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HISTOMORPHOMETRIC EVALUATION OF THE EFFECTS OF 1,25-DIHYDROXYCHOLECALCIFEROL, PARATHYROID HORMONE, AND THYROXINE ON CORTICAL AND TRABECULAR BONE IN ADULT DOGS

The Ohio State University PH.D. 1980

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HISTOMORPHOMETRIC EVALUATION OF THE EFFECTS OF
1,25-DIHYDROXYCHOLECALCIFEROL, PARATHYROID HORMONE,
AND THYROXINE ON CORTICAL AND TRABECULAR BONE
IN ADULT DOGS.

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Wanda Bernardette High, B.A., D.V.M.

* * * * *

The Ohio State University
1980

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CHAPTER I

HISTOMORPHOMETRIC EVALUATION OF THE EFFECTS OF INTERMITTENT 1,25-DIHYDROXYCHOLECALCIFEROL ADMINISTRATION ON CORTICAL BONE REMODELING IN ADULT DOGS

INTRODUCTION

Remodeling defects are important in the pathogenesis of skeletal disorders in both human beings and animals because the overall effect of the remodeling process is determined by the recruitment and function of bone cells (Frost, 1973). Critical in the pathogenesis of osteoporosis is a slowing of the remodeling rate which is characterized by a decrease in the recruitment of bone cells (Sedlin, 1964; Frost, 1973; Lindholm et al., 1977). Factors that influence the pathogenesis of metabolic bone disease produce quantitative and qualitative changes in bone by affecting these remodeling parameters (Frost, 1973).

It is well established that vitamin D can modify bone remodeling both experimentally and naturally in certain disease processes (Frost, 1973; Rasmussen, 1974a; Edelstein et al., 1976; Larsson et al., 1976; Raisz et al., 1978). 1,25-dihydroxycholecalciferol (1,25-(OH)2D3), the active metabolite of vitamin D, accumulates specifically in the nuclei of bone cells (Weber et al., 1971) and establishes normal mineralization (Rasmussen, 1974a). In addition, 1,25-(OH)2D3 mobilizes calcium from bone (Raisz et al., 1972; Rasmussen, 1974a)
induces osteogenesis, and under certain circumstances may result in osteosclerosis (Raisz et al., 1978).

The effects of 1,25-(OH)₂D₃ on bone are dose-dependent (Raisz et al., 1972, 1978; Rasmussen et al., 1974a, 1974b). Of great importance are the different effects of pharmacologic compared to near physiologic levels of the hormone. Pharmacologic levels of cholecalciferol congeners often cause interference with somatic growth and maintenance, including bone (Raisz et al., 1978). At moderate pharmacologic levels, 1,25-(OH)₂D₃ induces bone resorption by increasing osteoclasia which is independent of the osteocyte-osteoblast calcium pump, thereby producing a decrease in the mineral content of bone (Weisbrode et al., 1978).

In contrast, chronic intermittent pharmacologic doses of the parent vitamin D compound may result in surges of osteogenic activity and has resulted in cycles of matrix deposition, maturation and mineralization in rats (Storey, 1969). This osteogenic effect was interpreted as evidence of bone formation. Recent studies using low doses of 1,25-(OH)₂D₃ have also demonstrated an anabolic effect of the hormone on bone. The administration of continuous low doses of 1,25-(OH)₂D₃ to rats increased the cortical bone mass (Larsson et al., 1977). In dogs low doses of 1,25-(OH)₂D₃ resulted in a reduction of the trabecular bone loss associated with disuse osteoporosis (Caywood et al., 1979).

Most of the available data on the effects of 1,25-(OH)₂D₃ on bone have come from in vitro studies (Raisz et al., 1972; Chertow et al., 1975) or studies in young growing animals (Campbell and Douglas, 1965; Chineme et al., 1976; Reynolds et al., 1976). There is little known about the effects of 1,25-(OH)₂D₃ on the adult skeleton. In addition, the cellular mechanisms by which 1,25-
(OH)$_2$D$_3$ and other agents cause changes in bone remodeling are not well understood.

Tissue injury may increase bone remodeling by factors of 2- to 20-fold (Frost, 1973). Sola et al. (1976) demonstrated enhanced longitudinal bone growth in 85% of subjects following periosteal stripping. Yabsley and Harris (1965), and Jenkins et al. (1975) studied the effects of periosteal stripping on the epiphyseal plate and cortical bone and concluded that vascular hypertrophy induced by this technique was the mediator of enhanced osteogenesis. The anatomical distribution of the accelerated remodeling that follows tissue injury is known to be regional and due to an increased activation frequency (Frost, 1973). This is a reflection of an increased differentiation of mesenchymal cells into osteoclasts and osteoblasts with a corresponding increase in the number of remodeling centers, resorption and formation surfaces, and bone turnover.

In the present experiment, surgical elevation of the periosteum of the 9th rib was used to induce a "regional acceleratory phenomenon" to determine if this technique would provide a useful model for detecting and comparing in the same animal the effects of chemical agents on cortical bone remodeling within a reduced period of time. The objectives of this investigation were: (1) to determine the morphometric and dynamic effects of intermittently administered 1,25-(OH)$_2$D$_3$ on cortical bone remodeling in unaltered ribs and in combination with periosteal elevation in adult dogs; and (2) to correlate the alterations in bone remodeling with changes in serum calcium and phosphorus, and urinary hydroxyproline concentrations.
MATERIALS AND METHODS

Experimental Animals: Six female beagle dogs (Marshall Research Animals, North Rose, N.Y. 14516) each weighing approximately 20 pounds and between 10 to 12 months of age were acclimated to laboratory conditions for two weeks. The dogs were fed commercial Purina dog chow (Ralston Purina Co., Checkerboard Square, St. Louis, MO 63188) with free access to water. Blood and urine samples were collected during the acclimation period and biweekly, thereafter. The dogs were maintained in individual stainless steel metabolism cages for urine collection. Blood collection and intravenous 1,25-(OH)₂D₃ administration were performed using indwelling catheters placed in the jugular vein (epidural catheter, No. 8222, Becton-Dickinson & Co., Rutherford, NJ 07070).

Experimental Design: The study lasted for 60 days or one-half the lifespan of a bone forming site in the dog (Frost, 1973). Fifty units (1.25 µg/day) of 1,25-(OH)₂D₃ in ethanol was administered intravenously in equally divided doses twelve hours apart. The hormone was administered for 6 days, then withdrawn for 14 days. Three complete cycles of drug administration and withdrawal occurred within the 60-day period. The dose was selected from a pilot dose-response study with 1,25-(OH)₂D₃ and results of a study with the parent compound in dogs that determined the lowest dose that altered bone dynamics without resulting in persistent hypercalcemia.*

Each dog served as its own control by collecting a pre-treatment rib biopsy (left 9th, 10th and 11th), when the periosteal elevation of the right 9th rib was performed on day 0. The dogs were euthanized after 60 days and the

contralateral ribs (right 9th, 10th, and 11th) were removed and fixed in 70% ethanol.

Serum was collected twice weekly. Serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Model 303) (Willis, 1960). Serum phosphorus was determined by the method of Baginski and Zak (1960). Urine was collected under toluene twice weekly in metabolism cages. Urine was assayed for hydroxyproline by the method of Kivirikko et al. (1967) and creatinine by the method of Clark (1961). Urinary hydroxyproline was expressed as a ratio to creatinine.

Surgical Procedures

Anesthesia: The dogs were administered 0.25 mg/kg acepromazine maleate (Ayerst Laboratories, Inc., New York, NY 10017) and 0.20 mg atropine sulfate (Eli Lilly & Co., Indianapolis, IN 46232) intramuscularly. Anesthesia was induced with Surital (Thiamylal Sodium for injection, Parke-Davis & Co., Detroit, MI 48232) administered intravenously and was maintained using methoxyfluorane (Metafane, Pitman-Moore, Inc., Washington Crossing, NJ 08560).

Periosteal Elevation: The incision site for periosteal elevation of the right 9th rib was determined by measuring 5.0 cm vertically and proximally from the costochondral junction and 5.0 cm vertically and distally from the transverse process of the vertebra (Piermattei and Greeley, 1966). A skin incision approximately 5.0 cm in length was made directly over the right 9th rib and continued through the subcutaneous fat, fascia and muscle. The periosteum was incised vertically for a length of 3.5 cm, separated from underlying bone,
and reflected laterally from the rib using periosteal elevators. (Keys elevator-chisel, Nos. 5W500 and 6W501, Max Wocher & Sons, 315 Plum St., Cincinnati, OH 45202). A small curved periosteal elevator (Mackintz elevator No. 5W275, Max Wocher & Sons, 315 Plum Street, Cincinnati, OH 45202) was used to free the rib laterally and medially of its periosteum.

**Rib Biopsy:** The biopsy incision site was identified as described above (Piermattei and Greeley, 1966). The incision was made over the left 10th rib for a length of 6.0 to 7.0 cm. Blunt dissection of fat, fascia and muscle was accomplished with scissors to expose the left 11th, 10th, and 9th ribs. The periosteum was reflected and a 3.0 cm section of each rib was removed with a double-action bone cutter (Stille-Liston double action bone cutting forceps, No. 69-410, Misdom-Frank Corp., 860 Broadway, New York, NY 10003).

**Bone Histomorphometry**

Morphometric analysis was performed on ground bone sections stained with Osteochrome stain (Villaneuva, 1974) using both fluorescence and bright field light microscopy.

**Double Fluorochrome Bone Labelling**

Determination of the dynamics of appositional bone growth was facilitated by the intravenous administration of tetracycline and a second fluorochrome label DCAF (2,4 BIS) N,N' Di (carboxymethyl) (amino methyl fluorescein), (I.C.N. Pharmaceuticals, Inc., Life Science Group, Cleveland, OH 44128) (Suzuki and Mathews, 1966; Frost, 1969). Fluorochrome administration began 18 days before the start of the study and again on day 42 using the
schedule of 3 days on (tetracycline), 7 days off, and 3 days on (DCAF). The dosage was 15 mg/kg for both tetracycline and DCAF.

Osteochrome-stained ground bone sections (80 μ) were viewed under an ultraviolet microscope using a Wratten 18A filter at the source, a 2B filter at the eye piece, and a wavelength of 36 μm at both 10X and 25X magnification. Tetracycline (Oxytetracycline hydrochloride, "Liquamycin", Pfizer, Inc., New York, NY 10017) produced yellow (Frost, 1969) and DCAF a green fluorescence (Suzuki and Mathews, 1966) with ultraviolet light.

Ground Bone Sections

An average of 5 cross-sections per rib were cut at approximately 200 μ thickness using a jeweler's saw (Apparatus for Microstructural Analysis Buehler Ltd., 2120 Greenwood St. Evanston, IL 60204). The sections were ground to approximately 100 μ thickness (Frost, 1958). After staining for 48 hours with osteochrome the sections were reground to 75-90 μ. Section thickness was measured by a mikrokatıor (Mikrokatıor No. 7VZ509-4, C.E.J. Gage Co., Subsidiary of C.E. Johnson, Sweden, 10641 Haggerty, Dearborn, MI 48126). The sections were washed in 0.1% benzalkonium chloride to remove debris, rinsed in distilled water, and differentiated in 0.01% acetic acid in 95% methanol. When differentiation was completed they were dehydrated in ascending concentrations of alcohol, cleared in xylol, and mounted in Eukitt's mounting media (Calibrated Instruments, Inc., 731 Saw Mill River Road, Ardsley, NY 10502).

Morphometric Evaluation of Bone

Measurements: Morphometric measurements were performed with a calibrated micrometer and Zeiss integrator plate II, 100/25 ocular grid with a
0.6 aperture (Frost, 1962). A minimum of 5 cross-sections of each bone were measured in order to analyze $50 \text{ mm}^2$ of bone.

Cross sectional areas of cortical and total bone were measured separately by the grid method of Sedlin (1964). A square-ruled (10 X 10) grid of known area was superimposed optically upon a section measured under a light microscope. Intersections covered by the surface were counted as "hits" and those tangent were counted as "1/2 hits". The mean cortical and total cross sectional areas were calculated by averaging the results of separate determinations.

The circumference of bone surfaces was determined by measuring the perimeter intersects of the surface using the Zeiss integrator ocular grid in a 40X objective. The eyepiece grid contained 10 equally spaced parallel and perpendicular lines which were superimposed on the surface to be analyzed. The number of parallel lines intersecting the intima of the seam were counted as "hits". On each observation per field ("throw") the "hits" were counted, totaled, and the mean calculated per section. The distance between 2 parallel lines was measured and calibrated.

Active centers of bone formation were identified using the fluorescence microscope by the presence of yellow (tetracycline) or green (DCAF) labels at the periphery of an osteoid seam. Measurements for calculation of the appositional rates ($M_a$ and $M_T$) were taken at the site of localization of the fluorochrome labels. The distance between two labels was measured in millimeters by a calibrated eyepiece micrometer at four equidistant points around its circumference and mean values were calculated.
The following nine measurements and counts for cortical bone were made from cross-sections of rib from each dog:

(1) Cortical area ($A_C$)
(2) Total area ($A_T$)
(3) Total number of osteoid seams ($A_f$)

The mean number was expressed per mm$^2$ of cortical area ($A_C$). An osteoid seam was identified as a bone formation site by a green or red intimal band on sections stained with osteochrome.

(4) Total number of resorption cavities ($A_r$)

$A_r$ was measured by systematic scanning of the entire section using a 10 X objective. Resorption cavities were identified by the presence of scalloped edges.

(5) Total number of labelled osteoid seams ($L$)

(6) Mean distance between fluorochrome dyes within a single Haversian system (MAR)

(7) Mean wall thickness of finished osteons (MWT)

(8) Osteoid seam thickness (OST)

(9) Mean circumference of osteoid seams ($S_f$)

Calculations: From the nine basic measurements the quantitative morphometric analysis of bone consisted of the following stereological calculations (Frost, 1973):

(1) Total cortical cross sectional area ($A_C$) in mm$^2$

(2) Total cross sectional area ($A_T$) in mm$^2$

$$A_C \text{ or } A_T = \frac{\text{No. of Hits}}{(\text{Grid Factor})(\text{No. of Throws})(\text{No. of Intersections})}$$

(3) Mean number of osteoid seams per mm$^2$ ($A_f$)
(4) Mean number of resorption cavities per \( \text{mm}^2 \) (\( A_r \))

\[
A_f \text{ or } A_r = \frac{\text{Total No. of Osteoid Seams or Resorption Cavities}}{\text{Total Cortical Area} (A_C) \text{ mm}^2}
\]

(5) Percent labelled osteoid seams (\( \% L \))

\[
\% L = \frac{\text{Total No. Labelled Osteoid Seams}}{\text{Total No. of Osteoid Seams}}
\]

(6) Mean appositional rate (\( M \)) in \( \mu \text{/day} \)

\[
M = \frac{\text{Mean Distance Between Two Labels (MAR) in microns}}{\text{No. of Days given Label}}
\]

(7) Radial closure rate (\( M_f \)) in mm/yr.

\[
M_f = (M) \times (\% L) \times (365/1000^*)
\]

* reduces microns/day to mm/year.

(8) Osteoid seam circumference (\( S_f \))

\[
S_f = \frac{\text{(No. of Hits)}}{\text{(Distance Between Parallel Grid) (\( \pi \))}} \times (2) \times \frac{\text{(No. of Throws)}}{}
\]

(9) Ratio of cortical (C) area in mm\(^2\) (\( A_C \)) to total (T) area in mm\(^2\) (\( A_T \))

\[
C/T = \frac{A_C}{A_T}
\]

(10) Ratio of the numbers of resorption cavities (\( A_r \)) to osteoid seams (\( A_f \))

\[
\frac{A_r}{A_f} = \frac{A_r}{A_f}
\]

(11) Osteon formation time in years (\( \Theta_f \))

The time required to complete lamellar bone formation in a cross section of an average osteon.

\[
\Theta_f = \frac{MWT}{M_f}
\]

(12) Number of activation frequency foci in mm\(^2\)/year (\( \mu_f \))

\[
\mu_f = \frac{A_f}{\Theta_f}
\]
\( \mu_f \) represents the number of new osteons introduced per year in an average mm\(^2\) of cortical area.

(13) Bone formation rate in mm\(^2\)/mm\(^2\)/year \((V_f)\)

\[
V_f = (A_f) \times (S_f) \times (M_F)
\]

**Statistical Analysis:** Statistical significance of differences in the morphometric data comparing treatment effects between ribs were determined by the Nemenyi rank sum one-way classification test (Nemenyi, 1961). The Wilcoxon signed rank test was used to determine statistical significance of differences in paired data for each rib (Dixon *et al.*, 1977). The biochemical data were evaluated by Student t test.
RESULTS

Histomorphometric Evaluation

The bone measurements and calculated parameters are given (Table 1.1) as means ± standard error with their associated levels of significance.

Effects of 1,25-(OH)$_2$D$_3$ Alone - 11th Rib: Activation frequency foci ($\mu_f$), an index of the number of new remodeling sites per mm$^2$ per year, decreased 42% (P<0.01) after 1,25-(OH)$_2$D$_3$ compared to baseline values from 13.07 to 7.56 foci/mm$^2$/year. Osteoid seam thickness (OST) decreased 20% (P<0.05) from 8.95 to 7.15 µm and osteoid seam circumference ($S_f$) decreased 16% (P<0.05) from 0.12 to 0.10 mm. Dynamic data revealed that the mean appositional rate decreased 16% (P<0.05) from 0.81 to 0.68 µ/day and bone formation rate decreased 57% (P<0.01) from 0.14 to 0.06 mm$^2$/mm$^2$/year.

Effects of 1,25-(OH)$_2$D$_3$ and Periosteal Elevation - 9th Rib: The effects of 1,25-(OH)$_2$D$_3$ with periosteal elevation superimposed increased the activation frequency in cortical bone 41% (P<0.05) from 9.22 to 13.74 foci/mm$^2$/year. This was reflected in an increase (4.05 to 4.17/mm$^2$) in the numbers of osteoid seams ($A_f$) (3%, P<0.01), resorption cavities ($A_r$) (0.74 to 1.62/mm$^2$) (119%, P<0.01), and the ratio ($A_r$/A$_f$) of bone resorbing to bone forming sites (100%, P<0.01) (0.20 to 0.40). Static morphometric data demonstrated a 29% (P<0.01) increase in osteoid seam thickness and a 30% (P<0.01) increase in osteoid seam circumference. The mean diameter of the osteoid seams was 40 µ. There was a 47% (P<0.01) increase in the number of labelled seams. The mean radial closure rate ($M_r$) increased 42% (P<0.01) from 0.19 to 0.27 mm/year and compared to
Baseline values was associated with a 44% decrease (P<0.02) in the mean osteon formation time ($T_f$) from 0.56 to 0.33 years.

Bone formation rate ($V_f$) increased 75% (P<0.01) from 0.08 to 0.14 mm$^2$/mm$^2$/year. Cortical and total bone areas were significantly increased 10% and 15% (P<0.05), respectively. The increased bone area primarily consisted of new woven bone on the periosteal envelope.

1,25-(OH)$_2$D$_3$ and Regional Effects of Periosteal Elevation - 10th Rib: Only two significant changes were observed in the 10th rib. The mean activation frequency decreased 40% (P<0.05) from 13.11 to 7.89 foci/mm$^2$/year and there was a 137% increase (P<0.05) in the ratio of numbers of resorption cavities to osteoid seams.

Comparison of 1,25-(OH)$_2$D$_3$ Effects on the 9th, 10th, and 11th Ribs: Periosteal elevation superimposed upon the effect of 1,25-(OH)$_2$D$_3$ (9th rib) resulted in an increase in cortical bone area while the hormone alone (11th rib) produced a decrease. The difference in these effects was significant (P<0.02). 1,25-(OH)$_2$D$_3$ resulted in an increase in the number of bone forming sites both independently (11th rib) and with periosteal elevation superimposed (9th rib). The magnitude of the increase in the number of bone forming sites following treatment with the hormone alone (11th rib) was significantly greater than that observed when periosteal elevation was superimposed (9th rib) (P<0.05). The 10th rib exposed to both the hormone and the regional influence of surgery showed a trend towards a decrease in the number of bone forming sites.

