 hours post-operative. After this period, the values stabilize as indicated by the standard deviation, but tend to drop slightly. Since these changes were not produced in the nephrectomised animal, if parathyroidectomised, there can be little doubt that they indicate marked increases in the secretory activity of the parathyroids.

While experiments such as these do not necessarily indicate that the hormone works through the osteoclasts in achieving its results on bone, they do demonstrate that, following nephrectomy, there is a drastic increase in the rate of dissolution of bone. The significance of these data will be discussed later, when they will be used as evidence that an increased amount of calcium is returned to the plasma by the overactive parathyroid gland in nephrectomised animals.

The effects of renal failure or nephrectomy on plasma calcium and phosphate values are well established. There is an immediate rise in blood phosphate which reaches, in the rat, a value of well over 10 mg P/100 ml plasma in six hours after kidney removal. From that time until the death of the animal there is a progressive increase in this value. It is not unusual to obtain values of 20-30 mg P/100 ml for plasma phosphate by the third or fourth day. More often a plateau in the range of 18 mg P/ml is reached, usually by 48 hours, and is maintained until the death of the animal. Calcium values, on the other hand, remain relatively constant in the normal range throughout. The activity of the parathyroids following nephrectomy is shown by the fact that parathyroidectomy causes a
marked drop in plasma calcium with little or no effect on the phosphate values.

Increased secretory activity of the parathyroids has been indicated by Davis (1959) by changes in the gland itself, and in this work by the changes in the osteoclast count which has been established as a secretory index.

The works of Strates et al (1957) and those reported earlier (Talmage et al, 1960) confirm studies done in 1929 (Shear et al) showing that plasma is not normally saturated with Ca\(^{++}\) and HPO\(_4\)\(^{-2}\) ions. In the studies of Wimer (1959) and Talmage et al (1960) saturation of rat plasma was reached when the product of these two ions is between 50 and 60 (plasma ionized calcium x plasma phosphorus values.). If one were to assume that one half of the plasma calcium is ionized, it is easy to calculate that the product of these two ions in the plasma of nephrectomized rats surpasses this by a considerable sum. To illustrate that in the nephrectomized condition calcium and phosphate must exist in some other form, presumably as a colloidal complex, plasma taken from nephrectomized rats was centrifuged both at normal speeds and at 14,000 X gravity. The results are shown in Table IV. The higher speed of centrifugation removed from serum sufficient calcium and phosphate to reduce the product of these two ions to within the 50 to 60 range. The remaining calcium value is therefore lower than normal, and served, perhaps, as a stimulus for the increased parathyroid activity known to occur following nephrectomy.
TABLE IV

EFFECT OF HIGH-SPEED CENTRIFUGATION ON PLASMA CALCIUM AND PHOSPHATE VALUES*

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca</th>
<th>Ca</th>
<th>PO₄&lt;sub&gt;4&lt;/sub&gt;</th>
<th>PO₄&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BHSC***</td>
<td>ARSC***</td>
<td>BHSC</td>
<td>ARSC</td>
</tr>
<tr>
<td>Control</td>
<td>11.61±.15</td>
<td>11.67±.09</td>
<td>6.16±.29</td>
<td>6.42±.25</td>
</tr>
<tr>
<td>Nepx 36 hrs</td>
<td>11.07±.37</td>
<td>8.03±.36</td>
<td>17.29±.60</td>
<td>14.88±.64</td>
</tr>
<tr>
<td>Nepx 48 hrs 10 mg Ca/4 hr</td>
<td>11.16±.12</td>
<td>11.06±.10</td>
<td>8.89±.54</td>
<td>9.00±.54</td>
</tr>
</tbody>
</table>

