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The Effects Of Environmental Pollutants On Bone Remodelling

Kenneth David Danylchuk

"THE EFFECTS OF ENVIRONMENTAL POLLUTANTS ON BONE REMODELLING"

by

Kenneth D. Danylchuk, M.Sc.

Department of Pathology

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DEDICATION

TO KATHERINE AND WILLIAM,

MY PARENTS.

"When you cannot measure it, when you cannot express it in numbers, – you have scarcely in your thoughts advanced to the stage of science, whatever the matter may be".

William Thomson, Lord Kelvin
1844 – 1907

ABSTRACT

Using the fluorescent labelling technique bone remodelling rates were determined in beagle dogs treated for long periods of time with low doses of environmental pollutants - cadmium (Cd), lead (Pb), trisodium nitrilotriacetate (Na_3NTA) and zinc (Zn). The objective of this thesis was to determine if the administration of these contaminants over long periods of time - in sufficiently low doses so as not to induce compensatory action of the parathyroid gland, intestine and the kidney - had a first order effect on the rate of cortical bone turnover. Higher doses of the environmental pollutants were not used for fear of inducing hyperparathyroidism secondary to renal failure.

Although the possibility of subtle compensatory mechanisms mediated through the parathyroid gland, intestine and the kidney cannot be ruled out, - it is suggested that considering the post-experimental normal serum biochemistry, normal haematological profile, normal immunoreactive PTH and in the case of the Cd treated dogs normal levels of $1,25\text{-}(OH)_2D_3$ in the post treatment dogs - that the possibility of such mechanisms occurring is unlikely.

Measurement of bone Pb and Zn indicated that these metals were absorbed through the gastrointestinal tract and stored in the bone. Since others have reported that Na_3NTA is stored largely in bone, costly duplication of this procedure was not undertaken. Measurement of bone Cd concentrations confirmed the inability of this tissue to accumulate Cd.

Measurement of bone turnover rates in the Cd - treated dogs indicated a potent dose-related inhibition of bone formation at the cellular, tissue, and organ level. The administration of Pb to dogs resulted in a similar inhibition of bone remodelling although to a

lesser degree. The effect of the Zn treatment showed no such decrease in Haversian remodelling rates, in fact at the cellular level - taking into account the normal fall in apposition rate with age - there was a postponement of such a physiological decline, suggesting that excess Zn in extracellular fluid may enhance bone formation at the cellular level.

Considering the small population of Na_3NTA treated dogs, nevertheless, a downward trend did exist in remodelling rates in these animals.

The significance of depressed cortical bone remodelling rates is dependent upon the consequences of interruption of normal function, that is, firstly to allow foci of microscopical damage to persist for unusually long periods of time which then accumulate and coalesce until symptomatic complications occur, and secondly to allow the "mean skeletal age" of the average osteon to increase and the hypermineralization which follows results in a loss of normal mechanical properties which may render these individuals increasingly susceptible to suffer skeletal complications with time.

These results may be of greater importance in view of the possible effects of the lifetime chronic accumulation of these environmental pollutants from childhood onward.

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TABLE OF CONTENTS

LIST OF FIGURES

DESCRIPTION

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Cd	Cadmium
Ca	Calcium
DCAF	2,4 Bis. N, N^1 DI Carboxy-methyl-amino-methyl fluorescien
ECF	Extracellular Fluid
EDTA	Ethylenediamine Tetraacetic acid
Fe	Iron
iPTH	Immunoreactive Parathyroid Hormone
Pb	Lead
OS	Osteoid Seams
PO_4	Phosphate
RS	Resorption Space
Zn	Zinc
$1,25\text{-}(OH)_2D_3$	1,25 dihydroxycholecalciferol
$25\text{-}(OH)D_3$	25-hydroxycholecalciferol
CT	Calcitonin

Bone Parameters

A_t	Total Area/mm^2
A_c	Cortical Area/mm^2
C/T	Ratio Cortical/Total Area
A_f	Number of Osteoid Seams/mm^2
A_r	Number of Resorption Spaces/mm^2
S_f	Circumference of Osteoid Seams, mm
M	Appositional Rate, microns/day
M_f	Radial Closure Rate, mm/yr
U_f	Activation Frequency, foci/yr
O_f	Osteon Formation Time, yrs
A_r/A_f	Ratio Resorption/Formation
V_f	Bone Formation Rate, $mm^2/mm^2/yr$
W.O.S.	Width of Osteoid Seams, microns
%	Percent of Osteoid Seams Labelled

INTRODUCTION AND PURPOSE OF THIS THESIS

The homeostatic control of levels of ionic calcium in body fluids depends on a number of factors: the gastrointestinal tract, the kidneys, the skeleton, parathyroid hormone, calcitonin and vitamin D.

Adequate levels of ionic calcium in body fluids depend on the availability of calcium in the diet, and the integrity of the gastrointestinal and renal functions which in turn are secondarily influenced by parathyroid hormone and vitamin D. The ultimate economic conservation of calcium is dependent on bone and the action of calcitonin.

When a conflict arises between the maintenance of adequate levels of ionic calcium in body fluids and skeletal integrity, bone is sacrificed to provide the needed calcium.

Bone is endowed with a particular inherent mechanism through which it undergoes this sacrifice. This ranges through making calcium available from the rapidly exchangeable pool to cell mediated mechanisms including osteocytic osteolysis and osteoclastic resorption of bone.

But osteoclastic resorption of bone is the first in a series of steps of normal skeletal events, the object of which is to replace bone on an ongoing basis - or to remodel it - in order that the skeletal mass and structure continually meets the biological requirements of that animal.

The cells which participate in that remodelling of bone are under the influence of parathyroid hormone, calcitonin and vitamin D.

It stands to reason then that noxious stimuli which affect the normal function of the gastrointestinal tract, the kidneys, the parathyroid gland, the calcitonin secreting cells of the thyroid or

vitamin D metabolism will interfere with levels of ionic calcium in tissue fluids and also with the bone remodelling activity (numerous clinical situations attest to all these possibilities). But it also stands to reason that if the cells which are in charge of the normal bone remodelling activity are altered in some way by noxious stimuli, bone may not be able to accomodate its remodelling activity to meet the fluid calcium demands. It may be possible then to hypothesize that abnormalities in bone remodelling activity may occur in situations where fluid calcium levels are maintained within normal limits due to compensatory mechanism of the gastrointestinal tract and kidney, or even that abnormalities in fluid calcium levels may be a manifestation of abnormal bone remodelling activity in the absence of adequate compensation on behalf of the gastrointestinal tract or kidney.