1,25-(OH)$_2$D$_3$ increased the number of resorption sites over baseline values in all 3 ribs but the increase was significant only in the 9th rib where periosteal elevation was superimposed. The effect on the 9th rib was
significantly different from the effects observed in both the 10th (P<0.05) and the 11th (P<0.02) ribs.

**Biochemical Evaluation**

Serum calcium and phosphorus and urinary hydroxyproline concentrations are given as means ± standard error (Table 1.2). Serum calcium values were consistently increased above the baseline value of 8.6 ± 0.16 mg/dl following 1,25-(OH)₂D₃. The highest calcium concentrations were observed during the three intervals of 1,25-(OH)₂D₃ administration. During the first period of 1,25-(OH)₂D₃ calcium concentrations increased up to 103% (17.49 ± 0.57 mg/dl) (P<0.001) above baseline values. Serum calcium increased 56% to 13.40 ± 0.61 mg/dl (P<0.001) and 30% to 11.2 ± 0.62 mg/dl (P<0.01) during the second and third periods of 1,25-(OH)₂D₃ administration, respectively. During the three withdrawal periods serum calcium concentrations declined but reached the baseline range only during the first withdrawal period. Serum calcium values decreased during the second and third withdrawal periods with blood levels remaining 23% (10.57 ± 0.67 mg/dl (P<0.05) and 8% (9.30 ± 0.42 mg/dl) above mean baseline values, respectively.

Serum phosphorus was increased above the baseline value of 4.19 ± 0.19 mg/dl after 1,25-(OH)₂D₃. The highest serum phosphorus concentrations were observed during the first period of 1,25-(OH)₂D₃ when blood levels were increased 58% (6.62 ± 0.81 mg/dl) (P<0.05) above baseline values and at the termination of the experiment. During the second and third periods of 1,25-(OH)₂D₃ administration serum phosphorus increased 35% to 5.65 ± 1.38 mg/dl and 44% to 6.02 ± 0.39 mg/dl (P<0.01), respectively. Serum phosphorus
concentrations decreased during the three withdrawal periods. Serum phosphorus decreased to $4.37 \pm 0.28$ mg/dl during the first withdrawal period and decreased 12% ($3.75 \pm 0.51$ mg/dl) during the second withdrawal period.

Urinary hydroxyproline excretion was increased 90% above baseline values during the first period of $1,25-(OH)_2D_3$ administration to $13.53 \pm 0.82$ µg/mg CR ($P<0.01$). During the second interval of $1,25-(OH)_2D_3$ administration hydroxyproline excretion decreased 20% ($5.66 \pm 1.14$ µg/mg CR). Hydroxyproline excretion increased 43% ($10.72 \pm 3.07$ µg/mg CR) on the first day but had decreased 13% ($6.18 \pm 4.12$ µg/mg CR) on the sixth day during the third interval of $1,25-(OH)_2D_3$ administration. During the first, second and third withdrawal periods hydroxyproline excretion approached baseline values and was below baseline levels at the termination of the study.
DISCUSSION

The present histomorphometric study demonstrated that within the confines of the length of this study the intermittent administration of moderately low doses of 1,25-(OH)$_2$D$_3$ decreased the calculated activation frequency in cortical bone (10th and 11th ribs), increased osteon formation time, and decreased bone formation. Osteoid seam thickness and osteoid seam circumference decreased and resulted in a preponderance of nearly completed Haversian systems. There was a significant decrease in the mean appositional rate which correlated with changes in the character of the osteoid seam and probably reflected their smaller circumference. Marotti et al. (1976) demonstrated in young dogs that a mean Haversian canal radius of 65 μ correlated with an apposition rate of 1.8 μ/day while a mean radius of 16 μ correlated with an apposition rate of 0.4 μ/day. A similar finding has been reported in adult dogs by Anderson and Danylchuk (1979). The present study revealed the mean radius of Haversian canals in the rib of the dogs to be 15 μ.

Previously formed osteons appeared to continue to completion but at a slower rate following intermittent 1,25-(OH)$_2$D$_3$ administration in the present study. The increase in osteon formation time as well as the decrease in bone formation rate reflected the effects of a decrease in radial closure and apposition rates. Therefore, there appeared to be a slowing down of the measured process of osteoblastic bone formation over the relatively short time course of this study.

The effects of intermittent 1,25-(OH)$_2$D$_3$ shared certain features with the transient effects of estrogen treatment in human beings in which a suppressor
effect on activation led to a deficit of new resorbing centers and allowed older centers to continue to completion (Frost, 1973). This effect of estrogen temporarily lowered bone resorption and caused a temporary positive skeletal balance.

Recent studies reported by other investigators using continuous administration of $1,25-(OH)_2D_3$ to rats demonstrated that enhanced bone formation occurred in cortical and trabecular bone. Larsson et al (1977) using 0.03, 0.06, and 0.12 µg (1.20, 2.4, and 4.8 U, respectively) of $1,25-(OH)_2D_3$ per 0.5 kg adult rat, observed an increase in the trabecular bone mass of the femur. In addition, the low dose of $1,25-(OH)_2D_3$ caused an increase in the cortical thickness. Both the inner and outer diameters of cross-sections of the femur were decreased following the administration of $1,25-(OH)_2D_3$ at all three dose levels. These results were interpreted to suggest that low doses of the active metabolite of vitamin D increased cortical bone mass by stimulating bone formation and suppressing bone resorption at the endosteal surface. Higher dose levels caused a decrease in cortical thickness.

Intermittent administration of 50 U (1.25 µg) of $1,25-(OH)_2D_3$ to adult dogs in the present study resulted in a trend towards a decrease in the total area, cortical area and marrow area in the unaltered 11th rib. These changes reflected net bone resorption at the periosteal envelope and net formation at the endosteal envelopes. These effects were in contrast to the normal expansion of envelopes of compact bone throughout life (Frost, 1973).

The possibility that some of the effects of intermittent $1,25-(OH)_2D_3$ administration on bone of adult dogs were secondary to a relative hypoparathyroidism or increased secretion of calcitonin secondary to hypercalcemia
cannot be completely ruled out. Previous investigations have demonstrated that circulating levels of parathyroid hormone decrease following vitamin D₃ administration (Chertow, 1973; Larsson, 1976). Parathyroid hormone appears to have a permissive effect with regard to the action of 1,25-(OH)₂D₃ on bone and has an important role in the recruitment of new remodeling sites (Frost, 1973; Rasmussen, 1974b).

The combination of 1,25-(OH)₂D₃ and periosteal elevation in the 9th rib resulted in significantly different effects than those seen in the 10th and 11th ribs. The activation frequency increased, bone formation increased, and osteon formation time decreased. The combined effects of periosteal injury and 1,25-(OH)₂D₃ accelerated bone remodeling and appeared to enhance both osteoblastic and osteoclastic activity. Whether these changes were related to the effects of 1,25-(OH)₂D₃ at all is not known; however, they were different from those detected in the 10th and 11th ribs, and may only reflect a response to the regional acceleratory phenomenon (RAP) induced by the periosteal elevation. An alternative explanation that the results may represent a magnification as well as a compression in time of the effects of 1,25-(OH)₂D₃ cannot be completely excluded. A comparison of the effects of 1,25-(OH)₂D₃ with those of parathyroid hormone (High et al., 1980) and thyroxine (High et al., 1980) on the 9th rib of adult dogs in which a RAP had been induced demonstrated that data from this site do reflect in part the influence of the hormone. Thyroxine and 1,25-(OH)₂D₃ increased Aₛ, Sₛ, Aₙ, Mₛ, µₛ and Vₛ, and decreased Qₛ in the periosteally elevated 9th rib. By comparison, parathyroid hormone (PTH), produced a significant increase in cortical bone area and decreased osteoid seam circumference in the 9th rib but had no significant effect upon any of the
other parameters. If the changes observed following thyroxine and $1,25-(OH)_2D_3$ administration reflected only the effect of the RAP, similar changes should have been observed in the PTH study.

Evaluation of serum electrolytes demonstrated that serum calcium and phosphorus were elevated during the intervals of $1,25-(OH)_2D_3$ administration and approached baseline values during periods of withdrawal. These elevations in serum electrolytes probably reflected the action of $1,25-(OH)_2D_3$ on intestinal calcium and phosphorus absorption and renal clearance of phosphorus. The highest serum calcium values primarily were associated with the first interval of $1,25-(OH)_2D_3$. The initial period of hypercalcemia was associated with an increase in urinary hydroxyproline excretion which probably was the result of an increase in bone resorption induced by $1,25-(OH)_2D_3$.

The results of this investigation must be interpreted with caution because the length of the experiment was less than the time required to complete a new bone forming unit in adult dogs. However, a comparison of data from the 9th rib (periosteal elevated) to the 11th rib demonstrated that the RAP cannot be used effectively as a system in which to generate information on the skeletal effects of chemical agents in the absence of an untreated control group that received only the periosteal elevation. The results from the unaltered ribs demonstrated that $1,25-(OH)_2D_3$ administration for 60 days decreased both the activation frequency and the rate of new bone formed compared to baseline values.
SUMMARY

The effects of intermittent low doses of 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$; 1.25 μg daily, administered intravenously for 6 days and withdrawn 14 days for 3 complete cycles) on cortical bone were determined and compared in ribs with steady state and superimposed regionally accelerated remodeling using adult intact female beagle dogs. The bone changes were analyzed by dynamic bone histomorphometric methods using tetracycline and DCAF (2,4 B1S) N, N' di (carboxymethyl) (amino methyl fluorescein) In vivo double labelling of bones before treatment and after 60 days of intermittent 1,25-(OH)$_2$D$_3$ administration.

The histomorphometric profile of normal cortical bone following the intermittent administration of 1,25-(OH)$_2$D$_3$ was characterized by a marked decrease in the activation frequency, bone formation rate, osteoid seam thickness, seam circumference, and mean appositional rate.

The morphometric changes in cortical bone exposed to both 1,25-(OH)$_2$D$_3$ and periosteal elevation were characterized by a marked increase in both the activation frequency and bone formation rate, and associated with a decrease in the osteon formation time. Other morphometric parameters that were increased included radial closure rate, osteoid seam number, resorption cavity number, the ratio of bone resorbing sites to forming sites, percent labelled seams, osteoid seam circumference, and both the total and cortical bone areas.

Serum calcium and phosphorus levels increased during 1,25-(OH)$_2$D$_3$ administration. Urinary hydroxyproline excretion increased during the first interval of 1,25-(OH)$_2$D$_3$ administration but was not changed significantly during the last two intervals.
These results indicated that in normal cortical bone intermittent administration of 1,25-(OH)$_2$D$_3$ decreased activation frequency and slowed the remodeling rate. Although recruitment of new remodeling sites was decreased, previously existing remodeling units continued to completion. These effects resulted in a preponderance of mature osteons in normal cortical bone. The combined effect of periosteal elevation and 1,25-(OH)$_2$D$_3$ were markedly different from those observed with 1,25-(OH)$_2$D$_3$ alone. The directional change in the parameters evaluated reflected an increase in bone remodeling such as occurs regionally following tissue injury. These findings suggest that the rapid bone turnover induced by tissue injury could mask or alter the effects of hormones on bone remodeling when studied over a relatively short period of time.
Table 1.1: Effects of intermittent 1,25-dihydroxycholecalciferol (1,25(OH)$_2$D$_3$) on histomorphometric parameters in the 9th, 10th, and 11th ribs of dogs. (Mean S.E., N = 6 dogs.)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>9th Rib Periosteal Elevated</th>
<th>10th Rib Regional Effects</th>
<th>11th Rib 1,25-(OH)$_2$D$_3$ Effects</th>
<th>p Values Comparing Treatment Effects Between 9th, 10th and 11th Ribs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-1,25(OH)$_2$D$_3$</td>
<td>Baseline</td>
<td>Post-1,25(OH)$_2$D$_3$</td>
</tr>
<tr>
<td>BONE MASS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_C$ (mm$^2$)</td>
<td>7.49 (0.61)</td>
<td>$^{++}+$8.25* 7.08 (0.41)</td>
<td>7.29 (0.67)</td>
<td>7.31 (0.24)</td>
</tr>
<tr>
<td>$A_T$ (mm$^2$)</td>
<td>15.90 (0.87)</td>
<td>18.26* 17.21 (1.23)</td>
<td>18.05 (1.52)</td>
<td>17.47 (1.45)</td>
</tr>
<tr>
<td>C/T</td>
<td>0.48 (0.05)</td>
<td>0.47 (0.04)</td>
<td>0.43 (0.02)</td>
<td>0.42 (0.01)</td>
</tr>
<tr>
<td>MWT (mm)</td>
<td>0.09 (0.01)</td>
<td>0.09 (0.00)</td>
<td>0.09 (0.01)</td>
<td>0.09 (0.01)</td>
</tr>
<tr>
<td>BONE FORMATION and OSTEOID</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_f$ (no/mm$^2$)</td>
<td>4.05 (0.75)</td>
<td>$^{++}$4.17* 4.91 (0.83)</td>
<td>$^{+}$4.02 (0.35)</td>
<td>4.73 (0.68)</td>
</tr>
<tr>
<td>OST (μm)</td>
<td>7.78 (1.13)</td>
<td>10.02*** 8.03 (0.61)</td>
<td>7.18 (0.43)</td>
<td>8.95 (0.60)</td>
</tr>
<tr>
<td>$S_f$ (mm)</td>
<td>0.10 (0.01)</td>
<td>0.13*** 0.10 (0.01)</td>
<td>0.11 (0.01)</td>
<td>0.12 (0.01)</td>
</tr>
<tr>
<td>L (%)</td>
<td>0.66 (0.16)</td>
<td>0.97*** 0.79 (0.09)</td>
<td>0.70 (0.07)</td>
<td>0.79 (0.14)</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.025, *** p<0.01 for individual paired (pre versus post) rib observations using Wilcoxon Signed Rank Test
+ p<0.05, ++ p<0.025 for treatment effects comparing ribs using Nemenyi One-way Classification Rank Sum Test.
Table 1.1: (continued)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>9th Rib Periosteal Elevated</th>
<th>10th Rib Regional Effects</th>
<th>11th Rib 1,25-(OH)(_2)D(_3) Effects</th>
<th>p Values Comparing Treatment Effects Between 9th, 10th and 11th Ribs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-1,25(OH)(_2)D(_3)</td>
<td>Baseline</td>
<td>Post-1,25(OH)(_2)D(_3)</td>
</tr>
<tr>
<td><strong>BONE RESORPTION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A_r) (no/mm(^2))</td>
<td>0.74</td>
<td>+1.62***</td>
<td>0.88</td>
<td>+1.04</td>
</tr>
<tr>
<td></td>
<td>(0.20)</td>
<td>(0.28)</td>
<td>(0.14)</td>
<td>(0.40)</td>
</tr>
<tr>
<td>(A_r/A_f)</td>
<td>0.20</td>
<td>0.40***</td>
<td>0.19</td>
<td>0.45*</td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(0.05)</td>
<td>(0.02)</td>
<td>(0.12)</td>
</tr>
<tr>
<td><strong>BONE CELL ACTIVITY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR (μm)</td>
<td>9.12</td>
<td>9.53</td>
<td>9.18</td>
<td>8.68</td>
</tr>
<tr>
<td></td>
<td>(0.53)</td>
<td>(0.46)</td>
<td>(0.25)</td>
<td>(0.02)</td>
</tr>
<tr>
<td>M (μ/day)</td>
<td>0.77</td>
<td>0.78</td>
<td>0.77</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.09)</td>
<td>(0.01)</td>
<td>(0.12)</td>
</tr>
<tr>
<td>(M_f) (mm/yr)</td>
<td>0.19</td>
<td>0.27***</td>
<td>0.22</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>(0.02)</td>
<td>(0.05)</td>
<td>(0.02)</td>
<td>(0.05)</td>
</tr>
<tr>
<td>(\Theta_f) (time/yr)</td>
<td>0.56</td>
<td>0.33**</td>
<td>0.45</td>
<td>0.52</td>
</tr>
<tr>
<td></td>
<td>(0.25)</td>
<td>(0.05)</td>
<td>(0.13)</td>
<td>(0.11)</td>
</tr>
<tr>
<td><strong>BONE TURNOVER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\mu_r) (activation)</td>
<td>9.22</td>
<td>13.74*</td>
<td>13.11</td>
<td>7.89*</td>
</tr>
<tr>
<td>(\mu_f) (freq. foci/mm(^2)/yr)</td>
<td>2.68</td>
<td>(2.42)</td>
<td>(1.92)</td>
<td>(1.73)</td>
</tr>
<tr>
<td>(V_f) (mm(^2)/mm(^2)/yr)</td>
<td>0.08</td>
<td>0.14**</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(0.03)</td>
<td>(0.02)</td>
<td>(0.02)</td>
<td>(0.02)</td>
</tr>
</tbody>
</table>

* p<0.05,  ** p<0.025,  *** p<0.01 for individual paired (pre versus post) rib observations using Wilcoxon Signed Rank Test.
+ p<0.05,  ++ p<0.025 for treatment effects comparing ribs using Nemenyi One-way Classification Rank Sum Test.
Table 1.2: Serum calcium and phosphorus, and urinary hydroxyproline (HOP) in adult dogs following intermittent 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) (Mean ± S.E., N = 6 dogs).