NOTE: Nepx = Nephrectomized

* Modified from Wimer, 1959
** Before high-speed centrifugation
*** After high-speed centrifugation
The earlier portions of this section have demonstrated the increased activity of the parathyroids following nephrectomy. They have also suggested that this increased activity might be due to a decrease in circulating ionic calcium levels. If this be true, then it might be expected that marked reversal of these events might result from the administration of exogenous calcium to the nephrectomized animals. The following experiments were done to illustrate this point. Calcium was given by stomach tube in the amounts indicated, at four hour intervals commencing at nephrectomy. The changes in the sequence of events normally seen following nephrectomy are summarized in Table V. The largest dose of calcium used, 15 mg/4 hours, completely prevented the increase in osteoclasts, maintained slightly elevated plasma calcium values, and kept the phosphate in the normal range. High-speed centrifugation studies, as shown by Wimer (1959) in Table IV, demonstrated that no colloidal calcium phosphate existed in the serum, and that the ion product was well within the normal range. The only factor that was not completely normal was the mitotic count in the parathyroid tissue (Talmage et al., 1960). This count did, however, show a marked decrease over the control condition and thus indicates at least partial suppression of parathyroid stimulation (Table V).

Although the work presented in this section strongly indicates that calcium is the controlling factor in parathyroid stimulation, the question still remains as to the position of phosphate in this picture. The third section of this study deals with the problem of eliminating phosphate as a possible direct stimulating agent of
TABLE V

EFFECTS OF Ca ADMINISTRATION ON MITOSIS (PARATHYROID GLAND) AND OSTEOCLASTS
IN NEPHRECTOMIZED RATS

<table>
<thead>
<tr>
<th>Group</th>
<th>0/F*</th>
<th>Serum Ca</th>
<th>Serum PO₄</th>
<th>M/F**</th>
<th>X Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.55±.07(32)</td>
<td>11.0±.2(38)</td>
<td>7.3±.2(38)</td>
<td>0.141±.21(14)</td>
<td>1</td>
</tr>
<tr>
<td>NEPX 24 hrs</td>
<td>6.95±.32(16)</td>
<td>11.6±.4(16)</td>
<td>13.7±.5(16)</td>
<td>0.163±.03(5)</td>
<td></td>
</tr>
<tr>
<td>NEPX 48 hrs</td>
<td>5.91±.14(20)</td>
<td>12.3±.1(20)</td>
<td>16.6±.4(20)</td>
<td>4.11±.19(13)</td>
<td>29</td>
</tr>
<tr>
<td>NEPX 48 hrs 5 mg Ca/4 hrs</td>
<td>6.33±.36(7)</td>
<td>12.9±.2(7)</td>
<td>9.8±.3(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEPX 48 hrs 10 mg Ca/4 hrs</td>
<td>4.76±.24(10)</td>
<td>11.2±.1(10)</td>
<td>8.9±.4(10)</td>
<td>1.27±.20(7)</td>
<td>9</td>
</tr>
<tr>
<td>NEPX 48 hrs 15 mg Ca/4 hrs</td>
<td>3.69±.21(8)</td>
<td>13.2±.5(8)</td>
<td>7.6±.2(8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEPX 72 hrs</td>
<td>4.75±.69(5)</td>
<td>9.6±.8(5)</td>
<td>24.4±1.3(5)</td>
<td>4.04(2)</td>
<td>29</td>
</tr>
<tr>
<td>NEPX 96 hrs</td>
<td></td>
<td></td>
<td></td>
<td>4.29(1)</td>
<td>29</td>
</tr>
</tbody>
</table>

* Osteoclasts per field

** Number of mitoses per field
parathyroid secretion. It was felt that a discussion of the full significance of the data presented here should be deferred until the next section has been presented and then a general summation of all data with reference to the current concept of parathyroid action be presented.
Section III. Lavage Studies Utilizing Variable Calcium and Phosphate Levels.

As previously stated, the effects of phosphate alone have not been completely eliminated as possible stimulators of parathyroid secretion. A major difficulty has resulted from the relationship of calcium and phosphate in body fluids such that a change in the concentration of one ion in one direction is usually accompanied by an inverse change in the concentration of the other ion. Because of this phenomenon it was not possible to tell, by nephrectomy alone, whether it was a decreased calcium ion concentration which was stimulating the gland or the concurrently produced increased concentration of the phosphate ion. Even with calcium administration there was, with the elevated calcium level, a fall in the phosphate level. This section is directed, therefore, toward a study of the changes in secretory rates of the parathyroid produced by differing concentrations of one of these two ions in the presence of a controlled level of the other.