Thus it was decided to carry out investigations to test the hypothesis that abnormalities of bone remodelling activity may be detected before alterations in fluid levels of ionic calcium were noticed.

The medical literature was reviewed in order to find out whether substances have been found to accumulate in bone which reportedly caused no ill effect on bone affected bone remodelling, and whether substances could cause ill effects on bone affected bone remodelling, and whether substances could cause ill effects on bone which could be attributable to bone remodelling without accumulating in bone. The following interesting environmental pollutants were selected.

It has been known for a long time that certain metals affect bone without accumulating selectively in bone as is the case of cadmium (Friberg et al 1973) (Nordberg 1974).

It has also been known that certain heavy metals - such as lead - accumulate selectively in bone (Barry 1975) (Gross et al 1975) yet apparently caused no damage to bone remodelling activity.

More recently it has been discovered that certain environmental pollutants of industrial and domestic waste origin also accumulate selectively in bone without apparent undue effect. This is particularly the case of nitrilotriacetic acid (Budny 1966).

Experimental investigations with other metals have demonstrated an influence in bone formation at ectopic sites and in bone repair. This has been the case of zinc (Calhoun et al 1975).

To date, the relevant literature on these topics summarizes the analytical, biochemical and morphological findings, but there is very little evidence indicative of what influence, if any, that prolonged exposure to these elements may have on bone turnover rates over a period of time at dosages which would cause no otherwise acute untoward systemic reaction and without interference with the gastrointestinal tract or kidney.

The purpose of this thesis was to employ fluorescent labelling techniques to study what effects on cortical bone remodelling activity of dogs could be found after subjecting these experimental animals to very low doses of cadmium, lead, nitrilotriacetic acid and zinc over a prolonged period of time.

LITERATURE REVIEW

CADMIUM REVIEW

In recent years there has been an increasing number of articles in the medical literature concerning human and experimental observations on the possible mechanisms through which chronic cadmium (Cd) exposure may produce abnormalities in bone. The Japanese government has declared that "itai-itai" disease is an osteomalacia caused by chronic Cd poisoning (Tsuchiya 1969b) but there appears to be a controversy as to the real nature of the skeletal lesions (Tsuchiya 1976) (Abe et al 1976). In endemic regions in Japan rice has been shown to contain higher concentrations of zinc (Zn), lead (Pb) and Cd, than in non-endemic regions. Thus the possibility was suggested that all three metals may have had a role in the pathogenesis of this disease.

Friberg et al (1973), Nordberg (1974), Fassett (1975) and Abe et al (1976) have published reviews on the absorption, excretion, organ concentration and binding proteins involved in Cd metabolism in humans and in experimental animals.

HUMAN DATA

Tsuchiya (1969a,b), Murata et al (1970) and Nogawa et al (1975) have published clinical reviews of affected patients in Japan, while Friberg (1950) and Adams et al (1969) have described similar lesions in alkaline battery workers in Scandinavia. The most salient features with respect to bone reported by these authors include; lumbago, sharp bone pain, pain upon palpation of bone, a waddling gait from bone deformation, leg myalgia, rib fractures upon coughing, the presence of a number of Milkman's pseudofractures, scanty callus formation following fractures, radiological osteopenia, and a pecular osteoid

tissue that is markedly hyperplastic in the epiphyseal regions. The skull bones appear to be unaffected. The condition usually progresses rapidly.

Murata et al (1970) and Emmerson (1970) summarized the biochemical outline from patients with "itai-itai" disease as having a normal or somewhat lowered serum Ca, an abnormally low serum Fe, a low serum PO_4 and a markedly elevated alkaline phosphatase. Emmerson (1970) observed that the low serum PO_4 coincided with a normal urinary phosphate excretion.

The renal tubular dysfunction found in chronic Cd poisoning has been observed for a long time (Friberg 1950). Proteinuria was reported to occur when the Cd concentration in the kidney reached 200 ppm (Friberg et al 1973).

Nogawa et al (1975) reported urine Cd levels averaging 9.9 µg/l in "itai-itai" patients and an average level of 1.86 µg/l in age-matched women in a non-polluted area of Japan.

Larsson and Piscator (1971) and Itokawa et al (1973) have reported that the post-menopausal women who had developed "itai-itai" disease had a low intake of both Ca and vitamin D. Nogawa et al (1975) refuted these claims. Kobayashi (1971) described the bones from affected women as being "so soft one could cut them with a knife or scissors".

Murata et al (1970) have described the condition as histologically resembling osteomalacia. The cortical bone is fragile while the cancellous bone was reported to be sparse.

EXPERIMENTAL DATA

RATS

To date, the rat has been the most popular experimental animal

used in investigations of the toxic effects of Cd. The interaction between Cd and Ca in the gastrointestinal tract has been studied by several investigators. Cotzias et al (1961) found that rats absorbed from 0.5% to 8% of orally administered ^{109}Cd after four hours. Friberg et al (1971) reported that 10 to 40% of the airborne Cd is retained in the lung.

Investigating rats that were given 300 ppm Cd in the drinking water Sugawara (1974) found that the calcium binding activity of the duodenal mucosa was decreased to about one-fifth that of a control group. He also found that the Ca binding activity of the renal cortex was decreased to one-half that of the controls.

In in vitro experiments using the duodenum removed from rats fed diets containing 75 ppm Cd for 30 days Sugawara et al (1976) found that the absorption of ^{45}Ca in the duodenum was decreased in the experimental animals. They also found that the ^{45}Ca binding activity of Ca binding protein (CaBP) was decreased. They concluded that since the Cd and Ca dissociation constants for CaBP are similar, the effect of Cd on the activity of CaBP cannot be explained by the greater affinity of this protein for Cd. These same authors found that levels of mucosal ATPase and alkaline phosphatase were decreased in the Cd treated group. These investigators speculated that the decrease of calcium absorption may be the result of a change of cell membrane contractility or of the inhibition of brush border membrane-dependent enzymes by Cd.