<table>
<thead>
<tr>
<th>Interval (Day)</th>
<th>Serum Calcium (mg/dl)</th>
<th>Serum Phosphorus (mg/dl)</th>
<th>Urinary HOP:Creatinine (µg:mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>8.60 ± 0.16</td>
<td>4.19 ± 0.19</td>
<td>7.13 ± 0.64</td>
</tr>
<tr>
<td>1 1,25-(OH)$_2$D$_3$</td>
<td>9.93 ± 0.17**</td>
<td>5.53 ± 0.40*</td>
<td>6.79 ± 0.59</td>
</tr>
<tr>
<td>3 1,25-(OH)$_2$D$_3$</td>
<td>13.25 ± 0.90**</td>
<td>6.62 ± 0.81*</td>
<td>4.71 ± 0.44*</td>
</tr>
<tr>
<td>5 1,25-(OH)$_2$D$_3$</td>
<td>17.49 ± 0.57***</td>
<td>5.88 ± 0.49*</td>
<td>13.53 ± 0.82**</td>
</tr>
<tr>
<td>7 Withdrawal</td>
<td>13.23 ± 0.39***</td>
<td>4.75 ± 0.27</td>
<td>-</td>
</tr>
<tr>
<td>13 Withdrawal</td>
<td>8.40 ± 0.48</td>
<td>4.37 ± 0.28</td>
<td>8.13 ± 0.38</td>
</tr>
<tr>
<td>15 Withdrawal</td>
<td>-</td>
<td>4.65 ± 0.31</td>
<td>8.45 ± 0.32</td>
</tr>
<tr>
<td>22 1,25-(OH)$_2$D$_3$</td>
<td>11.69 ± 0.84*</td>
<td>5.25 ± 1.18</td>
<td>5.66 ± 1.14</td>
</tr>
<tr>
<td>25 1,25-(OH)$_2$D$_3$</td>
<td>13.40 ± 0.61***</td>
<td>5.65 ± 1.38</td>
<td>-</td>
</tr>
<tr>
<td>27 Withdrawal</td>
<td>11.95 ± 0.84**</td>
<td>4.10 ± 0.26</td>
<td>-</td>
</tr>
<tr>
<td>30 Withdrawal</td>
<td>10.57 ± 0.67*</td>
<td>3.75 ± 0.51</td>
<td>8.52 ± 1.36</td>
</tr>
<tr>
<td>40 1,25-(OH)$_2$D$_3$</td>
<td>11.20 ± 0.62**</td>
<td>5.05 ± 0.30</td>
<td>10.22 ± 3.07</td>
</tr>
<tr>
<td>45 1,25-(OH)$_2$D$_3$</td>
<td>10.56 ± 0.68*</td>
<td>6.02 ± 0.39**</td>
<td>6.18 ± 4.12</td>
</tr>
<tr>
<td>50 Withdrawal</td>
<td>10.62 ± 0.18***</td>
<td>4.48 ± 0.30</td>
<td>9.15 ± 0.63</td>
</tr>
<tr>
<td>55 Withdrawal</td>
<td>-</td>
<td>6.18 ± 1.31</td>
<td>-</td>
</tr>
<tr>
<td>60 Withdrawal</td>
<td>9.30 ± 0.42</td>
<td>7.19 ± 0.35***</td>
<td>4.14 ± 0.90</td>
</tr>
</tbody>
</table>

*p<0.05  **p<0.01  ***p<0.001
CHAPTER II

HISTOMORPHOMETRIC EVALUATION OF THE EFFECTS OF LOW DOSE PARATHYROID HORMONE ADMINISTRATION ON CORTICAL BONE REMODELING IN ADULT DOG

INTRODUCTION

Metabolic bone disease results when alterations occur in the recruitment or function of bone cells (4,7). The role of parathyroid hormone (PTH) on the skeleton has been studied experimentally and in naturally occurring diseases. Early studies in growing rats demonstrated an osteosclerotic effect of PTH on trabecular bone (9, 36). Studies by Parsons et al. (19) and Reeve et al. (27) demonstrated that chronic low doses of both bovine and human PTH to dogs and to osteoporotic women, respectively, produced increased calcium retention, bone mass and calcium accretion. In primary hyperparathyroidism of human beings studies of the effects of PTH upon the endosteal and Haversian envelopes have demonstrated a large variation in the frequency of activation and bone formation rate (4). However, both osteon formation and resorption time have been shown to be lengthened (4). Studies by Wilde et al. (4) revealed a trend towards depressed bone formation at the cellular level and acceleration of bone formation and resorption at the tissue level. The cellular mechanisms in the skeleton which respond to PTH and produce these changes have not been elucidated.
By studying the effects of PTH in a system of accelerated bone remodeling it may be possible to magnify the effects of the hormone and shorten the interval required to demonstrate its effects. Local tissue injury increases bone remodeling by a factor of 2 to 20 fold by increasing the activation frequency (4). The anatomic distribution of the accelerated remodeling that follows tissue injury is regional (4). In the present study surgical elevation of the periosteum of the 9th rib of the dog was used to induce a "regional acceleratory phenomenon" (4). The specific objectives of this investigation were: (1) to determine the morphometric and dynamic effects of low dose PTH administration on cortical bone remodeling in adult dogs; (2) to compare the effects of PTH on cortical bone in combination with periosteal elevated (accelerated remodeling) and in unaltered (steady-state) ribs; and (3) to correlate the alterations in bone remodeling with changes in serum electrolytes and urinary hydroxyproline concentrations.
MATERIALS AND METHODS

Experimental Animals:

Six female beagles (Marshall Research Animals, North Rose, NY 14516), weighing approximately 10 kg. and 12 to 20 months of age were acclimated to laboratory conditions for 2 weeks. The dogs were fed commercial Purina dog chow (Ralston Purina Co., Checkerboard Square, St. Louis, MO 63188) with free access to water. Blood and urine samples were collected during the acclimation period and weekly thereafter. The dogs were maintained in individual stainless steel metabolism cages for urine collection. Blood collection and intravenous administration of bovine parathyroid hormone (PTH, Parathyroid Injection, USP, Eli Lilly & Co., Indianapolis, IN 46206) were performed by indwelling catheters in the jugular vein (epidural catheter, No. 8222, Becton-Dickinson & Co., Rutherford, NJ 07070).

Experimental Design:

The study lasted for 60 days which is one-half the lifespan of bone forming sites in the dog (4). A PTH dose of 2.5 U per kg. body weight per day (1.25 μg per kg. per day) was administered intravenously in 4 equally divided doses every 6 hours for 60 days. This dose was selected from a previous pilot dose-response study and an earlier study that determined the lowest dose of PTH that altered bone dynamics in the dog without resulting in persistent hypercalcemia (Nagode, L.A., personal communication, 1976).
Each dog served as its own control by collecting a pre-treatment rib biopsy (left 9th, 10th and 11th) when the periosteal elevation of the right 9th rib was performed on day 0. The dogs were sacrificed after 60 days of PTH by an intravenous overdose of sodium pentabarbital and the contralateral ribs (right 9th, 10th, and 11th) removed and fixed in 70% ethanol.

Serum was collected weekly. Serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Model 303) (37). Serum phosphorus was determined by the method of Baginski and Zak (1). Urine was collected under toluene initially and thereafter weekly using metabolism cages. Urine was assayed for hydroxyproline by the method of Kivirikko et al (10) and creatinine by the method of Clark (2). Urinary hydroxyproline was expressed as a ratio to creatinine.

**Surgical Procedures**

**Anesthesia:** The dogs were administered 0.25 mg. per kg. acepromazine maleate (Ayerst Laboratories, Inc., New York, NY 10017) and 0.20 mg. atropine sulfate (Eli Lilly & Co., Indianapolis, IN 46232) intramuscularly. Anesthesia was induced with Surital (Thiamylal Sodium for injection, Parke-Davis & Co., Detroit, MI 48232) intravenously and maintained with methoxyfluorane (Metafane, Pitman-Moore, Inc., Washington Crossing, NJ 08560).

**Periosteal Elevation:** The incision site for periosteal elevation of the right 9th rib was determined by measuring 5.0 cm. vertically and proximally from the costochondral junction and 5.0 cm. vertically and distally from the transverse processes of the vertebrae (21). A skin incision approximately 5.0 cm. in length was made directly over the 9th rib and continued through the
subcutaneous fat, fascia and muscle. The periosteum was incised vertically for a length of 3.5 cm., separated, and reflected laterally from the rib using periosteal elevators (Keys elevator-chisel, Nos. 5W500 and 6W501, Max Wocher & Sons, 315 Plum St., Cincinnati, OH 45202). A small curved periosteal elevator (Mackintz elevator No. 5W275, Max Wocher & Sons, 315 Plum Street, Cincinnati, OH 45202) was used to free the rib laterally and medially of its periosteum.

**Rib Biopsy:** The biopsy incision site was identified as described above for periosteal elevation (21). The incision was made over the left 10th rib for a length of 6 to 7 cm. Blunt dissection of fat, fascia and muscle was accomplished with scissors to expose the left 11th, 10th and 9th ribs. The periosteum was reflected and a 3.0 cm section of each rib was removed with a double-action bone cutter (Stille-Liston double action bone cutting forceps, No. 69-410, Misdon-Frank Corp., 860 Broadway, New York, NY 10003).

**Bone Histomorphometry**

**Double Fluorochrome Bone Labelling:** The dynamics of appositional bone growth for each dog was evaluated by the intravenous administration of tetracycline (Oxytetracycline hydrochloride, "Liquamycin", Pfizer, Inc., New York, NY 10017) (4,30,33) and a second fluorochrome label, DCAF (2,4,BIS) N,N'-di-(carboxymethyl) amino methyl fluorescein, (I.C.N. Pharmaceuticals, Inc., Life Science Group, Cleveland, OH 44128) (33). The fluorochromes were administered initially 18 days before the start and then near the conclusion of the study (day 42) according to the schedule of 3-days-on, 7-days-off, and 3-days-on. The dosage was 15 mg/kg with tetracycline given the first 3 days and DCAF given the second 3 days.
Osteochrome-stained ground bone sections (80 µm) were viewed under an ultraviolet microscope using a Wratten 18A filter at the source, a 2B filter at the eyepiece, and a wavelength of 36 µm at both 10X and 25X magnification. Tetracycline produced yellow and DCAF green fluorescence with ultraviolet light in ground bone sections.

**Ground Bone Sections:** An average of 5 cross sections per rib were cut at approximately 200 µm thickness using a jeweler's saw (Apparatus for Micro-structural Analysis, Buehler Limited, 2120 Greenwood St., Evanston, IL 60204). The sections were ground to approximately 100 µm thickness according to the method of Frost (4). After staining for 48 hours with osteochrome (35) the sections were reground to 75-90 µm thickness. Section thickness was measured by a mikrokator (Mikrokator, No. 7VZ509-4, C.E.J. Gage Co., Subsidiary of C.E. Johnson, Sweden 10641 Haggerty, Dearborn, MI 48126). The sections were washed in 0.1% benzalkonium chloride to remove debris, rinsed in distilled water, and differentiated in 0.01% acetic acid in 95% methanol. When differentiation was completed they were dehydrated in ascending concentrations of alcohol, cleared in xylol, and mounted in Eukitt's mounting media (Calibrated Instruments Inc., 731 Saw Mill River Road, Ardsley, NY 10502).

**Morphometric Evaluation of Bone**

**Measurements:** Morphometric measurements were performed with a calibrated micrometer and Zeiss integrator plate II, 100/25 ocular grid with a 0.6 aperture (5). A minimum of 3 cross sections of each bone were measured in order to analyze a minimum of 50 mm² of bone.
The following nine measurements and counts for cortical bone were made from cross sections of ribs from each dog:

1. Cross sectional Cortical ($A_C$) and (2) Total bone areas ($A_T$) were measured separately by the grid method of Sedlin (31) at a magnification of 1 to 2X. In this point-count technique a square-ruled (10 X 10) grid of known area ("g") is superimposed optically upon a section measured under bright field light microscopy. Intersections covered by the surface were counted as "hits" and those tangent were counted as "1/2 hits" counting 3 field observations ("throws") per bone section. The mean cortical and total sectional areas were calculated by averaging the results of separate determinations.

3. The total number of osteoid seams ($A_P$) were measured by systematic scanning of the entire section using a 10X objective. An osteoid seam was identified as a bone formation site by a green or red intimal band on sections stained with osteochrome. The mean number of osteoid seams per mm$^2$ of bone was calculated from the total number of osteoid seams and the total cortical area ($A_C$).

4. The total number of resorption cavities ($A_P$) were measured similarly as the osteoid seams. Resorption cavities were identified by the presence of scalloped edges. The mean number was expressed per mm$^2$ of cortical area ($A_C$).

5. The total number of labelled osteoid seams (%L) were determined by counting active centers of bone formation under fluorescent microscopy. The number of active seams were expressed as a percentage of total seams. Active centers of bone formation were identified by the presence of a yellow (tetracycline) and green (DCAF) labels at the periphery of an osteoid seam.
(6) The mean distance between fluorochrome dyes within a single Haversian system (MAR) was measured at the site of localization of the fluorochrome labels. The distance between 2 labels was measured in millimeters by a calibrated eyepiece micrometer at 4 equidistant points around its circumference and mean values were calculated.

(7) The mean circumference of osteoid seams (S₁) was determined by measuring the perimeter intersects of surfaces using the Zeiss integrator ocular grid with a 40X objective. The eyepiece grid contained 10 equally spaced parallel and perpendicular lines which were superimposed on the surface to be analyzed. The number of parallel lines intersecting the intima of the seam were counted as "hits". On each "throw" the number of hits were counted, totaled, and the mean number calculated per section. The distance between 2 parallel lines ("a") was measured and calibrated as a reference.

(8) The mean wall thickness of finished osteons (MWT) and (9) the osteoid seam thickness (OST) were measured in microns and millimeters, respectively, with a calibrated micrometer by measuring at 4 equispaced radii the walls and seams, respectively. Approximately 50 measurements each per rib were done in this investigation.

Calculations: From the 9 basic measurements the quantitative morphometric analysis of bone consisted of the following stereological calculations (4):

1. Total Cortical Cross Sectional Area (A_C) in mm²
2. Total Bone Cross Sectional Area (A_T) in mm²

\[ A_C \text{ or } A_T = \frac{\text{No. of Hits}}{(\text{Grid Factor}) (\text{No. of Throws}) (\text{No. of Intersections})} \]

3. Mean Number of Osteoid Seams per mm² (A₁)
(4) Mean Number of Resorption Cavities per mm\(^2\) (\(A_r\))

\[
A_f \text{ or } A_r = \frac{\text{Total No. of Osteoid Seams or Resorption Cavities}}{\text{Total Cortical Area (}A_C\text{) mm}}
\]

(5) Percent Labelled Osteoid Seams (% \(L\))

\[
\%L = \frac{\text{Total No. Labelled Osteoid Seams}}{\text{Total No. of Osteoid Seams}}
\]

(6) Mean Appositional Rate (\(M\)) in \(\mu\)/day

\[
M = \frac{\text{Mean Distance Between Two Labels (MAR) in Microns}}{\text{No. of Days Administered Label}}
\]

(7) Radial Closure Rate (\(M_f\)) in mm/yr.

\[
M_f = (M) \times (%L) \times (365/1000^*)
\]

* reduces microns/day to mm/year.

(8) Osteoid Seam Circumference (\(S_f\))

\[
S_f = \frac{(\text{No. of Hits}) (\text{Distance Between Parallel Grid}) (\pi)}{(2)(\text{No. of Throws})}
\]

(9) Ratio of Cortical (\(C\)) Area in mm\(^2\) (\(A_C\)) to Total (\(T\)) Area in mm\(^2\) (\(A_T\))

\[
\frac{C}{T} = \frac{A_C}{A_T}
\]

(10) Ratio of the Numbers of Resorption Cavities (\(A_r\)) to Osteoid Seams (\(A_f\))

\[
\frac{A_r}{A_f}
\]

(11) Osteon Formation Time in Years (\(\Theta_f\))

The time required to complete lamellar bone formation in a cross-section of an average osteon.

\[
\Theta_f = \frac{MWT}{M_f}
\]

(12) Number of Activation Frequency Foci in mm\(^2\)/year (\(\mu_f\))

\[
\mu_f = \frac{A_f}{\Theta_f}
\]
\( \mu_f \) represents the number of new osteons introduced per year in an average mm\(^2\) of cortical area.

(13) **Bone Formation Rate** in mm\(^2\)/mm\(^2\)/year \( (V_f) \)

\[ V_f = (A_f) \times (S_f) \times M_f \]

**Statistical Analysis:** Statistical significance of differences in the morphometric data comparing treatment effects (11th rib - PTH and steady-state remodeling, 9th rib - PTH and periosteal elevation, 10th rib - PTH and regional influence of periosteal elevation) between ribs were determined by the Nemenyi Rank Sum One-Way Classification Test (3, 14). The Wilcoxon Signed Rank Test was used to determine statistical significance of differences in paired data for each rib (3). The biochemical data were evaluated by Student t test.
RESULTS

The bone measurements and calculated parameters are given (Table 2.1) as means ± S.E. with their associated levels of significance.

Effects of parathyroid hormone (11th rib)

The administration of parathyroid hormone to dogs in this study increased the radial closure rate \( (M_r) \) 13% \((p<0.05)\) from \(0.32 \pm 0.10\) to \(0.50 \pm 0.09\) mm per year in the 11th rib. The increase in the radial closure rate was accompanied by a 47% \((p<0.05)\) decrease in the osteon formation time \((\Theta_r)\) from \(0.27 \pm 0.19\) to \(0.10 \pm 0.12\) years. The ratio of cortical to total bone area \((C/T)\) increased 3% \((p<0.01)\).

Effects of parathyroid hormone and periosteal elevation (9th rib)

The combined treatment of parathyroid hormone and periosteal elevation in the 9th rib had no significant effect upon either radial closure rate or osteon formation time.

The cortical cross sectional area \( (A_{CO})\) was increased 22% \((p<0.01)\) from \(6.66 \pm 0.59\) to \(8.15 \pm 0.98\) mm\(^2\) of bone. The circumference of osteoid seams was decreased 20% \((p<0.025)\) from \(0.10 \pm 0.01\) to \(0.08 \pm 0.01\) mm.

Effects of parathyroid hormone and regional effects (10th rib)

The circumference of osteoid seams in the 10th rib increased 11% \((p<0.05)\) from \(0.09 \pm 0.01\) to \(0.10 \pm 0.01\) mm.
Comparison of parathyroid hormone on the 9th, 10th, 11th Ribs

In comparison, PTH alone (11th rib) resulted in a 2.6% increase in total bone area from $10.35 \pm 1.41$ to $10.62 \pm 1.34 \text{ mm}^2$ while PTH plus periosteal elevation (9th rib) caused a 3.7% decrease from $13.46 \pm 2.77$ to $12.95 \pm 0.98 \text{ mm}^2$ ($p<0.01$).

Biochemical Evaluation

Serum calcium, phosphorus and magnesium, and urinary hydroxyproline concentrations are given (Table 2.2) as means $\pm$ S.E. of the group. Serum calcium values were significantly elevated above baseline only on experimental day 5 to $9.97 \pm 0.17 \text{ mg/dl}$. Serum phosphorus and magnesium values were not changed significantly during the 60 days of PTH administration (Table 2.2). Urinary hydroxyproline was increased significantly above baseline only on experimental day 10 and was decreased on days 20 and 30.
DISCUSSION

The results of this study demonstrated that the effects of PTH on bone remodeling in the dog evaluated over the period of approximately one-half a sigma unit were different in the unaltered rib versus a rib in which a regionally accelerated remodeling state had been induced by tissue injury. There was an increased rate of measured bone accretion in cortical bone in the unaltered 11th rib when dogs were administered low daily doses of PTH for 60 days. This effect of PTH was demonstrated by the increase in the radial closure rate, which is an index of concentric Haversian bone formation. Parathyroid hormone appeared also to accelerate osteoblastic activity as reflected in the shortened osteon formation time in the 11th rib. This was associated with a downward trend in both the numbers of bone resorption and formation sites. The ratio of resorption to formation remained similar over the course of the experiment, indicating that the effect of PTH was similar upon both bone forming and bone resorbing sites. Over the course of this experiment, PTH appeared to increase bone formation at the osteoblastic level in previously existing Haversian units without altering the normal coupling of the resorptive and formative phases of bone remodeling. Parathyroid hormone had no significant effect upon bone turnover at the tissue level over the course of this experiment when given in a low daily dose.

The fractional cortical bone area was increased at the organ level. This was associated with a trend toward an increase in both the cortical and total
bone areas, and a decrease in marrow area. These findings suggested that net bone formation had occurred on both the periosteal and endosteal surfaces.

The results of increased bone accretion in cortical bone following PTH administration are directionally similar to the results of Kalu et al. (9) and Walker (36) who observed that PTH increased trabecular bone in the rat. The results of these studies suggested that PTH may have an anabolic effect on bone. Similarly, Parsons and Reit (19) demonstrated that chronic low dose infusions of bovine PTH to dogs resulted in calcium retention. Reeve et al. (27) also observed improved calcium balance and increased accretion of calcium in the skeleton when low doses of human PTH (1-34 peptide fragment) was administered to post-menopausal osteoporotic women. The results of the present study in adult dogs were interpreted to suggest that the immediate anabolic effect of PTH on the skeleton may be due to an accelerated accretion rate reflecting increased osteoblastic activity in established Haversian systems.

An effect of PTH on accretion rate in Haversian systems was not evident in the 9th rib in which the periosteum was elevated comparing pre- and post-treatment values. However, a trend towards a prolonged osteon formation time was evident, as was a decrease in osteoid seam circumference and thickness. Thus, the osteons appeared to have become more mature. In the periosteal elevated rib there was a suggestion of dissociation between the initiation of new bone resorbing and forming sites as the number of resorbing sites increased and the number of forming sites decreased after PTH administration. This observation may be an effect of PTH on the switch over from bone resorption to formation since the regional acceleratory phenomenon alone on the rib of the dog produces an equal increase in bone formation and resorption when studied
for a similar length of time (Black et al., personal communication, 1976). In the adjacent 10th rib subjected to the regional effects of periosteal elevation plus the effects of PTH, bone formation tended to be unaltered at the cellular, tissue and organ levels.

The administration of low continuous doses of PTH to dogs did not result in the development of hypercalcemia or hypophosphatemia compared to baseline values and produced only a transient rise in urinary hydroxyproline levels on day 10. These results were similar to those reported by Parsons et al. (18). Because urinary hydroxyproline is derived almost exclusively from collagen, the increased excretion on day 10 in conjunction with the elevated serum calcium levels on days 12 and 15 suggests increased bone mineral mobilization.