The lavage technique employed in this section is that of Talmage and has been described several times in past publications (Talmage, et al., 1957, 1957a) and was reviewed in the materials and methods.

Calcium- and Phosphate-free Lavage. In animals with intact parathyroids, the amount of calcium removed per hour averaged 1.7 mg, approximately 30% of which was due to the action of the parathyroids, and 70% due to the physico-chemical equilibrium processes. This procedure placed upon the animal what has been termed by Talmage and co-workers a "calcium stress", temporarily lowering the calcium
concentration of circulating body fluids below the point of basic equilibration. However, judging by the failure of this type of lavage, even when extended for 48 hours, to produce hypertrophy, detectable by present methods, or increased mitotic activity of the parathyroid glands (Davis, 1959), it is assumed that calcium removal in these experiments did not equal the turnover of calcium in bone caused by nephrectomy.

The results of the lavage studies utilizing calcium- and phosphate-free lavage solution can be summarized briefly as follows: 1) They established the different rates of calcium removal in control and parathyroidectomized rats; 2) They confirmed the concept of a basic physico-chemical equilibration process between bone and body fluid, represented by the parathyroidectomized condition; 3) They demonstrated that, if the concentration of calcium in the body fluids dropped below this level, bone was able, for extended periods of time, to continuously supply calcium to the circulating fluids; and 4) They showed that the effects of parathyroid hormone on bone and on circulating calcium levels were not dependent in any way upon a renal function.

For the specific purpose of this study these calcium- and phosphate-free lavage experiments are utilized to illustrate the increased activity of the parathyroids, as judged by the above-mentioned criterion of parathyroid activity: the osteoclast concentration. These data are summarized in Figure II. They illustrate the result of lavage continued for 36 hours in animals with intact parathyroids. The osteoclast count increased from the control value
FIGURE II

EFFECTS OF CONTINUOUS PERITONEAL LAVAGE ON OSTEOCLAST
COUNTS AND CALCIUM AND PHOSPHATE REMOVAL.

Note: All equilibration periods are for one hour.
of 3.5 to 6.2 during the experimental period, while the calcium value of the fluid remained constant. Phosphate values tended to gradually fall. Plasma values taken at various times during the experimental period showed slightly depressed calcium and phosphate values (9-10 mg Ca/100 ml and 5-6 mg P/100 ml). It must be emphasized, therefore, that this evidence of increased secretory activity of the parathyroids was obtained under conditions of low calcium and low phosphate concentrations in body fluids. This, then, indicates that it is low calcium and not high phosphate which is regulating secretion of the parathyroid glands.

**Calcium-free and High-phosphate Lavage**. The next step was to study the effect of high circulating phosphate levels on this system in the presence of low calcium concentrations. For this, phosphate ($Na_2HPO_4$) was added to the lavage solution to a concentration of 10 mg P/100 ml. If high phosphate were the controlling agent, the expected results would have been a further increase in the secretory index (the osteoclast count) and possibly higher calcium values. Figure III demonstrates, however, that the only difference noted was the higher phosphate values both in the fluid removed from the peritoneal cavity and in the urine.

**High-calcium and Phosphate-free Lavage**. The effect of calcium on this system was next studied by the use of a lavage fluid low in phosphate and high in calcium. Calcium was added to a concentration of 10 mg/100 ml of lavage solution. The results are shown in Figure III. The addition of calcium prevented the rise in the secretory index which normally resulted from these lavage procedures. This evidence would seem to point, again, to calcium as the controlling agent.
FIGURE III

EFFECTS OF ADDITION OF CALCIUM OR PHOSPHATE TO PERITONEAL LAVAGE SOLUTION

Notes:

1) Lavage run continuously for 12 hours with one-hour equilibration periods

2) Phosphate added to a concentration of 10 mg P/100 ml solution

3) Calcium added to a concentration of 10 mg Ca/100 ml solution
High-calcium and High-phosphate Lavage. It might still be argued that either low calcium or high phosphate could independently increase directly the secretory activity of the parathyroid glands. For this reason a final group of experiments was used in which an attempt was made to keep both the calcium and phosphate levels high. If high phosphate levels in the presence of high calcium levels could stimulate activity then the hypothesis of a double control might be tenable. The concentrations of calcium and phosphate desired were too high to permit the addition of both to the lavage fluid. Therefore, for these studies, some calcium (3 mg/100 ml) and all of the phosphate (10 mg P/100 ml) were added to the lavage fluid and the additional calcium (10 mg every two hours) was administered by stomach tube. The results are given in Figure IV. The complete suppression of any increases in the secretory index in the presence of high values of both of these ions indicate that phosphate concentrations of circulating fluids do not, as such, control the secretion of the parathyroid gland. The important indirect effects of high phosphate level were discussed earlier.

It will be noted that in this particular set of experiments the stimulation of osteoclasts was not as great as in the preceding series. The control group had an osteoclast concentration only slightly above that for normal animals. The other two groups were correspondingly lower. This phenomenon, though unexplainable at the present time, was due to the addition of potassium to the lavage fluid. It has been shown in subsequent work that potassium will cause a decrease in the stimulation of osteoclasts in both normal and parathyroidectomized
FIGURE IV

EFFECTS OF ADDITION OF CALCIUM AND PHOSPHATE TO

PERITONEAL LAVAGE SOLUTION

Notes:

1) Lavage run continuously for 20 hours with one-hour equilibration periods.

2) Calcium added to a concentration of 3 mg Ca/100 ml lavage solution. In addition, 10 mg Ca administered by stomach tube every 2 hours.

3) Phosphate added to a concentration of 10 mg P/100 ml solution
rats. This will be further discussed in a later section.

Through the number of osteoclasts was not as great as in other similar experiments, the differences between the groups was still very marked. The presence of calcium, or calcium and phosphate prevented the increase in osteoclasts seen in the control group. Thus the conclusion that low serum calcium rather than high phosphate is controlling parathyroid secretion seems inescapable.
DISCUSSION

The first objective of this work was to attempt a correlation between the amount of osteoclastic material in a given area of bone and a given condition of the parathyroid glands. The stability of the normal animals with regard to the numbers of osteoclasts per area allowed for further comparisons with other physiological conditions of the animal. It was heartening to find that treatments which are known to cause hypersecretion of the parathyroid glands also cause increases in osteoclast concentrations. Both the techniques of nephrectomy and peritoneal lavage are known to cause parathyroid secretion. Increases in osteoclasts accompanied both of these treatments when the parathyroid glands were intact. Since parathyroidectomy prevented the increases due to these stimuli it must be concluded that the osteoclast increases are mediated through the parathyroids.

The fact that parathyroid extract in as small a dose as 50 USP units could cause a significant increase in the osteoclast concentration lends further proof of parathyroid control of osteoclast formation. Another interesting point to be considered regarding the osteoclast concentration in the normal animal is that the data presented give strength to the concept of continuous parathyroid secretion. Since parathyroidectomy for 7 days caused a significant diminution in the numbers of osteoclasts from that of the normal animal it must be assumed that the osteoclasts are under a constant, low-level stimulation by the parathyroids. This is in keeping with the feed-back mechanism as proposed by Talmage earlier in this work.
Although there was no attempt made to correlate the increase in osteoclasts with a given titer of parathyroid hormone, the possibility exists that such a relationship might well be feasible. It can be noted that the amount of stimulation of the osteoclasts was about equal in the animals receiving 250 UBP units of parathyroid extract and those lavaged for 12 hours. A similar parallel can be drawn between those animals nephrectomized for 24 hours and those lavaged for 32 hours. What has not been determined, however, is the difference in effect between a short stimulus such as an injection of parathyroid extract and a continuous lower level stimulus such as the lavage. It might be that a continued supply of hormone might have some cumulative effect.