After studying uptake and excretion of Ca in rats pretreated with $CdCl_2$ Ando et al (1977) found a significant decrease in Ca absorption which coincided with a greater content of ^{47}Ca in the rat

feces. Compared to the controls, the uptake of ^{47}Ca in bone was decreased by 30% in rats pretreated with $CdCl_2$ for three months.

Hamilton and Smith (1977) have shown in the rat that Cd absorption is biphasic and similar to that of Ca with both a rapid and a time-dependent component. In the rat the time-dependent phase of calcium uptake was inhibited significantly by 0.5 mM Cd, while 10 mM Cd completely inhibited total Ca absorption. These same investigators stated that varying concentrations of Ca failed to influence Cd uptake. They also noted that when Cd remained constant the uptake of Ca could be modified. This suggested to these authors that these two metals entered the mucosa by different means. Nevertheless, they considered that Cd was an extremely potent non-competitive inhibitor of Ca transport. But Ingersoll and Wasserman (1971), also in the rat, had earlier suggested that several divalent cations including Cd were either in competition with ^{45}Ca for the binding site on protein molecules of the mucosa or that they depressed their protein binding by another unknown mechanism. The same authors stated that vitamin D enhanced the absorption of various ions of the alkaline earth series including Cd.

Experimentally induced bone lesions resulting from Cd administration have been reported in the rat by several research groups.

Using rats treated with 25 ppm Cd and a diet containing .04% Ca Larsson and Piscator (1971) detected decreases of inorganic matter in the tibia of 5.7% and 7.5% after one and two months respectively. They concluded that a reduced Ca intake coupled with an exposure to Cd gave rise to a decreased Ca accretion in bone. They claimed that the observed effect of Cd to inhibit new bone mineralization was an indirect one rather than a direct one. As plasma levels of Ca were not

affected by Cd exposure for as long as two months, they deduced that the decreased bone mass was caused by an increased bone resorption which would mobilize skeletal Ca to maintain plasma Ca. They suggested a parathyroid gland-mediated mechanism.

Kobayashi (1971) reported that the losses of mineral from bones of rats that he treated with Cd resulted in osteomalacic changes. Lordotic, kyphotic and scoliotic changes in rats treated with 50 ppm Cd in the drinking water were observed by Itokawa et al (1973). While the Cd concentration increased in bone, the concentration of Ca, Mg, and Zn were found to be decreased. Their histological description included: thinning of the cortex with a decrease in the number of osteocytes and an increase of fat in the femoral spongiosa. In rats fed a Cd diet with low Ca intake they found that 40 to 50% of trabecular bone consisted of osteoid.

Kimura et al (1974) reported that only a small amount (2.8 ppm) of Cd completely inhibited the in vitro 1-hydroxylation reaction of 25-(OH)D_3 by rat kidney tubule, while the in vivo 1-hydroxylation proceeded normally in rats up to a kidney Cd concentration of 40 ppm. They suggested that the toxic effect of Cd on bone was due to the fact that in bone only 20% of Cd is bound to metallothionein while most of the Cd in the kidney is. They proposed that the bone lesions resulted from a direct effect of the Cd ion on bone.

Bawden and Hammarstrom (1975) following the administration of intraperitoneal injections of ^{109}Cd to young rats reported that most of the activity was seen near the epiphyseal plates of bone in areas of osteoblastic activity as well as in the incisor odontoblasts. This suggested to these investigators that Cd could exert a direct effect in retarding deposition of new bone and dentin.

In their experiments with rats receiving from 10 to 300 ppm Cd Yoshiki et al (1975) expressed the opinion that inhibition of bone formation was due to a toxic effect of the metal directly on bone. They emphasized that none of their rats developed pathological changes in the kidneys. They stated that the bone lesions preceeded the kidney changes observed by other investigators and that osteomalacic features were preceeded by osteoporotic ones. This group of investigators cite the negligible accumulation of Cd in bone and state that other researchers have focussed more attention on the accumulation of Cd in the kidney and considered that the bone lesions may have been secondarily produced by renal damage.

Following the administration of 0, 5, 25 and 40 ppm Cd to one hundred rats either ad libitum or by equalized intake Cousins et al (1977) reported that the percentage of ash in the femur was not significantly changed. With this in mind, they then concluded that calcification was proceeding normally in these rats. Although still within the normal range they reported raised levels of alkaline phosphatase. The authors stated that the development of a demineralization defect with Cd excess reported by others may be due to the inhibitory action of Cd on 25-hydroxycholecalciferol-1-hydroxylase.

Kuboki et al (1977) found that the higher the concentration of the metal the greater were the associated qualitative changes in bone collagen crosslinkages following the administration to rats of diets that contained either 0, 30 or 200 ppm Cd.

OTHER SPECIES

Other species have been used to study toxic effects of Cd. Those which are potentially pertinent to bone metabolism and modulation are described below.

Suda et al (1973) reported the complete in vitro inhibition of the synthesis of 1,25-$(OH)_2D_3$ by 0.1 mM Cd in Leghorn chicks. Suda et al (1974) suggest that when Cd is deposited in the chick kidney bound to metallothionein, this storate is 'practically' without effect on vitamin D metabolism.

Also using the chick, Sakai et al (1975) demonstrated that when the tibia from 9 day old chick was cultivated for 5 days in a medium containing 10 ppm Cd as $CdCl_2$, a complete inhibition of the growth of this bone occurred. The histological changes observed were regressive changes in cartilage cells and necrosis of osteoblasts.

Using only radiographs, Nomiyama et al (1975) found no bone lesions in Cd treated rabbits given 300 ppm after 33 weeks.

Recently Abe et al (1976) have stated that of all the animal experiments on Cd there are very few studies in which large animals were used. Similarly, references to canine metabolism of Cd in the literature are scarce.

Loeser and Lorke (1976) observed no evidence of damage either at autopsy or at subsequent histological examination of tissues from male and female beagle dogs given 0, 1, 3, 10, 30 ppm Cd in the form of $CdCl_2$ in the diet. Cd was reported to accumulate in the kidneys and livers of these dogs. It was concluded that the beagle tolerated a concentration of Cd up to 30 ppm in the diet for 3 months without harm.