The findings observed in this study indicated that the effect of parathyroid hormone on bone remodeling is dependent upon the existing turnover rate in bone and is influenced by the microenvironment of bone cells. Previous studies have reported that the action of PTH on bone remodeling is associated with a decrease apposition rate and prolongation of osteon formation time (4). However, in the present study low doses of PTH in unaltered bone shortened the osteon formation time whereas in combination with periosteal elevation (accelerated remodeling) PTH tended to prolong the formation time of osteons.
SUMMARY

The effects of low dose levels of parathyroid hormone (PTH) on canine bone remodeling in ribs of adult dogs were evaluated under conditions of steady-state turnover and with accelerated remodeling. Six female beagles were administered PTH (2.5 U/kg./day) intravenously in equally divided doses 6 hrs. apart for 60 days. The level and dose schedule of PTH used in this study resulted in a significant elevation in blood calcium above baseline values only at experimental day 5, no change in serum phosphorus and magnesium, and a transient increase in urinary hydroxyproline excretion on day 10. Accelerated cortical bone remodeling was induced by surgical elevation of the periosteum of the 9th rib prior to PTH administration. Static and dynamic bone changes were analyzed using histomorphometric methods, including tetracycline and DCAF (2,4 BIS) N,N'di (carboxymethyl) (amino methyl fluorescein) in vivo double labeling.

The results of this study suggested that the response of cortical bone to PTH was dependent upon the existing remodeling rate in the cortical bone. Parathyroid hormone (PTH) increased the radial closure rate and decreased the osteon formation time in unaltered cortical bone. The ratio of cortical to total bone area was increased. These findings suggested that the initial anabolic effect of PTH may be due to increased bone formation at the osteoblastic level in existing Haversian units without altering the normal coupling of the resorptive and formative phases of bone remodeling. In periosteal-elevated
cortical bone the effect of an accelerated remodeling rate on PTH was a decrease in the circumference and thickness of osteoid seams. A trend was evident towards prolonged osteon formation time. The number of bone resorption sites was greater than the number of bone formation sites.

PTH administration did not increase activation frequency or remodeling in cortical bone of adult dogs.
Table 2.1: Effects of low dose parathyroid hormone (PTH) administration on histomorphometric parameters in the 9th, 10th, and 11th ribs of adult dogs. (Mean ± S.E., N = 6).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>9th Rib Periosteal Elevated</th>
<th>10th Rib Regional Effects</th>
<th>11th Rib PTH Effects</th>
<th>p-values Comparing Treatment Effects Between 9th, 10th, and 11th Ribs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Post-PTH</td>
<td>Baseline Post-PTH</td>
<td>Baseline Post-PTH</td>
<td></td>
</tr>
<tr>
<td><strong>BONE MASS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_C$ (mm$^2$)</td>
<td>6.66</td>
<td>6.62</td>
<td>6.16</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(0.59)</td>
<td>(0.50)</td>
<td>(0.44)</td>
<td></td>
</tr>
<tr>
<td>$A_T$ (mm$^2$)</td>
<td>13.46</td>
<td>11.82</td>
<td>10.35</td>
<td>p&lt;0.01, p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>(+) 12.95</td>
<td>(+) 11.44</td>
<td>(+) 10.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.77)</td>
<td>(1.32)</td>
<td>(1.41)</td>
<td></td>
</tr>
<tr>
<td>C/T</td>
<td>0.54</td>
<td>0.58</td>
<td>0.60</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.04)</td>
<td>(0.00)</td>
<td></td>
</tr>
<tr>
<td>MWT (mm)</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td></td>
</tr>
<tr>
<td><strong>BONE FORMATION and OSTEOID</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$A_f$ (No/mm$^2$)</td>
<td>7.77</td>
<td>8.04</td>
<td>7.31</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>(1.30)</td>
<td>(1.70)</td>
<td>(1.89)</td>
<td></td>
</tr>
<tr>
<td>OST (μm)</td>
<td>8.25</td>
<td>8.15</td>
<td>7.87</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>(0.39)</td>
<td>(0.37)</td>
<td>(0.58)</td>
<td></td>
</tr>
<tr>
<td>$S_f$ (mm)</td>
<td>0.10</td>
<td>0.09</td>
<td>0.09</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>(0.01)</td>
<td>(0.01)</td>
<td>(0.01)</td>
<td></td>
</tr>
<tr>
<td>L (%)</td>
<td>71.75</td>
<td>80.76</td>
<td>62.92</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>(0.14)</td>
<td>(0.15)</td>
<td>(0.18)</td>
<td></td>
</tr>
</tbody>
</table>

$p<0.05$, **p<0.025$, ***p<0.01 for individual paired (pre versus post) rib observations using Wilcoxon Signed-Rank Test

$+p<0.05$, ++p<0.025, +++p<0.01 for treatment effects comparing ribs using Nemenyi One-Way classification Rank Sum Test
Table 2.1: Continued

<table>
<thead>
<tr>
<th>Parameters</th>
<th>9th Rib Periosteal Elevated</th>
<th>10th Rib Regional Effects</th>
<th>11th Rib PTH Effects</th>
<th>p-values Comparing Treatment Effects Between 9th, 10th, and 11th Ribs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
<td>Baseline</td>
<td>Post-PTH</td>
<td>Baseline</td>
<td>Post-PTH</td>
</tr>
<tr>
<td><strong>BONE RESORPTION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( A_r ) (No/mm(^2))</td>
<td>1.58 (0.23)</td>
<td>2.01 (0.34)</td>
<td>1.09 (0.25)</td>
<td>1.16 (0.25)</td>
</tr>
<tr>
<td>( A_r/A_f )</td>
<td>0.22 (0.03)</td>
<td>0.56 (0.26)</td>
<td>0.18 (0.06)</td>
<td>0.17 (0.04)</td>
</tr>
<tr>
<td><strong>BONE CELL ACTIVITY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR (( \mu )m)</td>
<td>12.59 (1.34)</td>
<td>13.48 (1.07)</td>
<td>10.92 (1.48)</td>
<td>11.88 (1.48)</td>
</tr>
<tr>
<td>( M ) (( \mu )/day)</td>
<td>1.53 (0.23)</td>
<td>1.36 (0.12)</td>
<td>1.34 (0.24)</td>
<td>1.20 (0.16)</td>
</tr>
<tr>
<td>( M_f ) (mm/yr)</td>
<td>0.43 (0.12)</td>
<td>0.39 (0.08)</td>
<td>0.35 (0.08)</td>
<td>0.35 (0.10)</td>
</tr>
<tr>
<td>( \Theta_f ) (time/yr)</td>
<td>0.18 (0.16)</td>
<td>0.24 (0.07)</td>
<td>0.20 (0.03)</td>
<td>0.18 (0.13)</td>
</tr>
<tr>
<td><strong>BONE TURNOVER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \mu_f ) (Activation Freq Foci/mm(^2)/yr)</td>
<td>40.90 (10.87)</td>
<td>27.13 (8.73)</td>
<td>35.33 (10.56)</td>
<td>27.63 (6.96)</td>
</tr>
<tr>
<td>( V_f ) (mm(^2)/mm(^2)/year)</td>
<td>0.33 (0.09)</td>
<td>0.22 (0.09)</td>
<td>0.26 (0.10)</td>
<td>0.25 (0.08)</td>
</tr>
</tbody>
</table>

\*\ p<0.05, \**\ p<0.025, \***\ p<0.01 for individual paired (pre versus post) rib observations using Wilcoxon Signed-Rank Test

\+\ p<0.05, \;++\ p<0.025 \;+++\ p<0.01 for treatment effects comparing ribs using Nemenyi One-Way classification Rank Sum Test
Table 2.2: Serum calcium, phosphorus, magnesium, and urinary hydroxyproline (HOP) in adult dogs following low dose parathyroid hormone (PTH) administration for 60 days (Mean ± S.E., N = 6).

<table>
<thead>
<tr>
<th>Interval (Day)</th>
<th>Serum Calcium (mg/dl)</th>
<th>Serum Phosphorus (mg/dl)</th>
<th>Serum Magnesium (mg/dl)</th>
<th>Urinary HOP:Creatinine (µg:mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>9.17 ± 0.17</td>
<td>4.32 ± 0.28</td>
<td>1.35 ± 0.13</td>
<td>27.87 ± 1.04</td>
</tr>
<tr>
<td>1</td>
<td>8.67 ± 0.18</td>
<td>4.48 ± 0.60</td>
<td>1.21 ± 0.47</td>
<td>23.71 ± 1.44</td>
</tr>
<tr>
<td>5</td>
<td>9.97 ± 0.17*</td>
<td>4.28 ± 0.93</td>
<td>1.14 ± 0.39</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>8.53 ± 0.50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>9.04 ± 0.25</td>
<td>4.28 ± 0.29</td>
<td>1.09 ± 0.09</td>
<td>63.98 ± 3.90**</td>
</tr>
<tr>
<td>12</td>
<td>9.58 ± 0.16</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>9.67 ± 0.15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>8.56 ± 0.33</td>
<td>3.92 ± 0.32</td>
<td>1.05 ± 0.38</td>
<td>5.20 ± 0.27***</td>
</tr>
<tr>
<td>25</td>
<td>8.46 ± 0.35</td>
<td>-</td>
<td>-</td>
<td>26.40 ± 1.63</td>
</tr>
<tr>
<td>30</td>
<td>8.46 ± 0.35</td>
<td>-</td>
<td>-</td>
<td>18.77 ± 3.11*</td>
</tr>
<tr>
<td>40</td>
<td>9.38 ± 0.28</td>
<td>5.03 ± 0.37</td>
<td>1.21 ± 0.16</td>
<td>32.63 ± 2.53</td>
</tr>
<tr>
<td>50</td>
<td>9.77 ± 0.32</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>8.92 ± 0.17</td>
<td>4.40 ± 0.32</td>
<td>1.32 ± 0.17</td>
<td>28.57 ± 1.98</td>
</tr>
<tr>
<td>60</td>
<td>9.45 ± 0.12</td>
<td>5.09 ± 0.96</td>
<td>1.70 ± 0.44</td>
<td>28.20 ± 2.00</td>
</tr>
</tbody>
</table>

* p<0.1  ** p<0.01  *** p<0.001
CHAPTER III

HISTOMORPHOMETRIC EVALUATION OF THE EFFECTS OF
THYROXINE ON CORTICAL BONE REMODELING
IN ADULT DOGS

INTRODUCTION

Osteoporosis and hypercalcemia frequently are reported in association with thyrotoxicosis in human beings.\textsuperscript{1-5} Increased bone turnover has been observed both in humans\textsuperscript{1,2,4,5} with the naturally occurring disease and demonstrated in animals with experimentally induced hyperthyroidism by microradiography.\textsuperscript{3} Frost\textsuperscript{1} has reported that thyroxine elevates the frequency of activation of mesenchymal cells in bone four-fold, without changing other parameters of the bone metabolic remodeling unit. This increase in activation frequency is reflected in an increased differentiation of mesenchymal cells into osteoclasts and osteoblasts with a corresponding increase in the number of remodeling centers, resorption and formation surfaces, and bone turnover. In the hyperthyroid state there is an increased porosity of cortical bone due to longitudinal tunnelling by increased numbers of secondary Haversian remodeling centers. The increased bone turnover in thyrotoxicosis has an envelope expanding effect and produces increased porosity of cortical bone because of the lag phase between bone resorption and formation.\textsuperscript{1}
Thyroxine directly stimulates the metabolic activity of bone cells and profoundly affects bone turnover.\textsuperscript{9-11} Several investigators have demonstrated that thyroxine influences the responsiveness of bone cells to 1,25-(OH)\textsubscript{2}D\textsubscript{3},\textsuperscript{6} parathyroid hormone\textsuperscript{7-9} and calcitonin.\textsuperscript{10} The cellular mechanisms have not been elucidated whereby thyroxine alters the skeleton and produces the changes in bone cell metabolism and skeletal turnover. In this experiment the effects of thyroxine were evaluated under existing (steady-state) conditions and in combination with accelerated remodeling to investigate its mode and site of action on the remodeling process. Because increased skeletal turnover enhances the capacity of bone to respond to external influences,\textsuperscript{1} it may be possible to magnify the effects of the hormone and shorten the interval required to demonstrate its effects.

Surgical elevation of periosteum of the rib was used to induce a "regional acceleratory phenomenon" and thus locally increase bone turnover. The specific objectives of this investigation were: 1) to determine the morphometric and dynamic effects of daily moderate pharmacologic doses of thyroxine on cortical bone remodeling in unaltered ribs and in combination with periosteal elevation in adult dogs; and 2) to correlate the alterations in bone remodeling with changes in serum electrolytes and urinary hydroxyproline excretion.
MATERIALS AND METHODS

Experimental Animals: Six female beagle dogs, (Marshall Research Animals, North Rose, NY 14516 and locally from one closed colony) each weighing approximately 7 kg and between 12 and 18 months of age were acclimated to laboratory conditions for two weeks. The dogs were fed commercial Purina dog chow (Ralston Purina Co., Checkerboard Square, St. Louis, MO 63188) with free access to water. Blood and urine samples were collected during the acclimation period and at weekly or shorter intervals thereafter. The dogs were maintained in individual stainless steel metabolism cages for urine collection. Blood collection was performed using indwelling catheters (Epidural Catheter, No. 8222, Becton-Dickinson & Co., Rutherford, NJ 07070) placed in the jugular vein. L-thyroxine tablets (Soloxine, Daniels Pharmaceuticals, Inc., St. Petersburg, FL 33713) were administered orally in divided doses.

Experimental Design: The study lasted for 60 days which is equivalent to one-half the lifespan of a bone forming site in the dog. One mg per kilogram of l-thyroxine was administered orally in equally divided doses twelve hours apart daily for 60 days. The dose was selected from the results of an earlier pilot study that demonstrated this level was required to result in detectable changes in body weight, temperature, heart rate, serum calcium, and plasma proteins. Each dog served as its own control by collecting a pre-treatment rib (left 9th, 10th and 11th) biopsy. Periosteal elevation of the right 9th rib was
performed on day 0. The dogs were euthanized after 60 days and the contralateral ribs (right 9th, 10th, and 11th) were removed and fixed in 70% ethanol.

Serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Model 303). Serum phosphorus was determined by the method of Baginski and Zak. Urine was collected weekly under toluene from dogs kept in metabolism cages. Urine was assayed for hydroxyproline by the method of Kivirikko et al. and creatinine by the method of Clark. Urinary hydroxyproline was expressed as a ratio to creatinine.

Body weight, temperature, heart and respiratory rates were recorded at weekly intervals.

**Surgical Procedures**

**Anesthesia:** The dogs were administered 0.25 mg/kg acepromazine maleate (Ayerst Laboratories, Inc., New York, NY 10017) and 0.20 mg atropine sulfoxide (Eli Lilly & Co., Indianapolis, IN 46232) intramuscularly. Anesthesia was induced with Surital (Thiamyl Sodium for injection, Parke-Davis & Co., Detroit, MI 48232) administered intravenously and was maintained using methoxyfluorane (Metafane, Pitman-Moore, Inc., Washington Crossing, NJ 08560).

**Periosteal Elevation:** The incision site for periosteal elevation of the 9th rib was determined by measuring 5.0 cm vertically and proximally from the costochondral junction and 5.0 cm vertically and distally from the transverse process of the vertebrae. A skin incision approximately 5.0 cm in length was made directly over the 9th rib and continued through the subcutaneous fat, fascia, and muscle. The periosteum was incised vertically for a length of 3.5.
cm, separated from the underlying bone, and reflected laterally from the rib using periosteal elevators (Keys elevator-chisels, no. 5W500 and 6W501, Max Wocher & Sons, 315 Plum St., Cincinnati, OH 45202). A small curved periosteal elevator (Mackintz elevator no. 5W275, Max Wocher & Sons, 315 Plum St., Cincinnati, OH 45202) was used to free the rib laterally and medially of its periosteum.

**Rib Biopsy:** The biopsy incision site was identified as described above. The incision was made over the 10th rib for a length of 6 to 7 cm. Blunt dissection of fat, fascia and muscle was accomplished with scissors to expose the 11th, 10th, and 9th ribs. The periosteum was reflected and a 3.0 cm section of each rib was removed with a double action bone cutter (Stille-Liston double action bone cutting forceps, No. 69-410, Misdom-Frank Corp., 860 Broadway, New York, NY 10003).

**Bone Histomorphometry**

Morphometric analysis was performed on ground bone sections stained with Osteochrome stain using both fluorescence and bright field light microscopy.

**Double Fluorochrome Bone Labeling**

Evaluation of the dynamics of appositional bone growth was facilitated by the intravenous administration of tetracycline (Oxytetracycline hydrochloride, "Liquamycin", Pfizer Inc., New York, NY 10017) and a second fluorochrome label DCAF (2,4 Bis) N,N'-Di (carboxymethyl) (amino methyl fluorescein), I.C.N. Pharmaceuticals, Inc., Life Science Group, Cleveland, OH 44128). Fluorochrome administration began 18 days before the start of the study and on day 42
of the experimental period using the schedule of 3 days on (tetracycline), 7 days off, and 3 days on (DCAF). The dosage was 15 mg/kg for both tetracycline and DCAF.

Osteochrome-stained ground bone sections (80 μ) were viewed under an ultra violet microscope using a Wratten 18A filter at the source, 2B filter at the eyepiece, and a wavelength of 36 μm at both 10X and 25X magnification. Tetracycline produced yellow and DCAF a green fluorescence with ultraviolet light.18,19

Ground Bone Sections

An average of 5 cross-sections per rib were cut at approximately 200 μ thickness using a jeweler's saw (Apparatus for Microstructural Analysis; Buehler, Ltd., 2120 Greenwood St., Evanston, IL 60204). The sections were ground to approximately 100 μ thickness.20 After staining for 48 hours with osteochrome21,22 the sections were reground to 75-90 μ. Section thickness was measured by a mikrokator (Mikrokator, No. 7V2509-4, C.E.J. Gage Co., Subsidiary of C.E. Johnson, Sweden, 10641 Haggerty, Dearborn, MI 48126). The sections were washed in 0.1% benzalkonium chloride to remove debris, rinsed in distilled water, and differentiated in 0.01% acetic acid in 95% methanol.20-22 When differentiation was completed they were dehydrated in ascending concentrations of alcohol, cleared in xylol, and mounted in Eukitt's mounting media (Calibrated Instruments, Inc., 731 Saw Mill River Rd., Ardsley, NY 10502).

Morphometric Evaluation of Bone

Measurements: Morphometric measurements were performed with a calibrated micrometer and Zeiss integrator plate II, 100/25 ocular grid with a
A minimum of 3 cross-sections of each bone were measured in order to analyze 50 mm$^2$ of bone.\textsuperscript{1,23}

Cross-sectional areas of cortical and total bone were measured separately by the grid method of Sedlin.\textsuperscript{24} A square-ruled (10 X 10) grid of known area was superimposed optically upon a section measured under a light microscope. Intersections covered by the surface were counted as "hits" and those tangent were counted as "1/2 hits". The mean cortical and total cross-sectional areas were calculated by averaging the results of separate determinations.

The circumference of bone surfaces was determined by measuring the perimeter intersects of the surface using the Zeiss integrator ocular grid in a 40X objective.\textsuperscript{21,22,24} The eyepiece grid contained 10 equally spaced parallel and perpendicular lines which were superimposed on the surface to be analyzed. The number of parallel lines intersecting the intima of the seam were counted as "hits". On each observation per field ("throw") the "hits" were counted, totaled, and the mean calculated per section. The distance between 2 parallel lines was measured and calibrated.