Even though large changes in numbers of osteoclasts can take place under different stimuli, the number of nuclei per osteoclast remains constant. This indicates that the formation of new osteoclast material is accomplished by the addition of discrete units of cytoplasm with a single nucleus. The first possibility which comes to mind is the osteoblast and then the osteocyte. As pointed out in the literature, both of these cells may have the ability to dissolve bone provided that the proper stimulus is present. It is the opinion of the writer that the parathyroid hormone elicits two responses from bone cells. The first is the immediate shift in the metabolism of the cell to equip it for bone dissolution, either through the formation and release of citric acid or some collagenase, or both. Thus, the initial response to hormone release is a rapid mobilization of bone
NOTE TO USERS

Page(s) missing in number only; text follows. Microfilmed as received.

62

This reproduction is the best copy available.

UMI
calcium through existing cells (osteoclasts, osteoblasts, and osteocytes). If the stimulus is for a short time only, this response may suffice to satisfy the need for calcium and therefore inactivate the parathyroid before any other morphological changes of the bone cells could take place. If, however, the stimulus is continued for some time there is, in addition to the changes just mentioned, a morphological alteration in the cells tending to cause them to coalesce into osteoclasts. When the stimulus is removed the cells first cease their bone dissolving activities and then tend to dissociate once more into osteoblasts which are ready to rebuild what was torn down if the proper conditions exist.

It is felt that the data presented in the first section of results do demonstrate that the increase in osteoclasts is a reliable index of increased parathyroid secretion. The data further illustrate that exogenous parathyroid hormone is also effective in causing osteoclast increases. With the index of endogenous parathyroid activity established it is possible to assess changes in parathyroid activity under the influence of various experimental conditions.

Since the remainder of this thesis was concerned with separating the effects of calcium from those of phosphate with regard to parathyroid stimulation the question might be raised as to what the other principal ions present in the blood are doing as a result of varying parathyroid stimulation. This would bring to mind potassium, sodium, magnesium, and citrate, primarily. Toft (1957) showed that, in continuous peritoneal lavage, there was no significant difference
between control and parathyroidectomized animals with regard to either sodium or potassium content of the lavage fluid. Sodium was, of course, added as a constituent of the lavage fluid. There was no potassium added, however. Since there was no significant change in either of these two ions it is assumed that any change in parathyroid secretory rate could not be mediated directly through them.

In recent work (unpublished) Talmage and Toft noted that the administration of exogenous potassium to the lavage fluids caused a depression of osteoclasts in both control and experimental groups. Since this depression occurred in both groups of animals it is felt that this phenomenon is mediated through the osteoclasts themselves and not through the parathyroids. No explanation can be given at this time. It is hoped that further study will elucidate the cause of this, or at least give some more indication of what factors are involved.

In a series of experiments now under way in the laboratory the effects of exogenous magnesium are being studied. To date it has been noted that the endogenous magnesium is unaffected by parathyroidectomy in lavaged animals. There is no difference between the control and experimental groups with regard to magnesium level. The effects of calcium and phosphate administration on magnesium have not as yet been studied. However, since in the normal and parathyroidectomized animals there is no difference in magnesium level, it is felt that this ion could not have a controlling influence on the parathyroids, even though this view has been proposed at one time or another.
Another study under way at this time is to determine the effects of exogenous magnesium on calcium metabolism. There are no data available at the present time regarding this study.

The citrate ion presents a different problem. Since there has been adequate demonstration of the effects of citrate on bone dissolution and calcium mobilization the question of its action on the parathyroids might well be investigated. Exogenous citrate will cause calcium mobilization. However, this effect can be shown in parathyroidectomized as well as control animals indicating a direct effect of the citrate on bone rather than on the parathyroids. Talmage and Elliott (1956) showed that during peritoneal lavage the citrate level of the lavage fluid decreases rapidly and remains at a very low level. This is in contrast to the calcium level which is maintained constant throughout the lavage. If the citrate were stimulating the parathyroids, such a stimulation should decrease as the titer of citrate diminished. This is not the case and therefore it is felt that citrate is not a controlling factor of parathyroid stimulation.