Thind and Fischer (1975) described that Cd was preferentially retained by the kidneys and livers in mongrel female dogs with 2 mg Cd acetate/kg of body weight administered by intraperitoneal injections once a week for 18 weeks and then once every third week during the next 18 weeks. Zinc concentrations in the kidney, liver and sternum

increased concurrently with the Cd treatment.

Previous reports have demonstrated that Cd added to different lines of cells and bacteria in culture has a number of effects at the cellular level: (i) causing death of cells in a dose-related proportion (Heath et al 1961), (ii) resulting in an accomodation and proliferation of E coli only after an abnormal time-lag (Mitra and Bernstein 1977), (iii) causing single strand breaks in the DNA of E coli which must be repaired prior to cell proliferation through DNA ligase (Mitra and Bernstein 1977), (iv) interferring with keto-acid oxidation by interacting anaerobically with thiol groups of dihydrolipoic acid thus blocking Kreb's cycle (Heath 1961), (v) an inhibition of overall RNA synthesis (Hoffman and Niyogi 1977), (vi) producing mutagenic and carcinogenic alterations (Sirover and Loeb 1976) which have been observed in vivo.

SUMMARY

A prolonged period of human exposure to cadmium results in bone lesions. These lesions have been noticed after the patients presented clinical and laboratory evidence suggesting renal damage. The bone lesions have been qualified as being both osteoporosis and osteomalacia. It has also been suggested that there may be a dietary deficiency in Ca and vitamin D.

In the rat it has been found that exposure to relatively high doses of Cd results in an interference with vitamin D metabolism and intestinal absorption of calcium. Concomitantly it has been recorded that the ash content of the animals' bones is decreased. A structural abnormality of bone collagen has also been reported.

Studies employing the chick have also resulted in the observation of a decrease in bone formation and mineralization following Cd administration. Similar results have been reported in studies in

rabbits.

Studies in dogs exposed to different concentrations of Cd have shown no histological abnormality in bone.

In vitro studies on cell culture systems have demonstrated that Cd produced biochemical abnormalities at the molecular level.

Most of the experimental studies have been short term studies and were done with doses of Cd which interfered with intestinal or renal function.

No previous reports concerning chronic Cd exposure and bone remodelling have been found in the literature.

LEAD REVIEW

In recent years increasing concern has been expressed on the possible effects of Pb in the environment and its possible sources, routes of entry, distribution in the body, effects of acute and chronic intoxication, routes of excretion and possible therapy in humans (Goyer and Rhyne 1973, Barry 1975).

HUMAN DATA

Most of the ingested Pb is eventually concentrated in bone and may remain there for long periods of time (Westerman and Pfitzer 1965, Gross et al 1975, Barry 1975).

Westerman and Pfitzer (1965) have reported that the human intake of Pb may vary between 100 and 2000 µg Pb daily, with the high blood Pb levels being related to high bone Pb concentrations. It was suggested that Pb may interfere with phospholipid metabolism, iron transport, mitochondrial function, ribosomal function, heme synthesis, globin synthesis and the life span of red cells.

Variations in the metabolism of Pb in different animal models can in part be explained by the difference in absorption rates. Scharding and Oehme (1973) reported than man absorbs 10% of ingested Pb while cattle and sheep absorb only 1 to 2% ingested Pb.

According to Rosen and Roginsky (1973) no abnormality was detected in circulating levels of 25-(OH)D_3 in Pb poisoned children who had blood Pb concentrations up to 126 µg/dl. The mechanism postulated for the higher incidence of Pb poisoning in the summer was stated to be due to an increase of Pb absorption secondary to elevated circulating levels of vitamin D_3 due to an increase in sunlight. It was speculated that a major factor pertinent to Pb poisoning may be the competition by Pb and Ca for specific binding sites on proteins in the intestine.

Following Pb determinations on 129 autopsied individuals Barry (1975) concluded that although bone Pb increases with age in both sexes, this increase is greater in males. Bone Pb concentrations varied from 2.16 ppm in a child's rib to over 50 ppm in the petrous bone of elderly adult males. The body burden of Pb for an average male was listed at 164 mg while for the female it was 104 mg. Barry reported that ninety percent of the absorbed Pb was stored in bone. He also stated that the present levels of Pb in the environment "are not considered to be a hazard to the health of the population in general".

Gross et al (1975) analyzing autopsy samples from 46 white males reported increasing concentrations of Pb in cortical bone with age while Pb concentrations in cancellous bone increased, levelled off, and in later years declined with age. The latter phenomena may be related to the decrease of cancellous bone volume which occurs with age (Arnold 1973). Lower Pb concentrations in cancellous bone may in part be explained by the higher turnover activity of this envelope (Rasmussen and Bordier 1974).

Recently it has been reported (Bryce-Smith et al 1977) that the concentration of Pb and Cd in ribs and vertebrae of stillbirths is significantly increased, this notwithstanding the fact that Cd is not usually a bone-seeking metal.

EXPERIMENTAL DATA

RATS

Castellino and Aloj (1964) reported that only 40 to 50% of body Pb is stored in the skeleton. Following the intravenous administration of ^{210}Pb to rats they found that more of the Pb was excreted in the feces (35.7%) than in the urine (15.9%) during the fourteen days of

observation. In blood 96% of the absorbed Pb was cell-bound while only 4% was found in plasma.

In rats receiving only 5 ppm Fe in the diet and 200 µg/ml Pb in drinking water, Mahaffey and Goyer (1972) found that the symptoms of Pb-toxicity as illustrated by increases of Pb in liver, kidney and bone, are enhanced by a dietary deficiency of Fe. The only other parameter salient to bone that was recorded was the weight of the femur and in this experiment no change was observed. Mahaffey (1974) again outlined that diets low in Fe and Ca substantially increased Pb toxicity in rats. In this paper she also stated that the Pb concentration was increased in bone.

Little is known about the status of the parathyroid gland in Pb poisoning. Mahaffey (1974) described a histologically normal parathyroid gland with the normal number of cells per microscopic field in rats which were subjected to a combination diet characterized by an excess Pb, low Fe and low Ca.