Active centers of bone formation were identified using the fluorescence microscope by the presence of a yellow (tetracycline)\textsuperscript{1} or green (DCAF)\textsuperscript{1,18} labels at the periphery of an osteoid seam. Measurements for calculation of the appositional rates ($M$, and $M_f$) were taken at the site of localization of the fluorochrome labels. The distance between two labels was measured in millimeters by a calibrated eyepiece micrometer at four equidistant points around its circumference and mean values were calculated.
The following nine measurements and counts for cortical bone were made from cross-sections of rib from each dog:1,19,22,24:

1. Cortical area ($A_C$)
2. Total area ($A_T$)
3. Total number of osteoid seams ($A_f$)
   
   The mean number was expressed per mm$^2$ of cortical area ($A_C$). An osteoid seam was identified as a bone formation site by a green or red intimal band on sections stained with osteochrome.
4. Total number of resorption cavities ($A_r$)
   
   Measured by systematic scanning of the entire section using a 10 X objective. Resorption cavities were identified by the presence of scalloped edges.
5. Total number of labelled osteoid seams ($\%L$)
6. Mean distance between fluorochrome dyes within a single Haversian system (MAR)
7. Mean wall thickness of finished osteons (MWT)
8. Osteoid seam thickness (OST)
9. Mean circumference of osteoid seams ($S_f$)

Calculations: From the nine basic measurements the quantitative morphometric analysis of bone consisted of the following stereological calculations:

1. Total Cortical Cross Sectional Area ($A_C$) in mm$^2$
2. Total Bone Cross Sectional Area ($A_T$) in mm$^2$

\[
A_C \text{ or } A_T = \frac{\text{No. of Hits}}{\text{(Grid Factor) (No. of Throws) (No. of Intersections)}}
\]
(3) Mean Number of Osteoid Seams per mm² \( (A_f) \)

(4) Mean Number of Resorption Cavities per mm² \( (A_r) \)

\[
A_f \text{ or } A_r = \frac{\text{Total No. of Osteoid Seams or Resorption Cavities}}{\text{Total Cortical Area (A_c) mm}^2}
\]

(5) Percent labelled Osteoid Seams (%L)

\[
%L = \frac{\text{Total No. Labelled Osteoid Seams}}{\text{Total No. of Osteoid Seams}}
\]

(6) Mean Appositional Rate (M) in μ/day

\[
M = \frac{\text{Mean Distance Between Two Labels (MAR) in microns}}{\text{No. of Days Administered Label}}
\]

(7) Radial Closure Rate \( (M_f) \) in mm/yr.

\[
M_f = (M) \times (%L) \times (365/1000^*)
\]

* reduces microns/day to mm/year.

(8) Osteoid Seam Circumference \( (S_f) \)

\[
S_f = \frac{(\text{No. of Hits})(\text{Distance Between Parallel Grid})(\pi)}{(2)(\text{No. of Throws})}
\]

(9) Ratio of Cortical (C) Area in mm² \( (A_c) \) to Total (T) Area in mm² \( (A_T) \)

\[
C/T = \frac{A_c}{A_T}
\]

(10) Ratio of the Numbers of Resorption Cavities \( (A_r) \) to Osteoid Seams \( (A_f) \)

\[
A_r/A_f = \frac{A_r}{A_f}
\]

(11) Osteon Formation Time in Years \( (\Theta_f) \)

The time required to complete lamellar bone formation in a cross section of an average osteon.

\[
\Theta_f = \frac{\text{MWT}}{M_f}
\]
(12) Number of Activation Frequency Foci in mm²/year ($\mu_f$)

$$\mu_f = \frac{A_f}{\Theta_f}$$

$\mu_f$ represents the number of new osteons introduced per year in an average mm² of cortical area.

(13) Bone Formation Rate in mm²/mm²/year ($V_f$)

$$V_f = (A f) \times (S f) \times M_f$$

**Statistical Analysis:** Statistical significance of differences in the morphometric data comparing treatment effects between ribs were determined by the Nemenyi Rank Sum One-Way Classification Test.\(^{25,26}\) The Wilcoxon Signed Rank test was used to determine statistical significance of differences in paired data for each rib.\(^{25}\) The biochemical data were evaluated by Student t-test.
RESULTS

1-Thyroxine increased the mean body temperature 1.34°F (p<0.05) after 60 days of administration (Table 3.1). Both heart and respiratory rates were significantly increased (p<0.01, p<0.001, respectively) above baseline values. All six dogs showed a gradual increase (p<0.05) in body weight during and at the conclusion of the study.

Bone Histomorphometry

Effects of Thyroxine on Steady-State Bone Remodeling (11th Rib)

After 60 days of thyroxine the frequency of activation (μf) increased 113% (p<0.025) from 28.10 ± 8.73 to 50.85 ± 9.22 foci per mm² per year (Table 3.2). This increase in the recruitment of new remodeling centers was associated with an 89% increase (p<0.05) in the number of osteoid seams (Aₒ) and 165% increase (p<0.01) in the number of resorption cavities (Aᵣ). Numerically, the bone forming sites increased from 5.55 ± 1.29 to 10.47 ± 1.35, and the bone resorbing sites increased from 1.59 ± 0.53 to 4.22 ± 0.57 per mm² of bone.

The tissue level bone formation rate (Vₒ) increased 204% (p<0.01) from 0.27 ± 0.07 to 0.70 ± 0.16 mm² per mm² per year. Similarly, bone accretion increased as evidenced by an 80% increase (p<0.01) in osteoid seam circumference (Sₒ) and bone formation at the cellular and organ level remained unaltered.
Effects of Thyroxine on Accelerated Bone Remodeling Associated with Periosteal Elevation (9th Rib)

Thyroxine resulted in a greater (187%) increase (p<0.01) in activation frequency of new remodeling centers (μf) in bone with surgically induced accelerated bone remodeling than steady-state remodeling (Table 3.2). Associated with this increase in activation frequency was a 336% increase (p<0.01) in the number of resorption sites (A_r). The fractional resorptive to formative bone surface (A_r/A_f) increased 105% (p<0.025) from 0.22 ± 0.04 to 0.45 ± 0.06.

Bone accretion was enhanced as demonstrated by a 110% increase (p<0.025) in bone forming sites (A_f) from 5.15 ± 0.85 to 10.79 ± 1.21 per mm² (Table 3.2). The osteoid seam circumference (S_f) increased 90% (p<0.01) from 0.09 ± 0.02 to 0.19 ± 0.01 mm.

Bone formation increased significantly at the cellular and tissue levels with a trend towards an increase at the organ level. Osteon formation time (Ω_f) decreased 40% (p<0.025) and the closure rate (M_f) was increased 35% (p<0.05) after 60 days thyroxine administration (Table 3.2). The bone formation rate (V_f) at the tissue level was increased 453% (p<0.01) from 0.15 ± 0.04 to 0.83 ± 0.10 mm² per mm² per year.

Effects of Thyroxine on Bone Remodeling Associated with Regional Influences of Periosteal Elevation (10th Rib)

The effects of thyroxine combined with the regional influences of periosteal elevation on bone remodeling were similar in direction and magnitude to that observed with thyroxine alone (11th rib). Thyroxine resulted in an increase in activation frequency (p<0.05), numbers of bone forming and resorbing sites (p<0.01), circumference of osteoid seams (p<0.05), fractional
resorptive to formative bone surfaces (p<0.01), and the bone formation rate at the tissue level (p<0.01). Bone formation at the cellular and organ levels remained unaltered.

Comparison of the Effects of Thyroxine on the 9th and 11th Ribs

The incremental effect of thyroxine plus periosteal elevation on cortical (A_C) and total (A_T) cross-sectional areas of bone were significantly greater than that observed with thyroxine alone (p<0.05, p<0.02, respectively) (Table 3.2). The decrease in fractional cortical area (C/T) following administration of thyroxine and periosteal elevation was greater than that observed with thyroxine alone (p<0.05). Thyroxine plus periosteal elevation resulted in a significant decrease (p<0.05) in the osteon formation time and a greater increase in the mean appositional rate (M_A) compared to baseline values (p<0.05) than thyroxine alone.

Biochemical Evaluation

The serum calcium concentration was decreased below the baseline value of 9.6 ± 0.40 mg/dl (p<0.01) during the first 10 days of thyroxine administration but was subsequently increased until the last day of the study (Table 3.3). The greatest increase (11.91 ± 0.27 mg/dl) occurred on day 32. Serum phosphorus levels decreased below the baseline value of 5.78 ± 0.28 mg/dl (p<0.05) on the first day of thyroxine administration but were consistently elevated thereafter. The peak increase in serum phosphorus of 8.26 ± 0.33 mg/dl was detected on the 25th day of the study. Serum magnesium fluctuated around the baseline value of 2.08 ± 0.22 mg/dl but was decreased significantly only on experimental day 32 and 36 (Table 3.3).
Urinary hydroxyproline excretion had increased above the baseline value of $11.13 \pm 0.86$ on days 10, 22, 25, and 32 of thyroxine administration but had returned to pre-treatment levels by the termination of the experiment on day 60 (Table 3.3).
DISCUSSION

The results of the present study demonstrated an increase in bone turnover at the tissue level in cortical bone of adult dogs. This increase occurred under conditions of both steady-state and when locally accelerated bone remodeling was superimposed. The observed increase in bone turnover was similar to that reported in hyperthyroid patients with osteoporosis$^4,27$ and was in agreement with kinetic studies using radiocalcium in patients with hyperthyroidism.$^5,11,27-29$

The osteopenia that develops in humans with thyrotoxicosis is characterized by an increase in the activation frequency, number of resorption sites, ratio of resorptive to formative bone surfaces, and an increase in cortical porosity.$^1-5,10$ Similar bone lesions to those described in human patients with thyrotoxicosis occurred only in the ninth rib of experimental dogs in which bone remodeling had been locally accelerated by surgically elevating the periosteum. Moderate pharmacologic doses of thyroxine increased the activation frequency 1.8 times in cortical bone with steady-state remodeling and 2.9 times in cortical bone with thyroxine and accelerated remodeling compared to baseline values. This increased activation frequency was associated with a marked increase in the number of resorption sites (2.6 times greater in steady-state rib and 4.4 times greater in ribs with thyroxine and accelerated remodeling). Increased cortical porosity was demonstrated by an increase in the ratio of bone resorptive to formative surfaces and a trend toward a decrease in the fractional cortical area in ribs with thyroxine and accelerated remodeling. These findings
and those of other investigators indicate that increased cortical resorption is mainly responsible for bone mineral mobilization in hyperthyroidism.\(^{1,2,5,9,11,27,29,31}\)

Thyroxine administered daily for 60 days resulted in a 2-fold increase in the number of bone forming sites and the surface undergoing bone formation. This occurred under both steady-state conditions and with accelerated remodeling superimposed. These results suggest that thyroxine may enhance the switch-over from the resorptive to the formative phases of bone remodeling. Tissue level bone formation rate was similarly increased under both steady-state and when thyroxine was combined with accelerated remodeling. Thyroxine plus periosteal elevation appeared to enhance osteoblastic activity since, osteon formation time was decreased and the radial closure rate was increased in the 9th rib.

Thyroxine is known to influence calcium metabolism by increasing calcium compartment size and increasing the flow into and out of these compartments. Krane et al.\(^{11}\) demonstrated that these calcium fluxes were decreased in myxedema and increased in hyperthyroidism. It has also been reported that thyroid hormones can elevate serum phosphate levels by increasing the renal threshold for phosphate reabsorption.\(^{30}\) In the present study, serum calcium and phosphorus levels were increased at several intervals during the administration of 1.0 mg per kg of thyroxine. These results were consistent with the findings of Adams and Jowsey\(^3\) who experimentally induced hyperthyroidism in intact dogs using 1.5 mg per kg of thyroxine. The elevated serum calcium and phosphorus often correlated with an increased excretion of urinary hydroxyproline, suggesting increased bone matrix catabolism, that also has been reported in patients with thyrotoxicosis.\(^{31,32}\)
The results of this investigation suggested that thyroxine at the dose level and schedule used directly stimulated the metabolic activity of bone cells, altered the status of the calcium and phosphorus pools, and profoundly affected bone formation and resorption under conditions of steady-state and when accelerated remodeling was superimposed. The similar changes induced by thyroxine in bone with different remodeling rates were in contrast to the effects of parathyroid hormone (PTH) and 1,25-(OH)₂D₃ in adult dogs under identical experimental conditions. The intermittent administration of 1,25-(OH)₂D₃ decreased activation frequency and slowed the remodeling rate. The combined effect of periosteal elevation and 1,25-(OH)₂D₃ were markedly different from those observed with 1,25-(OH)₂D₃ alone and reflected an increased bone remodeling as would occur regionally following tissue injury. This change was associated with a corresponding increase in bone resorption and formation.

Parathyroid hormone (PTH) in low daily doses administered at 6 hr intervals for 60 days had no significant effect on the activation frequency in cortical bone of adult dogs with steady-state or when combined with accelerated remodeling. PTH increased the radial closure rate, ratio of cortical to total bone area, and decreased the osteon formation time under steady-state conditions. These results were interpreted to suggest that PTH under these conditions accelerated the accretion rate of bone in adult dogs. This may reflect an increased osteoblastic activity in already established Haversian systems. In contrast, PTH caused a decrease in the circumference and thickness of osteoid seams, and resulted in a greater number of bone resorption than formation sites in cortical bone when combined with
accelerated remodeling. This suggested a dissociation between bone resorption and formation. PTH may have uncoupled the switch-over from the resorptive to the formative phase of remodeling.¹,³³

Frost³⁵ has proposed that in order to restore bone mass in osteopenic states such as osteoporosis, one must be able to activate the skeleton then suppress the osteoclast population in bone to reduce the amount of mineral removed by each bone metabolic unit (BMU). Following depression of the resorptive phase, the formation phase is permitted to put back more bone than that which was removed. The process of Activation, Depression, Free, Repeat (ADFR) is continued until bone mass reaches the desired amount. The results of the present study demonstrate that the daily oral administration of thyroxine was a potent activator in an animal with a remodeling skeleton. Additional studies with other hormones (alone or in combination) under conditions of steady-state and accelerated remodeling should provide a useful model system for further developing and refining the ADFR concept.
**SUMMARY**

Histomorphometric evaluation was made on the effects of thyroxine on cortical bone remodeling under steady-state conditions and when combined with accelerated remodeling induced by surgical elevation of the periosteum of the rib. Six adult female beagles were administered orally 1.0 mg of l-thyroxine per kg body weight daily for 60 days. Static and dynamic changes were evaluated using tetracycline and DCAF (2,4 BIS) N,N'-Di(carboxymethyl) (amino methyl fluorescein) *in vivo* double labeling of bone specimens taken before treatment and after 60 days.

Thyroxine administered in moderate pharmacologic doses increased the activation frequency, number of bone forming and resorbing sites, and the osteoid seam circumference in unaltered bone. These results indicated that thyroxine by activating the skeletal remodeling process increased bone turnover and both formation and resorption at the tissue level under steady-state conditions.

In periosteal elevated cortical bone, accelerated remodeling and thyroxine increased the activation frequency, number of bone resorption and formation sites, and ratio of bone resorptive to formative surfaces. In addition, this combined treatment resulted in an increase in the osteoid seam circumference, radial closure rate, bone formation rate at the tissue level. The osteon formation time was decreased by periosteal elevation and thyroxine administration. Under conditions of accelerated remodeling, thyroxine administered to adult dogs increased osteoblastic and resorptive activity, to a greater degree
than in unaltered bone, and resulted in a bone lesion similar to that described in human beings with thyrotoxicosis. The increased serum calcium and phosphorus levels, and urinary hydroxyproline excretion at several intervals during thyroxine administration were consistent with the morphometric evidence of increased bone turnover and resorption. These findings suggest that thyroxine is a potent activator of skeletal remodeling and enhanced the switch-over from the resorptive to the formative phase under steady-state conditions.
Table 3.1: Effects of L-thyroxine (1 mg/kg/day) administration on metabolic parameters in experimental dogs. (Mean ± standard error, N = 6 dogs.)

<table>
<thead>
<tr>
<th>Days $T_4$</th>
<th>Body Weight (kg)</th>
<th>Body Temperature ($^\circ$F)</th>
<th>Heart Rate (Beats/min.)</th>
<th>Respiratory Rate (No./min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>7.39 ± 0.40</td>
<td>101.87 ± 0.21</td>
<td>101.66 ± 6.40</td>
<td>24.50 ± 2.33</td>
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<tr>
<td>1</td>
<td>-</td>
<td>101.40 ± 0.04***</td>
<td>90.50 ± 10.50</td>
<td>24.00 ± 0.00</td>
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<tr>
<td>2</td>
<td>-</td>
<td>101.75 ± 0.35</td>
<td>104.00 ± 2.83</td>
<td>19.00 ± 1.00*</td>
</tr>
<tr>
<td>7</td>
<td>6.93 ± 0.59</td>
<td>102.00 ± 0.06</td>
<td>135.00 ± 9.57*</td>
<td>31.75 ± 1.31***</td>
</tr>
<tr>
<td>11</td>
<td>7.98 ± 0.12</td>
<td>102.22 ± 0.19</td>
<td>125.33 ± 15.03</td>
<td>27.67 ± 2.70</td>
</tr>
<tr>
<td>16</td>
<td>7.70 ± 0.00</td>
<td>102.20 ± 0.28</td>
<td>131.00 ± 11.00</td>
<td>24.00 ± 0.00</td>
</tr>
<tr>
<td>20</td>
<td>8.08 ± 0.13</td>
<td>102.70 ± 0.21***</td>
<td>136.30 ± 4.01****</td>
<td>30.33 ± 2.85</td>
</tr>
<tr>
<td>25</td>
<td>-</td>
<td>102.28 ± 0.23</td>
<td>165.00 ± 5.00****</td>
<td>40.50 ± 2.63***</td>
</tr>
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<td>31</td>
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<td>102.40 ± 0.26</td>
<td>114.00 ± 11.68</td>
<td>31.33 ± 2.81*</td>
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<td>35</td>
<td>7.51 ± 0.43</td>
<td>102.67 ± 0.24*</td>
<td>149.30 ± 6.42****</td>
<td>33.33 ± 3.21*</td>
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<tr>
<td>45</td>
<td>8.21 ± 0.33*</td>
<td>101.98 ± 0.06</td>
<td>113.75 ± 9.05</td>
<td>30.33 ± 3.83</td>
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<tr>
<td>48</td>
<td>8.08 ± 0.47</td>
<td>102.45 ± 0.10***</td>
<td>135.00 ± 9.43*</td>
<td>33.83 ± 2.93**</td>
</tr>
<tr>
<td>55</td>
<td>8.34 ± 0.49*</td>
<td>102.00 ± 0.00</td>
<td>129.50 ± 13.50</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>8.03 ± 0.51</td>
<td>103.24 ± 0.47*</td>
<td>139.17 ± 9.22**</td>
<td>37.00 ± 5.46</td>
</tr>
</tbody>
</table>

*p<0.05  **p<0.02  ***p<0.01  ****p<0.001
Table 3.2: Effects of L-Thyroxine (T₄) on histomorphometric parameters in the 9th, 10th, and 11th ribs of dogs. (Mean ± S.E., N = 6 dogs.)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>9th Rib Periosteal Elevated</th>
<th>10th Rib Regional Effects</th>
<th>11th Rib Thyroxine Effects</th>
<th>p-Values Comparing Treatment Effects Between 9th, 10th, and 11th Ribs</th>
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</thead>
<tbody>
<tr>
<td>BONE MASS</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Aᵥ (mm²)</td>
<td>6.26 (0.50)</td>
<td>6.33 (0.72)</td>
<td>5.47 (0.56)</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>Aₜ (mm²)</td>
<td>9.15 (1.33)</td>
<td>10.08 (1.05)</td>
<td>8.47 (0.70)</td>
<td>p&lt;0.02</td>
</tr>
<tr>
<td>C/T</td>
<td>0.76 (0.11)</td>
<td>0.63 (0.02)</td>
<td>0.64 (0.02)</td>
<td>p&lt;0.05 (compared to 9th rib)</td>
</tr>
<tr>
<td>MWT (mm)</td>
<td>0.08 (0.00)</td>
<td>0.08 (0.01)</td>
<td>0.08 (0.00)</td>
<td>N.S.</td>
</tr>
<tr>
<td>BONE ACCRECTION</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aᵥ (no/mm²)</td>
<td>5.15 (0.85)</td>
<td>10.79 (1.21)</td>
<td>5.55 (1.29)</td>
<td>N.S.</td>
</tr>
<tr>
<td>OST (µm)</td>
<td>8.04 (0.47)</td>
<td>9.66 (0.78)</td>
<td>8.06 (0.40)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Sᵥ (mm)</td>
<td>0.09 (0.02)</td>
<td>0.19 (0.01)</td>
<td>0.10 (0.02)</td>
<td>N.S.</td>
</tr>
<tr>
<td>%L (%)</td>
<td>71.53 (10.88)</td>
<td>81.12 (5.13)</td>
<td>79.46 (9.84)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.025, *** p<0.01 for individual paired (pre versus post) rib observations using Wilcoxon Signed Rank Sum analysis