This leaves then, the two principal ions of the bone, calcium and phosphate. As further proof that one or the other of these two ions is responsible for controlling parathyroid secretion, the nephrectomy syndrome was studied. Although there is a transient accumulation of citrate in the blood following nephrectomy, this soon falls to normal in the face of a constantly increasing osteoclast concentration. Phosphate is increasing at the same time. It would seem at first glance that such a picture implicates phosphate as the controlling factor. Even when the osteoclast increases are
quantitatively altered by varying doses of calcium the phosphate may still be implicated since it, too, is diminished by calcium administration.

Though these nephrectomy studies do not, alone, point to calcium as the controlling agent they do show that variation in these two ions can change radically the degree of parathyroid stimulation. When the feed-back mechanism is considered it can be seen that the changes due to nephrectomy may be explained fully by a mechanism involving calcium as the stimulating substance for parathyroid secretion.

The technique of continuous peritoneal lavage has been very useful for two reasons. First, it mirrors, continuously, changes in the blood and extracellular fluids. Changes in the serum calcium or phosphate level are reflected by similar changes in the lavage fluids. The changes in the lavage fluids may not be the same in terms of milligrams/100 ml as those in the blood but they will be the same in percent change. The second advantage of the lavage is that it permits the addition of various concentrations of test substances to the physiological fluids of the animal in precise quantities and allows for the accurate measurement of the utilization of these materials by the animal. It further provides a method for creating a drain on the calcium stores of the body and thus allows for continual stimulation of the parathyroids. The degree of stimulation can be altered by the amount of exogenous calcium administered in the lavage.

The removal of calcium from control and parathyroidectomized animals demonstrates very well the ability of the physicochemical
mechanism to supply a constant level of calcium to the extracellular fluids. It also shows the ability of the parathyroids to maintain the serum calcium level above this equilibration level in the face of a continual drain of calcium from the animal.

The final proof of the control of the parathyroids must come through regulation of the level of one ion while changing the concentration of the other, and noting the difference in parathyroid activity as evidenced by changes in osteoclast concentration. The lavage studies were aimed at accomplishing this goal.

The two basic ideas concerning the control of parathyroid secretion have been the presence of either low serum calcium or high phosphate levels. The first lavage studies using a calcium- and phosphate-free solution caused stimulation of osteoclasts in the presence of a low calcium and low phosphate level. Thus the first evidence for calcium being the stimulating agent was presented. Certainly the stimulation could not have been caused by an elevated phosphate level since the phosphate was below normal.

In the second series of lavages the animals received a high-calcium and phosphate-free lavage solution. There was a definite suppression of osteoclast formation in these animals as compared to the controls which had received the saline lavage fluid only. Thus, low calcium was again implicated as the controlling agent. The phosphate remained low.

However, the possibility still existed that either a low calcium level or a high phosphate level could cause parathyroid stimulation. To test this possibility animals were lavaged with high phosphate
lavage while being administered calcium. The lavage fluid was
elevated with respect to both calcium and phosphate. Since there
was osteoclast inhibition in the experimental group in the presence
of high phosphate over the control group it was concluded that high
phosphate could not be the controlling factor and that a low serum
calcium must be the stimulatory agent.
SUMMARY

A method is described whereby the concentration of osteoclasts in a given area of rat femur is shown to be a quantitative index of endogenous parathyroid activity. Stimulation of parathyroid activity by nephrectomy or continuous peritoneal lavage causes a significant increase in osteoclast concentration. Parathyroidectomy for 7 days causes a significant fall in numbers of osteoclasts. Fifty units of parathyroid extract will return these parathyroidectomized animals to normal with regard to numbers of osteoclasts. The administration of parathyroid extract to normal animals causes an increase in osteoclasts within 18 hours.

Having established this index of endogenous parathyroid activity, the effects of nephrectomy are studied. Following nephrectomy there is a rapid rise in numbers of osteoclasts to double the normal number within 24 hours. Other changes following nephrectomy are also noted. The administration of graded doses of calcium to the nephrectomized animals beginning at the time of nephrectomy caused inhibition of osteoclast increase related directly to the dose administered. This indicates that calcium may be the controlling factor of parathyroid secretion; however, the changes in phosphate which accompanied this treatment do not eliminate it as a possibility.