Interference with bone cell function related to the use of Pb compounds as in vivo bone markers has recently been suggested by van Mullem and Stadhouders (1974). They detected intranuclear and cytoplasmic inclusion bodies in osteoclasts of rats given not more than a single dose of 4 mg/kg body weight. These inclusion bodies were similar in structure to those described in other organs in Pb poisoning and were characterized by a dense homogenous core with a less dense fibrillar periphery. These authors emphasize that osteoclasts may undergo impairment of function even when this dose of Pb is used and therefore that the use of Pb as a bone marker should be discontinued.

Other factors have been known to alter Pb metabolism. Cerklewski

and Forbes (1976) have demonstrated in rats that as the dietary Zn increases, the severity of Pb toxicity lessens. This is manifested by decrease in blood Pb, liver Pb, kidney Pb and tibial Pb concentrations in these animals. They found a decrease in the excretion of urinary delta-aminolevulinic acid, a decreased accumulation of free erythocyte porphyrins, a decreased inhibition of kidney delta-amino-levulinic acid dehydrase activity and a decrease in the absorption of Pb. The authors hypothesized that either a Pb-Zn complex was formed in the gut which was of low solubility, or that Pb and Zn competed for the same binding sites on a metallothionein-like protein.

Lead binding to the mitochondria of the renal tubular cell and the subsequent damage leading to a decrease in respiration through partial uncoupling of oxidative phosphorylation has been described by Goyer et al (1968). Abnormalities have also been demonstrated in kidney microsomal enzymes (Castellino and Aloj 1969) and (Alvares et al 1972). White (1977) could not rule out the possibility that the abnormalities he observed in microsomal and soluble fractions from kidney were secondary to damage to mitochondria. Similar enzymatic alteration of the Pb poisoned bone cells have not been demonstrated.

DOGS

Zook et al (1972a)has reported a high incidence of Pb poisoning in dogs less than one year old, which parallels the situation occuring in children one to three years of age. Canine Pb poisoning is increased in warmer weather and appears to occur in some breeds more than in others. They suggested that the increase of Pb poisoning found in the poodle may be related to this breed's tendency to "canine hysteria". In the dog, Pb poisoning was characterized by the presence

of nucleated red blood cells and stippled red blood cells in the absence of severe anemia. As in humans, canine blood Pb levels are the best solitary test of Pb toxicity.

Zook et al (1972b) reported that the Pb liver content of control animals was 11 ppm while the Pb liver content of Pb poisoned dogs was 66 ppm as determined by atomic absorption. Similarly, control dogs had a blood Pb level of 18 µg/dl while Pb poisoned dogs had values that averaged 92 µg/dl.

Lloyd et al (1975) reported that after intravenous administration of ^{210}Pb to 10 young beagle dogs, 99% of the absorbed Pb was found in red cells after 1 h. By 28 days the skeleton had 20% of the Pb and by the 1100th and 1497th days the skeleton had acquired 97% and 99% of body burden of Pb respectively.

Regardless of whether histological alterations were evident or not, White (1977) illustrated that beagle dogs less than 1 year old given dosages of 50 and 100 mg/kg/d of Pb carbonate, developed significant changes in enzyme activity in liver and kidney. The abnormality in enzyme function was still present in the dogs two months after cessation of Pb treatment.

RABBITS

After administration of a diet to rabbits containing 0.5% Pb, Hass et al (1964) observed a retardation of new bone formation, an increased bone resorption and a decrease osteoid production. The greatest examples of bone lesions were associated with the most severe renal lesions. The bone lesions were not described as rickets but the authors did relate them to renal dysfunction. The form in which Pb is transported, stored or mobilized in bone was unknown. These authors suggested that the associated hyperphosphaturia and hypophosphatemia

may contribute to the pathogenesis of the osteopathy. The Pb line seen in radiographs correlated to the prompt apposition of osteoid and its subsequent mineralization following cessation of exposure to the Pb.

Hass et al (1967) reported following the administration of a diet containing an excess of vitamin D_3 to rabbits that there was an enhancement of osteoclastic bone resorption, and an increased production of basophilic intercellular matrix in bone which subsequently was converted to eosinophilic osteoid matrix or mature bone, all of which almost completely obliterated the marrow space. In rabbits that received 0.5% Pb in their diets plus the vitamin D_3 supplement the stimulation of bone resorption induced by the vitamin D continued. However, these investigators noted a potent inhibitory action by the dietary Pb on the vitamin D_3 induced intercellular matrix formation. In this group of rabbits, although an enhanced bone resorption occured, the formation of basophilic matrix and its subsequent conversion to eosinophilic osteoid matrix was inhibited.

IN VITRO STUDIES

Rosen and Wexler (1975) have recently stated that the Pb stored bone is readily exchangeable and that this exchange is regulated by PTH, CT and ions in the extracellular fluid (ECF). Their studies were based on rat bone organ culture.

SUMMARY

Although it is now well established that the majority of the Pb ingested by humans is eventually stored in bone, the exact nature of the action of this metal on human bone metabolism remains obscure.

Humans absorb approximately 10% of ingested Pb. In children with

blood Pb levels to up to 126 µg/dl normal circulating levels of 25-(OH)D_3 have been noted. The bone Pb concentration in still births has been described to be significantly increased in one report.

The clinical outline of experimentally Pb poisoned animals resembles that of the human with the most accurate measure of toxicity being the blood Pb levels.

In rabbits given large amounts of Pb in their diet, a decreased intercellular matrix and osteoid production as well as the subsequent mineralization in bone have been found.

In rats Pb toxicity appears to be enhanced by a low Fe intake. Intranuclear inclusion bodies in osteoclasts have been observed in the rat following a single dose administration of Pb. A host of microsomal and mitochondrial enzyme defects have been observed in the Pb intoxicated rat.

In vitro experiments have illustrated that once Pb is stored in bone it is readily exchangeable and is under the influence of PTH, CT, and ions in the extracellular fluid.

To date the only effect of chronic low-dose administration of Pb on the Haversian bone remodelling rates have been described in a pilot study (Anderson and Danylchuk 1977).

NTA REVIEW

Trisodium nitrilotriacetate (Na_3NTA) is a chelating agent which is being used by the detergent industry as a partial replacement for sodium tri-polyphosphate in liquid and granular detergent formulations. Na_3NTA is currently not used in the United States' detergent industry but this chelating agent is used in Canada. NTA is found in the environment as Na, Ca, Fe salts, etc.

HUMAN DATA

Few references on the effect of NTA on human metabolism can be found in the literature.