+ p<0.05, ++ p<0.025 for treatment effects comparing ribs using Nemenyi One-way Rank Sum analysis
Table 3.2: (Continued)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>9th Rib Periosteal Elevated</th>
<th>10th Rib Regional Effects</th>
<th>11th Rib Thyroxine Effects</th>
<th>p-Values Comparing Treatment Effects Between 9th, 10th, and 11th Ribs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-(T_4)</td>
<td>Baseline</td>
<td>Post-(T_4)</td>
</tr>
<tr>
<td><strong>BONE RESORPTION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A_r) (No/mm)</td>
<td>1.06</td>
<td>4.62***</td>
<td>1.05</td>
<td>4.42***</td>
</tr>
<tr>
<td></td>
<td>(0.25)</td>
<td>(0.30)</td>
<td>(0.15)</td>
<td>(0.47)</td>
</tr>
<tr>
<td>(A_r/A_f)</td>
<td>0.22</td>
<td>0.43**</td>
<td>0.21</td>
<td>0.51***</td>
</tr>
<tr>
<td></td>
<td>(0.04)</td>
<td>(0.06)</td>
<td>(0.04)</td>
<td>(0.07)</td>
</tr>
<tr>
<td><strong>BONE CELL ACTIVITY</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAR ((\mu)m)</td>
<td>14.24</td>
<td>14.18**</td>
<td>16.36</td>
<td>16.83</td>
</tr>
<tr>
<td></td>
<td>(1.03)</td>
<td>(1.42)</td>
<td>(0.90)</td>
<td>(1.66)</td>
</tr>
<tr>
<td>(M) ((\mu)/day)</td>
<td>1.19</td>
<td>+1.40</td>
<td>1.39</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>(0.10)</td>
<td>(0.11)</td>
<td>(0.15)</td>
<td>(0.11)</td>
</tr>
<tr>
<td>(M_f) (mm/yr)</td>
<td>0.31</td>
<td>0.42**</td>
<td>0.39</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>(0.05)</td>
<td>(0.06)</td>
<td>(0.04)</td>
<td>(0.03)</td>
</tr>
<tr>
<td>(\Theta_f) (time/yr)</td>
<td>0.30</td>
<td>+0.18**</td>
<td>0.21</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>(0.06)</td>
<td>(0.02)</td>
<td>(0.04)</td>
<td>(0.01)</td>
</tr>
<tr>
<td><strong>BONE TURNOVER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\mu) (activation freq. foci/mm(^2)/yr)</td>
<td>21.66</td>
<td>62.27***</td>
<td>27.35</td>
<td>46.47*</td>
</tr>
<tr>
<td></td>
<td>(6.38)</td>
<td>(7.81)</td>
<td>(8.68)</td>
<td>(7.82)</td>
</tr>
<tr>
<td>(V_f) (mm(^2)/mm(^2)/year)</td>
<td>0.15</td>
<td>0.83***</td>
<td>0.22</td>
<td>0.66***</td>
</tr>
<tr>
<td></td>
<td>(0.04)</td>
<td>(0.10)</td>
<td>(0.07)</td>
<td>(0.16)</td>
</tr>
</tbody>
</table>

* \(p<0.05\), ** \(p<0.025\), *** \(p<0.01\) for individual paired (pre versus post) rib observations using Wilcoxon Signed Rank Sum analysis
** \(p<0.05\), **p\<0.025 for treatment effects comparing ribs using Nemenyi One-way Rank Sum analysis
Table 3.3: Serum calcium and phosphorus, and urinary hydroxyproline (HOP) in adult dogs following L-thyroxine administration (Mean ± standard error, N = 6 dogs).

<table>
<thead>
<tr>
<th>Days $T_4$</th>
<th>Serum Calcium (mg/dl)</th>
<th>Serum Phosphorus (mg/dl)</th>
<th>Serum Magnesium (mg/dl)</th>
<th>Urinary HOP:Creatinine (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>9.6 ± 0.40</td>
<td>5.78 ± 0.28</td>
<td>2.08 ± 0.22</td>
<td>11.13 ± 0.86</td>
</tr>
<tr>
<td>1</td>
<td>8.93 ± 0.08**</td>
<td>5.32 ± 0.15*</td>
<td>2.28 ± 0.20</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>9.03 ± 0.52</td>
<td>7.14 ± 0.85</td>
<td>1.90 ± 0.16</td>
<td>12.22 ± 0.85</td>
</tr>
<tr>
<td>8</td>
<td>8.80 ± 0.89</td>
<td>6.38 ± 0.52</td>
<td>2.07 ± 0.87</td>
<td>12.53 ± 1.99</td>
</tr>
<tr>
<td>10</td>
<td>8.40 ± 1.64</td>
<td>-</td>
<td>-</td>
<td>17.64 ± 1.22*</td>
</tr>
<tr>
<td>13</td>
<td>9.70 ± 0.14</td>
<td>7.20 ± 0.58*</td>
<td>1.97 ± 0.21</td>
<td>10.31 ± 1.41</td>
</tr>
<tr>
<td>22</td>
<td>9.87 ± 0.48</td>
<td>6.67 ± 0.61</td>
<td>1.98 ± 0.27</td>
<td>20.83 ± 1.31***</td>
</tr>
<tr>
<td>25</td>
<td>11.80 ± 1.05*</td>
<td>8.26 ± 0.33***</td>
<td>2.19 ± 0.15</td>
<td>28.65 ± 2.00**</td>
</tr>
<tr>
<td>32</td>
<td>11.91 ± 0.27***</td>
<td>6.99 ± 0.47*</td>
<td>1.77 ± 0.05**</td>
<td>22.49 ± 2.00**</td>
</tr>
<tr>
<td>36</td>
<td>10.72 ± 1.03</td>
<td>6.77 ± 0.51</td>
<td>1.59 ± 0.15*</td>
<td>15.60 ± 2.06</td>
</tr>
<tr>
<td>50</td>
<td>11.18 ± 0.40*</td>
<td>6.55 ± 0.64</td>
<td>2.06 ± 0.16</td>
<td>19.38 ± 3.49</td>
</tr>
<tr>
<td>55</td>
<td>10.35 ± 0.46</td>
<td>6.86 ± 0.43*</td>
<td>2.39 ± 0.71</td>
<td>14.53 ± 1.65</td>
</tr>
<tr>
<td>60</td>
<td>8.60 ± 0.76</td>
<td>6.36 ± 0.40</td>
<td>2.17 ± 0.04</td>
<td>10.07 ± 1.38</td>
</tr>
</tbody>
</table>

*p<0.05    **p<0.01    ***p<0.001
CHAPTER IV

HISTOMORPHOMETRIC EVALUATION OF THE EFFECTS OF
1,25-DIHYDROXYCHOLECALCIFEROL, PARATHYROID HORMONE, AND
THYROXINE ON TRABECULAR BONE REMODELING IN ADULT DOGS

INTRODUCTION
The most dramatic bone loss in several metabolic bone diseases occurs in trabecular bone and results in an osteoporosis (1-5). This is particularly true when osteoporosis arises quickly (1). Osteoporotic bone is characterized either by an excessively widened marrow cavity, thinned trabeculae, insufficient cross-sectional diameter, and thin cortices (1-5). This bone loss is confined to the endosteal envelope in senile and post-menopausal osteoporosis (1,2). Current evidence indicates that any change in endosteal remodeling caused by experimental condition or disease process which acts to increase bone loss per unit area of the endosteal envelope will cause a much larger percentage and more rapid loss of trabecular than cortical bone (1). In part, this arises from trabecular bone having the highest surface-to-volume ratio of all bone envelopes and accordingly is subjected to the greatest remodeling activity (1). In disuse and senile osteoporosis slowing of the remodeling rate is critical and due to a decrease in the recruitment of bone cells (1). Accelerated bone remodeling characterized by an initial resorptive phase of bone loss is observed in osteoporosis associated with thyrotoxicosis in human beings (1). This bone
loss is accentuated because of the time lag between the removal of bone during the resorptive phase and the complete replacement of it during the formative phase of remodeling. Trabecular bone remodeling depends upon a constant supply of bone cells that remove and form bone. They are derived ultimately from primitive mesenchymal cells following an activating stimulus (1).

The observation of thinned trabeculae enclosed by the endosteal envelope associated with an expanding marrow cavity in several forms of osteoporosis suggests that local mediating factors within the marrow cavity may play an important role in the pathogenesis of these disorders (1). The influence of bone modifying agents such as 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃), parathyroid hormone (PTH), and thyroxine (T₄) on the trabecular skeleton has been studied experimentally and in naturally occurring diseases. Histomorphometric evaluation of low and intermediate doses of 1,25-(OH)₂D₃ demonstrated osteosclerotic changes in trabecular bone of rodents (6,7). High levels of vitamin D also may produce osteosclerotic changes in trabecular bone (8). In addition, current studies using histomorphometric evaluation of bone in patients with renal failure (9) and senile osteoporosis (10) demonstrated a restorative effect towards normal remodeling following administration of 1a-hydroxy-vitamin D₃. Senile osteoporotic patients treated with low doses of vitamin D had an improvement in calcium balance with evidence of decreased bone resorption (10). Patients with chronic renal failure had an enhanced calcification rate associated with a decrease in unmineralized bone (9). Ultrastructural evaluation of the effects of 5 units of 1,25-(OH)₂D₃ on metaphyseal trabeculae of thyroparathyroidectomized rats revealed a marked
increase in osteoclasts and numbers of osteoblasts which were interpreted to be active in matrix production and mineralization (11).

In studies using physiological dose levels of parathyroid hormone (PTH) an anabolic effect has been reported without evidence of increased bone resorption (12-16). Studies by Parsons et al. (17) and Reeve et al. (18) demonstrated that administration of chronic low dose levels of both bovine and human PTH to dogs and to osteoporotic women produced increased calcium retention, bone mass, and calcium accretion.

Increased cortical and trabecular porosity may be a clinically debilitating feature of thyrotoxicosis in man (1,19-21). The administration of thyroxine in pharmacological doses results in osteoporotic bone under experimental conditions (22). However, lower levels of thyroxine have been reported to exert a direct growth-stimulatory effect on bone and other tissues (22,23). It is not clear if the effect of thyroxine on bone is direct or indirect. Recent studies on thyrotoxicosis using bone histomorphometric analysis demonstrated increased bone turnover with an increase in both bone resorption and formation (1,21). Anti-thyroid treatment resulted in a reversal of these effects (25) and trabecular bone surfaces were inactive with respect to bone formation and resorption. In addition, there was an increase in the amount, extent, and width of osteoid seams that was attributed to an increase in the life span of bone formation sites and a prolongation of the maturity of osteoid. These studies suggest that thyroxine acts on the remodeling process at the microenvironmental level around bone cells.
The cellular mechanisms in the trabecular skeleton that is affected by 1,25-(OH)\(_2\)D\(_3\), PTH, and T\(_4\) and which produce these reported changes have not been fully delineated. The iliac crest is the most frequent trabecular bone biopsied for evaluation in human beings with metabolic bone diseases (25). The present study is designed to evaluate the effects of three hormones on trabecular bone remodeling in adult dogs in order to characterize their mode and site of action. The hormonal effects on bone turnover in dogs should have direct applicability to osteopenic disorders of human beings because bone turnover in both human beings and adult dogs is primarily remodeling. The specific objectives of this investigation were: (1) to determine the effects of 1,25-(OH)\(_2\)D\(_3\), PTH, and T\(_4\) on remodeling in trabecular bone from the iliac crest by histomorphometric evaluation, and (2) to correlate these changes in bone turnover in adult dogs with alterations in serum electrolytes and urinary hydroxyproline.
MATERIALS AND METHODS

Experimental Animals

Six female beagles (Marshall Research Animals, North Rose, NY 14516) weighing approximately 10 Kg and 10 to 22 months of age were acclimated to laboratory conditions for two weeks. The dogs were fed commercial Purina dog chow (Ralston Purina Co., Checkerboard Square, St. Louis, MO 63188) with free access to water. The dogs were maintained in individual stainless steel metabolism cages for urine collection. Blood collection and intravenous administration of parathyroid hormone (PTH, Eli Lilly & Co., Indianapolis, IN 46206) and 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) were performed using indwelling catheters (epidural catheter, No. 8222, Becton-Dickinson & Co., Rutherford, NJ 07070) placed in the jugular vein. L-Thyroxine tablets ("Soloxine", Daniels Pharmaceuticals, Inc., St. Petersburg, FL 33713) were administered orally.

Experimental Design

The study lasted for 60 days or one-half the lifespan of a bone forming site in the dog (1). Fifty units (1.25 µg/day) of 1,25-(OH)$_2$D$_3$ in ethanol was administered intravenously in equally divided doses twelve hours apart. The hormone was administered for 6 days, then withdrawn for 14 days. Three complete cycles of 1,25-(OH)$_2$D$_3$ administration and withdrawal occurred within the 60-day period. The dose of 1,25-(OH)$_2$D$_3$ was selected from a pilot dose-response study that determined the lowest dose that altered bone dynamics.
without resulting in a persistent hypercalcemia. PTH was administered at a
dose of 2.5 U per Kg of body weight per day (1.25 μg per Kg per day)
intravenously in 4 equally divided doses every 6 hours for 60 days. This dose
was selected from a previous pilot dose-response study and results of an earlier
investigation that determined the lowest dose that altered bone dynamics
without resulting in persistent hypercalcemia (Nagode, L.: Personal communi-
cation, 1976). One (1.0) mg per kilogram of l-thyroxine was administered orally
in equally divided daily doses twelve hours apart. The dose was selected from
the results of an earlier study (Bishop, S.F., Robinson, W.F.: Personal
communication, 1976) that demonstrated only minimal changes in body weight
and temperature, heart rate, serum calcium, and plasma protein levels with this
dose of thyroxine in the dog.

Each dog served as its own control by collecting a pre-treatment biopsy of
the left iliac crest on day 0. The dogs were euthanized after 60 days and the
contralateral right iliac crest was removed and fixed in 70% ethanol.

Blood and urine were collected during the acclimation period for baseline
values and at variable intervals during administration of the hormone. In the
1,25-(OH)_{2}D_{3} study blood was collected on days 1,3,5,7,13,15,22,25,27,30,40,
45,50,55 and 60, and urine on days 1,3,5,13,15,22,30,40,45,50 and 60. Blood was
collected on days 1,5,7,10,12,15, 20,25,30,40,50,55 and 60, and urine on days
1,10,20,25,30,40,55 and 60 in the PTH study. In the T_{4} study blood was
collected on days 1,2,8,10,13,22,25,32,36,40,50,55 and 60, and urine on days
2,8,10,13,22,25,32,36,50,55 and 60. Serum calcium was determined by atomic
absorption spectrophotometry (Perkin-Elmer Model 303) (26). Serum phosphorus
was determined by the method of Baginski and Zak (27). Urine was collected under toluene at least weekly in metabolism cages. Urine was assayed for hydroxyproline by the method of Kivirikko et al. (28) and creatinine by the method of Clark (29). Urinary hydroxyproline was expressed as a ratio to creatinine. Body weight and temperature, heart and respiratory rates were recorded at weekly intervals during the administration of thyroxine. The daily mean values of serum calcium and phosphorus, urinary hydroxyproline concentrations, and body metabolic parameters of the 6 dogs of each hormonal group were averaged over 1-2, 3-5, and 6-8 weeks of the experiment.

**Surgical Procedures**

**Anesthesia:** The dogs were administered 0.25 mg/kg acepromazine maleate (Ayerst Laboratories, Inc., New York, NY 10017) and 0.20 mg atropine sulfa (Eli Lilly & Co., Indianapolis, IN 46206) intramuscularly. Anesthesia was induced with Surital (Thiamyl Sodium for injection, Parke-Davis & Co., Detroit, MI 48232) administered intravenously and was maintained using methoxyfluorane (Metafane, Pitman-Moore, Inc., Washington Crossing, NJ 08560).

**Iliac Crest Biopsy:** The skin incision commenced at the crest of the ilium and ran vertically down and caudally over the body of the ilium as it progressed toward the ischium (32,33). The cutaneous trunci muscle, subcutaneous fat, and deep gluteal fascia were incised in line with the skin incision to expose the middle gluteal muscle and the iliac crest. The middle gluteal muscle was freed from the ilium by the incision of its origin along the crest and dorsal edge of the body of the ilium. The incision was continued posteriorly and ventrally in the
same line to sever those fibers of the middle gluteal muscle that originated dorsal, medial and anterior to the ilium. The middle gluteal muscle was elevated from the wing of the ilium and retracted laterally. Complete exposure of the ilium required elevation of some insertions of the iliocostalis and longissiumus muscles on the medial surface of the crest and wing. An 8 mm thick biopsy of the antero-superior part of the iliac crest was removed with double action bone cutters (Stille-Liston double action bone cutting forceps, No. 69-410 Misdom-Frank Corp. 860 Broadway, New York, NY 10003). The middle gluteal muscle was sutured back into position by using mattress sutures in the muscle sheath and in the gluteal fascia to pull the muscle against the ilium. The bone sections were fixed in 70% ethanol.

Bone Histomorphometry
Morphometric analysis was performed on methyl methacrylate-embedded hand-ground and microtome-cut bone sections. The hand-ground sections were stained with osteochrome (32) and the microtome-cut sections with Goldner's (33). Morphometric evaluation utilized both fluorescence and bright field light microscopy.

Microtome-Cut-Bone Sections
One half of the iliac crest biopsy was embedded in methyl methacrylate and cut at 5 and 10 microns with a Jung "K" microtome (R.Jung, A.G. Heidelberg, No. 29331, William J. Hacker Co., Inc., P.O. Box 646, W. Caldwell, NJ 07006) and floated to a gelatin-coated slide with 70% ethanol (33). Gelatin (Haupt's Adhesive) was used to affix sections to microslides (33). Following drying the
plastic was removed and the slides were stained with Goldner's. When staining was completed the microslides were dehydrated in ascending concentrations of alcohol, cleared in xylol, and mounted in Eukitt's mounting media (Calibrated Instruments, Inc., 731 Saw Mill River Road, Ardsley, NY 10502).

**Ground Bone Sections**

An average of 5 cross-sections per iliac crest were cut at approximately 200 μ thickness using a jeweler's saw (Apparatus for Microstructural Analysis; Buehler, Ltd., 2120 Greenwood St., Evanston, IL 60204). The sections were stained for 72 h with osteochrome stain (32). When staining was complete, the sections were embedded in methyl methacrylate (33) and ground to 75-90 μ thickness (32). Section thickness was critically measured by a mikrokator (Mikrokater, No. 7VZ509-4, C.E.J. Gage Co., Subsidiary of C.E. Johnson, Sweden, 10641 Haggerty, Dearborn, MI 48126). The sections were washed in 0.1% benzalkonium chloride to remove debris, rinsed in distilled water, and differentiated in 0.01% acetic acid in 95% methanol (32,33). When differentiation was completed they were dehydrated in ascending concentrations of alcohol, cleared in xylol, and mounted in Eukitt's mounting media.

**Double Fluorochrome Bone Labeling**

Evaluation of the dynamics of appositional bone growth was facilitated by the intravenous administration of tetracycline (Oxytetracycline hydrochloride, "Liquamycin", Pfizer, Inc., New York, NY 10017) and a second fluorochrome label DCAF (2,4 BIS N,N'-di-(carboxymethyl) (aminomethyl fluorescein) (I.C.N. Pharmaceuticals, Inc., Life Science Group, Cleveland, OH 44128) (34-36).
Fluorochrome administration began 18 days before the start of the study and again on experimental day 42 using the schedule of 3-days-on, 7-days-off, and 3-days-on. The dosage was 15 mg/kg with tetracycline given the first 3 days and DCAF the second 3 days.