Another series of experiments utilized the technique of continuous peritoneal lavage to study the effects of varied levels of calcium and phosphate on the parathyroid stimulation as measured by the osteoclast index. With a low calcium, low phosphate condition there was stimulation of osteoclasts. When calcium alone was added
to the lavage fluid in the presence of low phosphate there was an inhibition of increase, thus indicating again that the calcium ion was the stimulating factor. Since this series did not eliminate high phosphate as a possible stimulating influence a third series was run.

In this series conditions of high calcium and high phosphate were maintained throughout the lavage. There was inhibition of osteoclast formation indicating a lack of parathyroid stimulation. This, of course, eliminated the possibility of phosphate being the controlling factor and also pointed again to the low calcium as being the factor controlling parathyroid secretion.

A discussion of calcium control of parathyroid stimulation is tied in with the concept of a feed-back mechanism for calcium metabolism. The effects of phosphate in such a system are important but are secondary to calcium and operate through the changes in calcium brought about by changes in phosphate.

Further work on this project will include a study of the effects of exogenous magnesium on the parathyroid output and on the release of radioactive calcium and phosphorus from bone. A study of potassium infusion and its effects on parathyroid physiology and osteoclast increases will be undertaken.
BIBLIOGRAPHY


Carneiro, J. and C. P. Leblond 1959 Role of osteoblasts and odontoblasts in secreting the collagen of bone and dentin as shown by radioautography in mice given tritium-labelled glycine. Exp. Cell Res. 18:291.


Collip, J. B. 1925 The extraction of a parathyroid hormone which will prevent or control parathyroid tetany and which regulates the level of blood calcium. J. Biol. Chem. 62:395.


---------- and ---------- 1956b Relative effect of vitamin D and parathyroid extract on plasma calcium and citric acid of normal and thyroparathyroidectomized dogs. Endo. 59:196.


---------- and ---------- 1958 Removal of Ca$^{40}$ and Ca$^{45}$ from bone by citrate as influenced by the parathyroids. Endo. 62:709.


1959 Parathyroid gland and bone in vitro, VI. Developmental Biology 1:152.


1942b The solubility of calcium phosphate. II. The solubility product. J. Biol. Chem. 143:711.


, and 1925b Studies in calcification. II. Delayed equilibrium between phosphates and its biological significance. J. Biol. Chem. 64:567.


, and A. Bodansky 1930a Experimental fibrous osteodystrophy (Osteitis Fibrosa) in hyperparathyroid dogs. J. Exp. Med. 52:669.


Kenny, A. D., P. R. Draskoczy, and P. Goldhaber 1959 Citric acid production by resorbing bone in tissue culture. Am. J.


Logan, M. A. 1940 Recent advances in the chemistry of calcification. Physiol. Revs. 20:522.


, M. A. Lipton, W. Bloom, and E. S. G. Barron 1946 Biological factors in calcification in bone. Trans. 14th Conf. on Metabolic Aspects of Convalescence 14:9.


Pugsley, L. I. 1932 The effect of parathyroid hormone and of irradiated ergosterol on calcium and phosphorus metabolism in the rat. J. Physiol. 76:316.


and H. Selye 1933 The histological changes in the bone responsible for the action of parathyroid hormone on the calcium metabolism of the rat. J. Physiol. 79:113.


Selye, H. 1932 Action of parathyroid hormone on the epiphyseal junction of the young rat. Arch. Pathol. 14:60.

1932 On the stimulation of new bone formation with parathyroid extract and irradiated ergosterol. Endo. 16:547.


Talmage, R. V. and J. R. Elliott 1956 Changes in extracellular fluid levels of calcium, phosphate and citrate ions in nephrectomized rats following parathyroidectomy. Endo. 59:27.

and 1958 Removal of calcium from bone as influenced by the parathyroids. Endo. 62:717.

and A. C. Enders. 1957 Parathyroid function as studied by continuous peritoneal lavage in nephrectomized rats. Endo. 61:256.


Tweedy, W. R. 1937 Further studies on the action of parathyroid extract in the dog following total and partial ablation of the kidneys. Endo. 21:55.