Budny and Arnold (1973) reported a poor absorption of Na_3NTA in the human based on an experiment in which 8 male subjects ingested 10 mg of ^{14}C-Na_3NTA in gelatin capsules. They recorded that only 12% of the ^{14}C-Na_3NTA appeared in the urine, and tha 87% of the urinary ^{14}C-Na_3NTA appeared during the first 24 hours. They also reported that the greatest concentration of ^{14}C-Na_3NTA in the blood appeared between one and two hours following ingestion.

Extrapolating the results of his experiments in rodents (vide infra) to man, Michael (1971) suggested that there would be no adverse interference by Na_3NTA on the skeleton of people with metabolic bone disease. He postulated that NTA may actually work to the advantage of osteopenic patients by stimulating calcium absorption.

EXPERIMENTAL DATA

RATS

Kidney and bone lesions resulting from the experimental administration of Na_3NTA to the rat are well documented.

Nixon (1971) reported that after administering concentrations

of Na_3NTA ranging from 7,500 to 20,000 ppm to rats for 90 days he observed renal lesions consisting of hydronephrosis and renal tubular cell damage.

Mahaffey and Goyer (1972) administered Na_3NTA in the drinking water to rats at concentrations of 0.01, 0.10, and 1.00% with or without 200 µg/ml of Pb in the drinking water. They found that the animals on the highest doses of Na_3NTA became ill, acquired a vacuolar tubular nephropathy, hyperglycemia and a marked glycosuria. Elevated blood glucose was seen in the other groups. Na_3NTA alone lowered the Pb concentration in the kidney. The mechanism whereby Na_3NTA influences blood glucose is unknown.

Nixon et al (1972) found an increase in the incidence of nephritis and nephrosis in rats fed for two years with Na_3NTA at concentrations of 0.03, 0.15 and 0.50% of the diet. An increase of bone Zn concentration was reported. The authors calculated that he considered a realistic human intake of NTA to be .0025 mg/kg/d, which was considerably below the lowest test level employed by him.

Michael (1971) concluded after administering diets containing three levels of Zn (10, 18, 25 ppm) and four levels of Na_3NTA (0, 0.03, 0.15, and 0.50%) to rats for 91 days that: (i) neither diets had any effect of the fat free weight, ashed content or percent ash of the tibia, (ii) Na_3NTA increased the Zn content of the tibia in all groups (the increase being in direct proportion to the dietary concentration of Zn), (iii) Na_3NTA increased Ca and Zn balances by increasing their absorption, (iv) Na_3NTA does not bind Zn as tenaciously as do Zn containing enzymes, (v) Na_3NTA is deposited in bone the steady state reached being dependent upon the dietary level of Na_3NTA,

(vi) Na_3NTA does not alter the mineral content, breaking strength or shearing stress of bone, (vii) microscopic examination using both mineralized and unmineralized bone sections revealed no changes in osteoid seams or width of epiphyseal plates.

After feeding 2% Na_3NTA in diets to rats Michael and Wakim (1971) also reported increases in Ca and Zn absorption. They stated that Na_3NTA enhanced the growth of the animals, decreased RBC count, hematocrit and hemoglobin. In their report skeletal Zn concentration was shown to be increased. However, they did not find any changes in the weight of the tibia, percent ash of the bones or serum alkaline phosphatase levels. From these results the authors inferred that the metabolic activity of the skeleton was normal. Although Na_3NTA binds readily to Ca and Zn, it was postulated that sufficient quantities of the latter existed in the milieu for normal biological processes to continue unhindered.

DOGS

Budny and Arnold (1973) reported that the beagle absorbs four times more Na_3NTA than the human.

Budny (1972) found that a rapid injection of 40 mg/kg Na_3NTA to beagles resulted in a temporary reduction in blood pressure which lasted 2.5 minutes. He attributed this change to an uncompensated reduction of ionic serum calcium. In determining tissue concentrations of Na_3NTA he found that the highest levels were found in bone.

Budny (1972) reported that following the administration of ^{14}C-Na_2NTA per os, to female beagles, the highest tissue concentrations of Na_2NTA were found in bone and kidney. He also reported that the beagle absorbed four times more Na_2NTA than the human. The effect of Na_2NTA on canine bone metabolism was not discussed.

Budny et al (1973) reported that following the feeding of dogs with diets containing 0.03, 0.15 and 0.50% Na_3NTA there was no change in general appearance, survival time, haematology, serum and urine chemistry, fecal cation concentration and microscopic examination of tissues including bone. Urinary Zn concentration was increased, but no clinical Zn dificiency resulted. After 90 days of treatment the bone Na_3NTA concentration varied between 123 and 142 ppm in the dogs recieving the 0.5% Na_3NTA diet. Using routine histological techniques no difference in the amount of osteoid tissue or width of epiphyseal plate was recorded. Budny (1973) concluded that when Na_3NTA is administered to dogs as 0.5% of the diet, this was not toxic.

SUMMARY

Investigation of the effect of Na_3NTA on human metabolism has to date been restricted to absorption and excretion studies. Na_3NTA is not readily absorbed in the gut. Following the injection of radioactive NTA in the dog, the highest levels of Na_3NTA are found in kidney and bone. Urinary Zn levels increased in dogs following administration of 0.5% NTA in the diet, while no other biochemical measurement was altered. Large amounts of NTA given to rats have produced hydronephrosis, renal tubular cell damage, vacuolar tubular nephropathy, hyperglycemia, glycosuria and nephritis. Abnormalities relative to bone were found when high doses of NTA were given to rats. These included; increase in bone Zn, increase in bone NTA, and an enhanced growth of the animals.

Although there is no experimental evidence relating the results of exposure of Na_3NTA to humans with bone diseases it has been suggested that this chelating agent may affect osteopenic patients advantageously.

The effect of NTA on the bone remodelling process has not been described.

ZINC REVIEW

The biological significance of zinc (Zn)-including the influence that this metal exerts on enzymes and enzymatic function, protein synthesis, carbohydrate metabolism and bone formation has only recently been more fully appreciated. Zinc has been the subject of several recent reviews (Sandstead 1968) (Mikac-Devic 1970) (Spivey Fox 1970) (Spencer et al 1974) (Calhoun et al 1974) (Seeling 1975).