Osteochromestained ground bone sections (80 µ) were viewed under an ultraviolet microscope using a Wratten 18A filter at the source, a 2B filter at the eyepiece, a wavelength of 36 µm at both 10X and 25X magnification. Tetracycline produced yellow and DCAF green fluorescence with ultraviolet light (34-36).

**Morphometric Evaluation of Bone**

**Measurements:** Morphometric measurements were performed with a calibrated micrometer and Zeiss integrator plate II, 100/25 ocular grid with a 0.6 aperture (25,37). A minimum of 3 cross-sections of each bone were measured in order to analyze 50 mm² of bone (37).

To compute values for trabecular bone dynamics, twelve basic parameters must be measured or counted (37). These include: a) fractional trabecular bone volume \( tV_{\text{frac}} \); b) specific surface of trabecular bone \( tS_{sp} \); c) fractional osteoid volume \( tV_{\text{frac(o)}} \); d) fractional formation surface \( S_{\text{frac(f)}} \); e) fractional resorptive surface \( S_{\text{frac(r)}} \); f) apparent separation between fluorochrome bone labels (µm); g) fractional labelled surface \( S_{\text{frac(lab)}} \); h) apparent mean wall thickness (µMWT); i) osteoblasts per unit length of active formation perimeter \( C_{f(\text{act)}} \); j) osteoclasts per unit length of resorptive
perimeter (Nc); k) osteoclasts per unit active resorption perimeter (C_{r(\text{act})}); and
l) fractional active resorptive surface (actS_{fract(r)}).

Basic Parameters

a). Fractional trabecular bone volume \( tV_{\text{fract}} \) was determined using a
grid and "point-count technique" to measure the decimal fraction of the area in
the section of whole trabecular bone tissue (WTBT) which comprises only
trabecular bone. All grid-point "hits" on bone are \( h_b \) and all other hits \( h_a \).
The decimal fraction of the volume of WTBT composed of trabecular bone alone
is: \( tV_{\text{fract}} = h_b/h_b + h_a \). It is expressed as a ratio with the dimensions: mm\(^3\) of
trabecular bone/mm\(^3\) WTBT.

b). Fractional trabecular osteoid volume \( tV_{\text{fract(o)}} \) was determined
similarly as the fractional trabecular bone volume: \( tV_{\text{fract(o)}} = h_o/h_o + h_a \).

c). Specific surface of trabecular bone \( tS_{sp} \) was determined by
measuring the perimeter intersects of trabecular edges on the lines of a parallel
eyepiece grid. By designating \( \Sigma h = \) total number of perimeter "hits" in a
number of throws, \( t = \) number of throws, \( A = \) area of the grid in object space,
and \( a = \) the perpendicular separation between two lines of the grid in object
space, the trabecular specific surface in a unit volume of a biopsy can be
calculated: \( tS_{sp} = (\Sigma h) (a) (\pi)/ (2) (A) (t) \) in mm\(^2\) trabecular surface/mm\(^3\) of
WTBT.

d). Fractional formation surface \( S_{fract(f)} \) was determined by measuring
the perimeter intersects of seams and non-seams covered perimeters of
trabecular bone on the lines of a parallel grid system. By designating \( h_o = \) hits
on osteoid (seam) perimeter, \( h_a \) = hits on all other trabecular perimeters, and
\[ \Sigma h = \text{all hits combined}, \quad S_{\text{fract}} = \frac{h_o}{h_o + h_a} \text{ as mm of osteoid perimeter/mm of total trabecular perimeter}. \]

e). **Fractional resorption surface** \( S_{\text{fract(r)}} \) was determined similarly as
\[ S_{\text{fract(r)}} \] The value for \( S_{\text{fract(r)}} = \frac{h_r}{h_r + h_a} \) as mm of resorptive perimeter/mm of total trabecular perimeter.

f). **Apparent separation between markers** (\( \mu M \)). Measurements were made from the middle of one label to the middle of the adjacent label. The mean separation between labels was calculated as
\[ \mu M = \frac{\text{sum of all measurements of all pairs}}{\text{the number of pairs measured}} \text{ as \( \mu m \).} \]

g). **Fractional labelled surface** \( S_{\text{fract(lab)}} \) was determined by using the perimeter intersect method under fluorescence microscopy. That fraction of the total trabecular surface was determined that lies over the double fluorochrome label. Counts are tallied separately: \( h_{\text{lab}} \) = intersects on labelled surface and \( h_a \) = intersects on all other trabecular surface.
\[ S_{\text{fract(lab)}} = \frac{h_{\text{lab}}}{h_{\text{lab}} + h_a} \text{ as a ratio comprising mm of double labelled perimeter/mm of total trabecular perimeter in thin sections in the solid WTBT.} \]

h). **Apparent mean wall thickness** (\( \mu MWT \)) was determined on ground thick sections (50 - 90\( \mu \)) by measuring the distance from cement line to free trabecular surface in completed bone remodeling sites with a calibrated eyepiece micrometer. Measurements were made each at 3 equally spaced points along its free perimeter and the mean determined. The means of 20-35 bone remodeling sites were averaged and expressed as mm.
i). Osteoblasts per unit length of active formation perimeter \( (C_{f(\text{act})}) \) was determined in thin sections lightly stained with Goldner's. The number of osteoblasts on a unit length of the perimeter of active formation surface was calculated: \( C_{f(\text{act})} = \left( \frac{10 \times n}{\Sigma x} \right) \cdot a \), where \( n \) - number of observations, \( \Sigma x \) = sum of all measured osteoid perimeter covered by 10' osteoblasts, and \( a = \) micrometer constant.

j). Osteoclasts per unit active resorption perimeter \( (C_{r(\text{act})}) \) was determined in thin sections by locating an osteoclast and at 150X - 250X measuring the length of perimeter of the resorption surface on the trabecula covered by the osteoclast. If \( \Sigma x \) designated the length of the perimeter covered by a particular osteoclast, \( n \) the number of osteoclasts measured, and \( a = \) the micrometer constant, then \( C_{r(\text{act})} = \frac{n}{(\Sigma x)} \cdot a \) as osteoclasts per mm of active resorption perimeter. At least 30-50 such measurements were made.

k). Fractional active resorptive surface \( (\text{act}S_{\text{frac}(r)}) \) was done similarly as the fractional resorption surface. A separate tally was made of those intersects of the parallel grid lines which cross any part of a trabecular surface lying beneath an osteoclast. If \( hc \) equals the number of intersects and \( ha \) signifies all other intersects, then \( \text{act}S_{\text{frac}(r)} = \frac{hc}{hc + ha} \) as \( \text{mm}^2 / \text{mm}^2 \) total trabecular surface.

l). Nuclei per osteoclast \( (N_c) \) were determined by counting the average number of nuclei observed in the typical osteoclast on a thin section.

From the twelve basic measurements and counts, the quantitative histomorphometric analysis of trabecular bone consisted of the following
sterological calculations according to Frost (25): (1) Fractional trabecular bone volume: \( V_v = \frac{t V_{\text{frac}}}{t} \) (2) Specific surface of trabecular bone: \( t S_{\text{sp}} = (\Sigma h) \) (n)/(2) \((\Lambda) \) (t); (3) Surface-to-volume ratio \( S/V = \frac{t S_{\text{sp}}}{t V_{\text{frac}}} \), which is the amount of trabecular surface that exists on the typical cubic mm of trabecular bone as \( \text{mm}^2/\text{mm}^3 \); (4) Fractional formation surface: \( S_{\text{frac}}(t) = \frac{h_0}{\Sigma h} \); Appositional rate: \( M_D \) and \( M_Y = (0.73) \) (\( \mu \text{M})/(T) \), where \( T \) is the time interval expressed in days and years, respectively, 0.73 the numerical value of the "orientation correction factor", and \( uM \) the uncorrected mean distance between labels: (5) Fractional labelled surface: \( S_{\text{frac}}(\text{lab}) = \frac{(h_{\text{lab}})}{(h_{\text{lab}}) + (h_{a})} \); (6) Bone formation rate (active surfaces): \( S_{V_f} = (M) \) (S_{\text{frac}}(\text{lab})) ; (7) Bone formation rate (total surface): \( S_{V_f}(\text{BMU}) = (S_{\text{frac}}(\text{lab})) \) (M)/(S_{\text{frac}}(t)) ; (8) Bone formation rate per unit active formation surface: \( S_{V_f}(\text{act}) = M \); (9) Fractional resorption surface: \( S_{\text{frac}}(r) = \frac{(h_r)}{(\Sigma h)} \); (10) Bone resorption rate (active surface): \( S_{V_r} = \frac{S_{V_f}}{S_{\text{frac}}(r)} \); (11) Bone resorption rate (total surface): \( S_{V_r}(\text{BMU}) = \frac{S_{V_f}(\text{BMU})}{S_{\text{frac}}(t)} \) ; (12) Sigma: \( \Theta_f \) (formation) = \( \mu \text{MWT}/M \), and \( \Theta_r \) (resorption) = \( (S_{\text{frac}}(r)) \) (\( \Theta_f \)) (S_{\text{frac}}(t)) ; (13) Bone formation per osteoblast: \( V_f(\text{obl}) = \frac{S_{V_f}(\text{act})}{C_f(\text{act})} \); (14) Bone resorption per osteoclast: \( V_r(\text{ocl}) = \frac{S_{V_r}(\text{act})}{C_r(\text{act})} \); (15) Volume-to-Surface ratio: \( V/S = \frac{t V_{\text{frac}}}{t S_{\text{sp}}} \) ; (16) Resorption/formation surface ratio: \( R/F = \frac{S_{\text{frac}}(r)}{S_{\text{frac}}(t)} \); (17) Fractional active resorptive surface: \( \text{act} S_{\text{frac}}(r) = \frac{h_c}{h_c + h_a} \); (18) Fractional trabecular osteoid bone volume: \( t V_{\text{frac}}(o) = \frac{h_0}{h_0 + h_a} \); (19) Nuclei per osteoclast: \( N_c \); (20) Thickness index of osteoid seams: \( \text{TIO} = \frac{t V_{\text{frac}}(o)}{S_{\text{frac}}(t)} \); (21) Mineralization lag-time: \( \text{M.L.T.} = \text{TIO}/M_D \); (22) Surface-to-volume ratio: \( S/V = \frac{t S_{\text{sp}}}{t V_{\text{frac}}} \); (23) Resorption per osteoclast nucleus: \( V_r(\text{nuc}) = \frac{V_r(\text{ocl})}{N_c} \); (24) Trabecular bone balance: \( t, v, B = (t V_{\text{frac}})2 - (t V_{\text{frac}})1/T_2-T_1 \).
represents duration between first and second bone sample in years. \(25\)

\[
\text{Tracecicular bone balance: } \left(t_{s.B.} = \frac{t_{v.B.}}{1} \right) \times \left( \frac{1}{\left[ \frac{1}{2} t_{sp}^{2} \right] + \left[ t_{sp}^{1} \right]} \right)
\]

**Statistical Analysis**

The Student "t" test was used to statistically evaluate the morphometric, biochemical and metabolic data before and following administration of each of the three hormones. The evaluations on biochemical and metabolic parameters were done on pooled means over the designated intervals of the 6 dogs for each hormone and compared to baseline values.
RESULTS

Bone Histomorphometry

Effects of 1,25-Dihydroxycholecalciferol on Trabecular Bone

The administration of 1,25-(OH)$_2$D$_3$ to dogs increased the fractional resorption surface ($S_{\text{frac}(r)}$) 30% ($P < 0.01$) and the fractional formation surface ($S_{\text{frac}(f)}$) 33% ($P < 0.001$) (Table 4.1). This increase in the formation surface was associated with a 55% increase ($P < 0.001$) in the fractional trabecular osteoid volume ($tV_{\text{frac(o)}}$); however, the number of osteoblasts per mm ($C_{f(\text{act})}$) of osteoid perimeter was increased by only 8% ($P < 0.05$). The amount of new bone made per osteoblast per year ($V_{f(\text{obl})}$) was decreased 16% ($P < 0.05$), while the appositional rate remained unaltered.

Bone formation rate ($S_V$) was depressed 13% from 0.08 ± 0.01 to 0.07 ± 0.03 mm$^3$/mm$^2$/yr ($P < 0.05$) (Table 4.1). The bone resorption rate ($S_V$) was increased 40% by 1,25-(OH)$_2$D$_3$ from 0.05 ± 0.01 to 0.07 ± 0.01 mm$^3$/mm$^2$/yr ($P < 0.05$). 1,25-dihydroxycholecalciferol resulted in a negative bone balance of -0.15 mm$^3$/mm$^2$/yr which correlated with a 12% decrease ($P < 0.01$) in the fractional trabecular bone volume ($tV_{\text{frac}}$). Mineralization lag-time (M.L.T.) was increased by approximately 5 days ($P < 0.001$), the thickness index of osteoid seams (TIO) was increased 36% from 10.66 ± 1.70 to 14.50 ± 0.50 ($P < 0.001$).
The fractional resorptive surface \( (S_{frac(r)}) \), an index of the activation frequency in trabecular bone, was increased by PTH compared to the baseline value. This increase was associated with a 51% increase in the number of osteoclast nuclei \( (N_{c}) \) from 3.00 ± 0.60 to 4.54 ± 0.54 \( (P < 0.01) \) (Table 4.1) but with a 43% decrease \( (P < 0.001) \) in the amount of bone resorbed per osteoclast nucleus per year \( (V_{r(nuc)}) \). However, the amount of bone removed per osteoclast per year remained unaltered after PTH compared to baseline values. Data for the active fractional resorptive surface \( (actS_{frac(r)}) \) suggested a downward trend whereas the ratio of resorptive to formative surfaces \( (R/F) \) tended to increase. The lifespan of resorbing sites was increased 2-fold \( (P < 0.05) \).

Bone formation rate \( (\dot{V}_f) \) increased (25%) from 0.08 ± 0.01 to 0.10 ± 0.02 mm\(^3\)/mm\(^2\)/yr \( (P < 0.001) \) by PTH in adult dogs (Table 4.1). Parathyroid hormone resulted in an overall increase in bone formation rate, which was associated with increased fractional formative surface \( (S_{frac(f)} ) \) and fluorochrome labelled surface, also defined as the mineralization front \( (f(4)) \), which increased 34% and 44%, respectively \( (P < 0.001) \) after PTH administration (Table 4.1). The fractional trabecular osteoid volume \( (\dot{V}_{frac(o)}) \) also increased (69%) from 3.98 ± 0.75 to 5.75 ± 0.48 \( (P < 0.001) \), which suggests a downward trend whereas the ratio of resorptive to formative surfaces \( (R/F) \) tended to increase. The lifespan of resorbing sites was increased 2-fold \( (P < 0.05) \).
positive bone balance \((t, sB)\) of \(+0.003\, \text{mm}^3/\text{mm}^2/\text{yr}\). This positive bone balance was reflected in a 21% increase \((P < 0.01)\) in the surface-to-volume ratio \((S/V)\) and a 50% increase \((P < 0.001)\) in the mean wall thickness \((\mu MWT)\).

**Effects of L-Thyroxine on Trabecular Bone**

Thyroxine resulted in a 60% increase \((P < 0.001)\) in the active fractional resorptive surface \((\text{act}S_{\text{fract(r)}})\) of trabecular bone from the iliac crest of adult dogs (Table 4.1). This was associated with a trend towards an increase in the fractional resorptive surface. The number of nuclei per osteoclast was increased (18%) from \(3.44 \pm 0.61\) to \(4.05 \pm 0.87\) \((P < 0.01)\).

The fractional formative and fluorochrome labelled surfaces were increased 30% \((P < 0.001)\) and 18% \((P < 0.05)\), respectively, above baseline value by thyroxine. Mineralization lag-time (M.L.T.) decreased 36% \((P < 0.001)\). A prolonged osteon formation time \((\Theta_t)\) was detected \((P < 0.001)\); however, other alterations in osteoblasts were not apparent after thyroxine administration.

Bone formation \((\delta V_f)\) and resorption \((\delta V_r)\) rates were increased 13% and 14%, \((P < 0.05)\), respectively, by thyroxine (Table 4.1). The thickness index of osteoid seams \((TIO)\) decreased (35%) from \(11.00 \pm 1.70\) to \(7.16 \pm 3.34\) \((P < 0.001)\). Thyroxine resulted in an overall positive bone balance \((t, sB)\) of \(+0.04\, \text{mm}^3/\text{mm}^2/\text{yr}\) which was reflected in a 13% increase in the fractional trabecular bone volume (from \(25.00 \pm 3.50\) to \(28.16 \pm 4.90\) \((P < 0.05)\). The mean wall thickness \((\mu MWT)\) also increased from \(0.04 \pm 0.01\) to \(0.06 \pm 0.01\) \((P < 0.05)\).
Biochemical Evaluation

Serum calcium and phosphorus, and urinary hydroxyproline excretion are given as pooled means ± the standard error over the designated experimental interval for each group of dogs administered either 1,25-(OH)₂D₃, PTH or T₄ (Table 4.2).

The active metabolite of vitamin D₃ (1,25(OH)₂D₃) elevated serum calcium above mean baseline levels of 8.60 ± 0.16 mg/dl during all three time intervals (Table 4.2). The greatest increase (45% above baseline) occurred during the first two weeks of the experiment. Subsequently, calcium levels remained elevated 38% at experimental weeks 3 to 5 and 21% above baseline values during experimental weeks 6 to 8. 1,25-dihydroxycholecalciferol significantly elevated (38%) serum phosphorus above a mean baseline value of 4.19 ± 0.19 mg/dl (P <0.05) only during the experimental weeks 6 to 8. Urinary hydroxyproline excretion was elevated 16% by 1,25-(OH)₂D₃ during the first two weeks of the study; however, this change in pooled mean values was not significant.

There were no significant alterations in the pooled mean serum calcium and phosphorus, and urinary hydroxyproline excretion following PTH administration during the designated time intervals (Table 4.2). However, urinary hydroxyproline excretion increased 57% over baseline values during the first two weeks of the study but decreased 40% during the third to fifth weeks, and approached baseline values during the last interval.

Following thyroxine administration urinary hydroxyproline excretion increased from 11.13 ± 0.86 to 23.99 ± 1.73 (P <0.01) during experimental weeks
3 to 5. Thyroxine also elevated serum calcium 17% during this interval. Serum phosphorus was elevated following thyroxine administration but these changes were significantly different from baseline values at the last 2 experimental intervals (Table 4.2). During experimental weeks 3 to 5 serum phosphorus was increased 26% above baseline values (P < 0.05) and remained elevated (15%) during the last interval.

Metabolic Evaluation

Body weights and temperatures, heart and respiration rates are given as pooled means ± the standard error over the designated experimental interval for dogs administered T₄ (Table 4.3). The body weight of dogs administered l-thyroxine increased from 7.39 ± 0.40 to 8.17 ± 0.02 (P < 0.05) during experimental weeks 6 to 8 (Table 4.3). Body temperature increased from 101.87 ± 0.21 to 102.45 ± 0.51 (P < 0.05) during experimental weeks 3 to 5 and 6 to 8, respectively. The heart rate was significantly increased above baseline values following l-thyroxine administration during all three time intervals (Table 4.3). The heart rate increased 12% (P < 0.05), 37% (P < 0.001), and 27% (P < 0.001) during experimental weeks 1 to 2, 3 to 5, and 6 to 8, respectively. The respiratory rate was significantly increased 33% and 38% (P < 0.05) above baseline values by l-thyroxine during experimental weeks 3 to 5 and 6 to 8, respectively (Table 4.3).
DISCUSSION

The results of this investigation indicated 1,25-(OH)₂D₃ increased the fractional resorption and formation surfaces of trabecular bone in adult dogs. The active surface resorption rate increased and the active surface formation rate decreased during the course of the study. These changes resulted in a loss of fractional trabecular bone volume and a negative bone balance. The alterations in trabecular bone were associated with an increased serum calcium during all 3 time intervals and an elevation of serum phosphorus during the last interval. The coincidental increase in urinary hydroxyproline excretion and elevation in serum calcium during the first interval suggested 1,25-(OH)₂D₃ increased bone resorption. The elevations of serum calcium and phosphorus during the latter two intervals probably reflected the known effect of 1,25-(OH)₂D₃ on the intestine since hydroxyproline excretion was not changed significantly.

These findings are consistent with Bordier et al. (39) who observed increased intestinal calcium absorption, serum calcium and osteoclastic bone resorption in vitamin D-deficient patients following the administration of 1,25-(OH)₂D₃ (2.5 μg/day). However, Larsson et al. (6) reported an increase in trabecular bone mass of adult rats following 1,25-(OH)₂D₃ administration. Caywood et al. (40) presented data suggesting a sparing effect of 1,25-(OH)₂D₃ on bone loss in the disease osteoporosis of disuse in dogs. Recently, Norrdin et al. (41) reported a greater amount of trabecular bone associated with an increased bone apposition rate in growing rats fed Solanum malacoxylon, a plant
a glycoside of $1,25-(OH)_2D_3$. These discrepancies on the reported effects of $1,25-(OH)_2D_3$ on bone are probably attributed to species, age and dose differences, as well as the duration of the studies and methods used to evaluate changes in bone. Studies utilizing rodents and young dogs evaluated the effect of $1,25-(OH)_2D_3$ primarily on bone modeling and not bone remodeling.

$1,25$-dihydroxycholecalciferol in adult dogs appeared to be a potent stimulator of trabecular bone remodeling. Over a 60 day time period (one-half of sigma in the dog), the active metabolite of vitamin D$_3$ decreased trabecular bone mass by altering the rates of bone resorption and formation in favor of net resorption. Because the study did not last over a complete lifespan of a remodeling unit, it is possible that the bone loss may only be temporary.

The intermittent administration of $1,25-(OH)_2D_3$ to adult dogs also increased the extent and thickness of osteoid seams in trabecular bone. This was detected by an increase in the fractional trabecular osteoid volume and surface, and the thickness index of osteoid seams. The mineralization lag-time was similarly increased. Storey (7) increased bone formation in rats with intermittent large dose levels of the parent compound of vitamin D. Initially, an increase in osteoid was observed that was later mineralized. It is known that alterations in osteoid seam thickness are due to a time-lag between the osteoblastic matrix apposition rate and the mineralization rate (4). The matrix appositional rate and mineralization lag time significantly increased in the experimental dogs. The number of osteoblasts per mm osteoid perimeter also was increased by $1,25-(OH)_2D_3$. These findings suggested that $1,25-(OH)_2D_3$
extended the osteoblastic foci by increasing the number of osteoblasts and resulted in a temporary lag in mineralization.

Parathyroid hormone increased bone remodeling and trabecular surface-to-volume ratio in adult dogs. This resulted in a modest positive bone balance. Reeve et al. (18) reported a similar anabolic effect of human PTH (1-34 fragment) in patients with osteoporosis. Although bone resorption and formation rates were increased on active surfaces, only the fractional active bone forming surface was increased in adult dogs. Urinary hydroxyproline excretion increased during the first interval of the study but approached baseline values at the last interval. The results of our study in adult dogs and the data of Reeve et al. (18) in human patients suggests that low doses of PTH may exert an anabolic effect on trabecular bone in an adult remodeling skeleton by increasing the active formation surfaces.

Parathyroid hormone at the dose and schedule used in experimental dogs increased the trabecular osteoid volume and surface. Alterations were not detected in the thickness index of osteoid seams. The mineralization front (42) increased, whereas the linear appositional rate was decreased after PTH administration. Mineralization lag time was increased only 3 days. Associated with these changes was a decrease in the numbers of osteoblast per mm osteoid perimeter. Similarly, the lifespan of bone forming sites was increased. These findings in adult dogs are consistent with those of Frost (1) who observed a prolonged lifespan of bone forming sites in patients with hyperparathyroidism. Parathyroid hormone appeared to increase the extent of osteoid in trabecular bone of adult dogs without interfering with mineralization.
Thyroxine increased trabecular bone remodeling in adult dogs that was characterized by an increase in the bone resorption and formation rates of active trabecular surfaces. This was associated with an elevation of urinary hydroxyproline excretion and serum calcium and phosphorus during the last five weeks of the study. Thyroxine appeared to enhance bone resorption at the cellular level by increasing the number of osteoclast nuclei after 60 days in adult dogs. These results were consistent with findings reported in experimentally-induced hyperthyroidism in the dog (19) and in naturally occurring hyperthyroidism in human beings (21, 24).

Thyroxine increased bone mass and bone balance in adult dogs over the 60 day period of this study. The dose and schedule of thyroxine used was sufficient to increase the heart and respiratory rates, and elevate the body temperature. The increased bone remodeling following thyroxine administration resulted only in a significant increase in bone forming surfaces. These findings were interpreted to suggest that thyroxine at the tissue level increased bone mass by enhancing the switch-over from the bone resorptive to the formative phase of remodeling.

At the cellular level thyroxine prolonged the lifespan of bone forming sites and increased the mineralization front. Associated with these changes were a decrease in the thickness of osteoid seams and mineralization lag time. Thyroxine appeared to enhance the coupling between osteoid matrix apposition and mineralization by recruiting bone forming sites and prolonging their lifespan.
The present study demonstrated that at the dose and schedule used 1,25-(OH)$_2$D$_3$, PTH, and thyroxine were potent stimulators of bone remodeling in trabecular bone of adult dogs. Over the 60 day experiment 1,25-(OH)$_2$D$_3$ decreased the trabecular bone volume by increasing the bone resorption rate and decreasing the formation rate. 1,25-(OH)$_2$D$_3$ also increased osteoid in trabecular bone of adult dogs by increasing the number of osteoblasts and the mineralization lag time. Parathyroid hormone resulted in a modest positive bone balance and an increase in trabecular osteoid without interfering with mineralization. It enhanced both bone resorption and formation rates but resulted in a significant increase only in the active formation surface. Parathyroid hormone prolonged both osteon formation and resorption times by decreasing the number of osteoblasts and increasing the osteoclast nuclei, respectively. Thyroxine increased trabecular bone volume by prolonging the lifespan of bone forming sites and enhancing the switch-over from bone resorption to formation as well as the coupling between osteoid apposition and mineralization.
SUMMARY

The effects of 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃), parathyroid hormone (PTH), and l-thyroxine on trabecular bone remodeling were evaluated by histomorphometric methods in adult female beagles. 1,25-(OH)₂D₃ (1.25 μg/day in equally divided doses) was administered intravenously and intermittently for 6 days and withdrawn 14 days for 3 complete cycles. PTH was administered intravenously (2.5 U/Kg/day) in equally divided doses 6 hours apart. Thyroxine was given orally (1.0 mg/kg/day) in equally divided doses. Static and dynamic changes were evaluated using tetracycline and DCAF (2,4 BIS) N, N', Di (carboxymethyl) (amino methyl fluorescein) in vivo double labelling of bone specimens taken before treatment and after 60 days.

1,25-dihydroxycholecalciferol, PTH and l-thyroxine each stimulated trabecular bone remodeling in adult dogs. The intermittent administration of 1,25-(OH)₂D₃ decreased the trabecular bone formation rate of active surfaces and decreased bone volume. 1,25-(OH)₂D₃ increased trabecular bone resorption rate of active surfaces, osteoid surface, volume and thickness, mineralization lag-time, and osteoblast number. 1,25-(OH)₂D₃ in adult dogs stimulated bone remodeling in favor of resorption by enhancing the resorption rate and depressing the formation rate.

Parathyroid hormone increased trabecular surface-to-volume ratio, bone resorption and formation rates, active bone forming surfaces, trabecular osteoid
volume and surface, lifespan of bone forming and resorbing sites, and the number of osteoclast nuclei. It decreased the linear appositional rate and number of osteoblasts. Low doses of PTH may exert an anabolic effect on trabecular bone in an adult remodeling skeleton by increasing the active formation surfaces and trabecular osteoid without interfering with mineralization.

Thyroxine increased bone resorption and formation rates of active trabecular surfaces, trabecular volume, bone balance, the number of osteoclast nuclei, lifespan of bone forming sites, and bone forming surfaces. The osteoid seam thickness and mineralization lag-time were decreased. Thyroxine appeared to increase bone mass by enhancing the switch-over from the bone resorptive to the formative phase of remodeling. In addition, coupling between osteoid apposition and mineralization was increased by recruiting more forming sites and prolonging their lifespan. The present study demonstrated that at the dose and schedule used 1,25-(OH)₂D₃, PTH, and thyroxine were potent stimulators of bone remodeling in trabecular bone of adult dogs.
Table 4.1: Histomorphometric evaluation of the effects of 1,25-(OH)$_2$D$_3$, PTH, and T$_4$ on trabecular bone from iliac crest. (mean ± S.E.), N = 6 dogs per hormone.

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<th>1,25-(OH)$_2$D$_3$ Baseline</th>
<th>1,25-(OH)$_2$D$_3$ Post-1,25-(OH)$_2$D$_3$</th>
<th>PTH Baseline</th>
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<td>$t_V^{\text{fract}}$ (%)</td>
<td>26.60 (2.49)</td>
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<td>$t_S^{\text{sp}}$ (mm$^2$/mm$^3$)</td>
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<td>4.10 (0.30)</td>
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<td>4.02 (0.50)</td>
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<td>0.04 (0.01)</td>
<td>0.04 (0.00)</td>
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<td>$S/V$ (mm$^2$/mm$^3$)</td>
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<td>$t_V^{\text{fract(o)}}$ (%)</td>
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<td>10.71 (2.39)</td>
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<td>12.46* (2.70)</td>
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Table 4.1 (Continued)

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<tr>
<td></td>
<td>39.50 (5.94)</td>
<td>52.67***</td>
<td>40.20 (4.47)</td>
<td>53.80***</td>
<td>43.90 (8.88)</td>
<td>61.17***</td>
</tr>
<tr>
<td>$TIO$ (µm)</td>
<td>10.66 (1.70)</td>
<td>14.50***</td>
<td>11.80 (1.70)</td>
<td>11.00 (1.94)</td>
<td>11.00 (1.70)</td>
<td>7.16***</td>
</tr>
<tr>
<td>$C_{f(act)}$ (No. Cells/mm)</td>
<td>11.84 (0.54)</td>
<td>13.78*</td>
<td>11.69 (0.74)</td>
<td>9.19**</td>
<td>11.75 (0.90)</td>
<td>11.68</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_f$ (obl) (µ²/cell/yr)</td>
<td>32.00 (5.00)</td>
<td>26.80*</td>
<td>31.06 (4.90)</td>
<td>31.85 (4.30)</td>
<td>32.04 (4.90)</td>
<td>33.03</td>
</tr>
</tbody>
</table>

**BONE CELL ACTIVITY**

| µM (µm)                         |                  | 17.00 (1.80)     | 18.00 (1.83) | 12.64*** | 17.22 (1.94) | 17.22 |
|                                 |                  | (2.80)           | (0.80)       |       | (4.14)       |       |
| $M_D$ (µ/day)                   | 1.02 (0.01)      | 0.97 (0.04)      | 1.00 (0.14)  | 0.89 (0.05) | 1.05 (0.16)  | 1.02 |
|                                 |                  |                  |     |     |       |       |
| $M_y$ (mm/yr)                   | 0.37 (0.04)      | 0.35 (0.08)      | 0.39 (0.04)  | 0.32**  | 0.38 (0.05)  | 0.38 |
|                                 |                  |                  |     |     |       |       |
| $\Theta_f$ (years)              | 0.11 (0.05)      | 0.11 (0.05)      | 0.10 (0.04)  | 0.19*** | 0.11 (0.05)  | 0.16*** |

*(Standard errors in parentheses)*
Table 4.1 (Continued)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(1,25-(OH)_2D_3) Baseline</th>
<th>(1,25-(OH)_2D_3) Post-</th>
<th>PTH Baseline</th>
<th>PTH Post-PTH</th>
<th>(T_4) Baseline</th>
<th>(T_4) Post-</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BONE RESORPTION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S_{\text{frac}}(r)) (%)</td>
<td>11.50 (1.56)</td>
<td>15.00** (1.93)</td>
<td>12.00 (1.96)</td>
<td>16.75** (1.74)</td>
<td>14.00 (5.08)</td>
<td>16.00 (4.51)</td>
</tr>
<tr>
<td>(\text{actS}_{\text{frac}}(r)) (mm(^2)/mm(^2))</td>
<td>0.70 (0.03)</td>
<td>0.77 (0.06)</td>
<td>0.65 (0.04)</td>
<td>0.47 (0.05)</td>
<td>0.67 (0.06)</td>
<td>0.27*** (0.05)</td>
</tr>
<tr>
<td>(C_{r} \text{ (act)}) (No. Cells/mm)</td>
<td>3.11 (0.58)</td>
<td>3.22 (0.14)</td>
<td>3.30 (0.58)</td>
<td>3.39 (0.30)</td>
<td>3.18 (0.58)</td>
<td>3.23 (0.50)</td>
</tr>
<tr>
<td>(N_{c}) (No.)</td>
<td>3.45 (0.69)</td>
<td>4.00 (0.91)</td>
<td>3.00 (0.60)</td>
<td>4.54** (0.54)</td>
<td>3.44 (0.61)</td>
<td>4.05** (0.87)</td>
</tr>
<tr>
<td>(V_{r} \text{ (nuc)}) (u(^2)/clast/yr.)</td>
<td>5.80 (0.65)</td>
<td>5.00 (0.71)</td>
<td>6.66 (0.39)</td>
<td>4.91*** (0.501)</td>
<td>5.80 (0.81)</td>
<td>4.94 (0.59)</td>
</tr>
<tr>
<td>(V_{r} \text{ (ocl)}) (mm(^2)/clast/yr.)</td>
<td>0.02 (0.01)</td>
<td>0.02 (0.01)</td>
<td>0.02 (0.01)</td>
<td>0.02 (0.01)</td>
<td>0.02 (0.01)</td>
<td>0.02 (0.01)</td>
</tr>
<tr>
<td>R/F</td>
<td>0.30 (0.11)</td>
<td>0.28 (0.06)</td>
<td>0.29 (0.10)</td>
<td>0.34 (0.09)</td>
<td>0.32 (0.09)</td>
<td>0.30 (0.05)</td>
</tr>
<tr>
<td>(\Theta_{r})</td>
<td>0.03 (0.01)</td>
<td>0.03 (0.01)</td>
<td>0.03 (0.00)</td>
<td>0.06* (0.02)</td>
<td>0.04 (0.01)</td>
<td>0.05 (0.01)</td>
</tr>
</tbody>
</table>
Table 4.1 (Continued)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1,25-(OH)(_2)D(_3)</th>
<th>PTH</th>
<th>(T_4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Post-1,25-(OH)(_2)D(_3)</td>
<td>Baseline</td>
</tr>
<tr>
<td>(sV_f) (mm(^3)/mm(^2)/yr)</td>
<td>0.08 (0.01)</td>
<td>0.07* (0.03)</td>
<td>0.08 (0.01)</td>
</tr>
<tr>
<td>(sV_r) (mm(^3)/mm(^2)/yr)</td>
<td>0.05 (0.01)</td>
<td>0.07* (0.01)</td>
<td>0.05 (0.01)</td>
</tr>
<tr>
<td>(sV_f) (BMU) (mm(^3)/mm(^2)/yr)</td>
<td>0.20 (0.03)</td>
<td>0.14** (0.06)</td>
<td>0.21 (0.04)</td>
</tr>
<tr>
<td>(sV_r) (BMU) (mm(^3)/mm(^2)/yr)</td>
<td>0.69 (0.01)</td>
<td>0.54* (0.03)</td>
<td>0.70 (0.01)</td>
</tr>
<tr>
<td>(t), vB (mm(^3)/mm(^3)/yr)</td>
<td>-0.30</td>
<td>+0.02</td>
<td>+0.19</td>
</tr>
<tr>
<td>(t), sB (mm(^3)/mm(^2)/yr)</td>
<td>-0.15</td>
<td>+0.003</td>
<td>+0.04</td>
</tr>
</tbody>
</table>

\* \(P < 0.05\)  \** \(P < 0.01\)  \*** \(P < 0.001\)
Table 4.2  Serum calcium and phosphorus, and urinary hydroxyproline (HOP) in adult dogs following 1,25-dihydroxycholecalciferol, parathyroid hormone, and thyroxine administration for 60 days (values represent pooled means ± S.E. over the designated time interval). N = 6 dogs for each hormone.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Interval (Weeks)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>1-2</td>
<td>3-5</td>
<td>6-8</td>
<td></td>
</tr>
<tr>
<td>Urinary HOP:Creatinine (µg:mg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25-(OH)(_2)D(_3)</td>
<td>7.13 ± 0.64</td>
<td>8.29 ± 1.88</td>
<td>7.54 ± 0.94</td>
<td>7.42 ± 1.39</td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>27.87 ± 1.04</td>
<td>43.85 ± 20.14</td>
<td>16.79 ± 6.20**</td>
<td>29.80 ± 1.42</td>
<td></td>
</tr>
<tr>
<td>T(_4)</td>
<td>11.13 ± 0.86</td>
<td>13.18 ± 1.57</td>
<td>23.99 ± 1.73*</td>
<td>14.90 ± 1.92</td>
<td></td>
</tr>
<tr>
<td>Serum Calcium (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25-(OH)(_2)D(_3)</td>
<td>8.60 ± 0.16</td>
<td>12.46 ± 1.57*</td>
<td>11.90 ± 0.58**</td>
<td>10.42 ± 0.40*</td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>9.17 ± 0.17</td>
<td>9.16 ± 0.27</td>
<td>8.79 ± 0.29</td>
<td>9.38 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>T(_4)</td>
<td>9.60 ± 0.40</td>
<td>8.97 ± 0.21</td>
<td>11.19 ± 0.66</td>
<td>10.21 ± 0.56</td>
<td></td>
</tr>
<tr>
<td>Serum Phosphorus (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25-(OH)(_2)D(_3)</td>
<td>4.19 ± 0.19</td>
<td>5.43 ± 0.40</td>
<td>4.68 ± 0.35</td>
<td>5.78 ± 0.47*</td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>4.32 ± 0.28</td>
<td>4.35 ± 0.07</td>
<td>3.92 ± 0.32*</td>
<td>4.84 ± 0.18*</td>
<td></td>
</tr>
<tr>
<td>T(_4)</td>
<td>5.78 ± 0.28</td>
<td>6.51 ± 0.44</td>
<td>7.31 ± 0.48</td>
<td>6.64 ± 0.11</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05  ** P < 0.01
Table 4.3: Metabolic parameters in adult dogs following l-thyroxine administration for 60 days (values represent pooled means ± standard error over the designated time interval of the experiment). N = 6 dogs.

<table>
<thead>
<tr>
<th>Metabolic Parameters</th>
<th>Experiment Interval (Weeks)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>1-2</td>
<td>3-5</td>
<td>6-8</td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>7.39 ± 0.40</td>
<td>7.46 ± 0.53</td>
<td>7.67 ± 0.29</td>
<td>8.17 ± 0.02*</td>
</tr>
<tr>
<td>Body Temperature (°F)</td>
<td>101.87 ± 0.21</td>
<td>101.84 ± 0.28</td>
<td>102.45 ± 0.20**</td>
<td>102.42 ± 0.51**</td>
</tr>
<tr>
<td>Heart Rate (Beats/Min)</td>
<td>101.66 ± 6.40</td>
<td>113.71 ± 17.47*</td>
<td>139.12 ± 17.19***</td>
<td>120.36 ± 9.64***</td>
</tr>
<tr>
<td>Respiratory Rate (No./Min)</td>
<td>24.50 ± 2.33</td>
<td>25.36 ± 4.61</td>
<td>32.70 ± 5.37***</td>
<td>33.72 ± 2.72***</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001
LIST OF REFERENCES

CHAPTER I


LIST OF REFERENCES

CHAPTER II


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CHAPTER IV