HUMAN DATA

Reports describing human industrial or accidental exposure to high concentrations of Zn compounds are extremely rare (McLord 1926) (Callender and Gentzkow 1937) (Laurence 1958) (Hamdi 1969) (Csata et al 1968) (Greaves and Skillen 1970) (Murphy 1970) (Gallery et al 1972). Symptoms attributed to Zn toxicity drawn from the above reports include: anorexia, epigastric discomfort, abdominal pain, nausea, vomiting, diarrhoea, dehydration and weight loss. Also reported are: mental fatigue, difficulty in concentration, irritability, dizziness, lethargy and muscular incoordination. The clinical biochemical abnormalities reported include elevated plasma and blood Zn levels, increases in serum amylase and lipase and electrolyte imbalance.

No references were found in the medical literature suggesting the possibility that an excess of Zn may have a primary or secondary effect on bone remodelling.

Primary Zn deficiency has been described by Prasad et al (1961) who reported retarded growth and open epiphysis in young boys in Egypt and Iran. Retarded growth, hypogonadism, hypogammaglobulinemia,

and chronic infections were symptoms of a Zn deficient patient reported by Caggiano et al (1969).

A group of children from Denver, Colorado, U.S.A., who exhibited low hair Zn content and lowered taste acuity were reported by Hambridge et al (1972) to have had an inadequate dietary intake of Zn.

According to Calhoun et al (1974), the recommended human intake for Zn has been established at 15 mg/d for adults, 20 mg/d during pregnancy and 25 mg/d during lactation.

Aitken (1976) reported that the decline in Ca found in cortical and cancellous bone commencing in the fourth decade is not accompanied by a similar fall in skeletal Zn. The greater metabolic activity of cancellous bone was cited as the reason why the Ca/Zn ratio was lower in this type of bone when compared to cortical bone.

The concentration of Zn in surface enamel of teeth of individuals of various ages reported by Brudevold et al (1963) ranged from 430 to 2100 ppm. Aitken (1976) found the Zn concentration in human femur samples obtained from an autopsy population of different age groups to be 200 mg/cm^{-3}.

Recently, Alhava et al (1977) determined the bone Zn concentration from the human iliac crest using an X-ray fluorescence analysis method, and found that the Zn concentration was related to age, reaching a maximum peak during the fifth decade. They reported lower bone Zn levels in men with chronic diseases. A significant correlation between the bone Zn concentration and bone strength was found in both men and women.

Briggs et al (1971) reported a marked decrease in plasma Zn in women who were given ethinyl-estradiol either by itself or in

conjunction with progesterone in comparison to normal women. They also found decreased plasma Zn levels in pregnancy. These authors postulated that the decrease in plasma Zn during pregnancy and in normal women taking oral contraceptives reflected either an important qualitative change in plasma proteins or a true Zn depletion. These investigators stated that due to the apparent lack of effect of endogenous estrogens on plasma Zn in non-pregnant women, it would be improbable that an estrogen induced abnormality of Zn metabolism could be regarded as a contributory factor in the pathogenesis of post-menopausal osteopenia.

Aitken et al (1976) also suggested that the fall in plasma Zn concentration with menstranol therapy could be either due to a qualitative change in plasma proteins or to a relative Zn depletion.

A hereditary hyperzincemia was described by Smith et al (1976) in a report of a black American family that presented with plasma Zn levels that averaged 315 $\pm$ 17 µg/100 ml. The normal concentration of Zn in plasma obtained from a reference population was reported to be 81 $\pm$ 13 µg/100 ml. A bone biopsy obtained from one member of this family had a Zn concentration of 191 µg/g while the bone Zn concentrations in 18 control subjects was 140 $\pm$ 25 µg/g. Although the bone Zn was increased it was only slightly so. These same authors reported a person with normal plasma Zn but with a bone Zn concentration of 187 µg/g.

Using sixteen patients who had undergone surgical excision of a chronically draining pilonidal sinus, Pories (1966) reported that wounds healed three to four times faster when Zn was supplemented orally in the diet. The precise mechanism by which Zn stimulated wound

healing was unknown, but an action of Zn on enzymatic and coenzymatic function was suggested by this author.

EXPERIMENTAL DATA

RATS

Following an analysis of retrieved polyvinyl sponges implanted subcutaneously in Zn deficient rats, Fernandez-Madrid et al (1971) reported a decreased Zn content, a decreased total collagen content, a relative increase in soluble collagen and no change in the concentration of non-collagenous proteins when compared to normal rats. These authors concluded that Zn deficiency in the rat is accompanied by defective collagen deposition.

Hegsted (1976) reported that when pregnant rats were fed a Zn deficient diet one to three days prior to parturition a reduction in birth weight of the offspring was noted. When twenty one day old rats were nursed by Zn deficient mothers there was a significant growth retardation and reduced levels of Zn in the tibia of the sucklings.

Bone lesions resulting from Zn toxicity in the rat have been known for a number of years. Sadivisian (1951) observed a reduction of weight and total ash content of bone in the rat following the administration of excessive amounts of Zn in the diet.

Huxley and Leaver (1966) maintained weanling rats on diets containing a normal (47 ppm) and a high (4340 ppm) Zn concentration with either a normal (.56%) or a low (.03%) Ca concentration in the diet. They demonstrated that both a dietary deficiency in Ca and an excess of Zn increased the concentration of Zn in bone and dentine. The high Zn diets depressed the total ash content of bone. The capacity of bone to accumulate Zn has been confirmed.

Ferguson and Leaver (1971) demonstrated in the rat that a low Ca/high Zn diet supplemented with vitamin D produced a marked decrease in the quantity of bone when compared to a low Ca and vitamin D supplement alone. They also reported mineralization defects in conjunction with osteopenia when the vitamin D supplement was removed from the low Ca and high Zn diet.

Calhoun et al (1975) demonstrated that during ectopic bone formation in the rat the bone Zn concentration increased concomitantly with that of Ca. Dietary Zn deficiency in their experiments resulted in a retardation of ectopic bone growth associated with a decrease in the Zn and Ca concentrations in the ectopic bone. They also reported a restored Zn concentration in ectopic bone in the previously Zn deficient animals following Zn repletion in the diet.

Recently Hallmans (1977) described that in the rat Zn is absorbed from excisional wounds treated with adhesive Zn tape and that this absorbed Zn is reflected in an increased concentration of Zn in serum, granulation tissue, pancreatic tissue and bone.

The half-time of ^{65}Zn in the femur, pelvis, and humerus following intravenous injections in male rats were 598, 752 and 941 days respectively according to Taylor (1961).

Bergman et al (1974) detected a 5-fold increase in tibial Zn levels in normal rats on normal diets from 3 to 32 weeks of age. In the younger rats from 12 to 24 weeks they reported a steady increase of bone Zn levels till the latter age when bone Zn concentrations levelled off.

DOGS

Using a histochemical method employing a dithizone solution

Haumont (1961) detected high Zn concentrations in a young adult dog at several sites of mineralization including developing osteons at the junction of mineralized and non-mineralized tissue, in cartilagenous partitions of hypertrophic cells and in endochondral bone freshly deposited in the metaphysis. This dog had not previously been exposed to high levels of Zn in the diet.

OTHER SPECIES

After an intraosseous injection of Zn-berryllium silicate in the rabbit Schneider et al (1973) reported a 9-fold increase in bone formation, as determined by the distance between tetracycline labels. This occurred during the first four weeks post-injection.

Haumont and Vincent (1961) found radioactivity only limited to the mineralization front five days after the administration of ^{65}Zn to young monkeys.

Summarizing data from rats, cats and dogs, Ashling and Hurley (1963) reported bone Zn values ranging from 100 to 300 µg/g of fresh bone.

Walters and Roe (1965) reported that mice given up to 5000 ppm Zn as $ZnSO_4$ in drinking water did not develop any increase in the incidence of tumours at any site.

After giving Zn deficient diets (15 ppm) to male day-old chicks for 4 to 6 weeks O'Dell et al (1958) described slow growth, shortening and thickening of long bones and keratosis of skin. They also reported poor calcification of bone, failure of cartilage cell development in the epiphyseal plate and decreased osteoblastic activity.

More recently, Westmoreland (1971) investigated connective tissue alterations in the zinc deficient chicken. Near blood vessels,

normal appearing chondrocytes were described but cellular changes were observed at a distance from the blood vessels. The latter consisted of differences in shape, the cells being surrounded by more extracellular matrix, and the cells did not stain normally for alkaline phosphatase. He concluded that zinc deficiency may affect cell maturation and that degenerative changes in these cells could ultimately affect calcification.

Battislone et al (1972) found histological features of accelerated bone healing following intraperitoneal injections of zinc cysteamine-N-acetic acid and zinc sulfate to previously experimentally bone injured guinea pigs. Acceleration of healing was correlated to increased levels of zinc in the repairing tissues.

IN VITRO

Samachson et al (1967) using bovine tissue showed that the in vitro uptake of ^{65}Zn by demineralized bone was greater than that of bone powder, and that the uptake by both components was considerably reduced by ethylenediamine-tetraacetic acid (EDTA). They postulated that the "presence of natural chelating agents binding serum Zn" explains the relative low concentration of Zn in bone despite a considerable preference of demineralized bone for ^{65}Zn over ^{47}Ca.

An inhibitory effect of various metals, including Zn, on the mineralization of rachitic cartilage matrix and on apatite crystal formation was demonstrated by Bird and Thomas (1963). The source of the inoculated cartilage was not mentioned.

Enzymatic abnormalities associated with excess Zn have been reported. Hove et al (1940) found that the activity of crude bone alkaline phosphatase could be progressively inhibited by increasing the concentration of Zn ion, while there was a small effect on kidney

alkaline phosphatase and no effect on intestinal alkaline phosphatase activity.

SUMMARY

Although human Zn deficiency has produced lesions in bone, the few reports of human Zn toxicity do not mention the mechanism of production of these lesions. Hyperzincemia has been reported as a heritable anomaly. Although Zn levels in bone are increased in this family, no bone lesions due to Zn have been reported. In the human being circulating levels appear to be influenced either directly or indirectly by estrogens. Improved wound healing has also been reported following treatment with Zn.

Exogenous estrogens have been reported to decrease bone Zn concentration.

Zn deficiency in the rat has produced bone abnormalities. Zn toxicity is associated with a decrease in the weight and total ash content of bone. Zn is required for normal formation of ectopic bone.

Zinc is necessary for the normal functioning of a wide variety of metalloenzymes but enzymatic abnormalities have been reported to occur in vitro when excessive Zn is used.

No report concerning chronic Zn exposure and Haversian bone remodelling has been found in the literature.

MATERIALS AND METHODS

THE FLUORESCENT MARKERS

The study of variations in bone remodelling parameters involves the labelling of bone with fluorescent markers which deposit at the appositional site of new bone formation along with the mineralization of the osteoid seam. There are numerous elements which could serve this purpose but among the cheapest and less toxic, allowing for the reliable microscopical visualization of crisp markers are the tetracyclines, and 2,4 Bis. N, N^1 Di Carboxy-methyl-amino-methyl fluorescien (DCAF). In this study the following fluorescent bone markers were used: (1) Tetracycline Hydrochloride (Achromycin, Lederle, Montreal, Quebec, Canada) which gives a yellow fluorescence when viewed with violet light at a wave-length of 405 nm. (2) Oxytetracycline (Terramycin, Pfizer Co. Ltd., Montreal, Quebec, Canada) which gives a green-yellow fluorescence when viewed with violet light at wave-length of 405 nm and gives an orange fluorescence when viewed with blue light at a wave-length of 490 nm. (3) DCAF (I.C.N. Pharmaceuticals, Inc., Cleveland, Ohio, U.S.A.) which gives a green fluorescence when viewed with blue light at a wave-length of 490 nm.

These fluorescent markers may be administered by mouth or may be injected intramuscularly, intravenously or intraperitoneally. In order to avoid interference with the intestinal flora, and thus running the risk of secondarily interfering with intestinal absorption none of the labels were given to the experimental animals by mouth. Because of the slight possibility of a systemic reaction following the intravenous injection of tetracyclines and DCAF, this route of administration was not attempted. In adequate solvents, both tetracyclines and DCAF may be injected intramuscularly with no observable deletereous side-effects

and they are adequately absorbed.

THE METHOD OF FLUORESCENT LABELLING (see Appendix to Methods I and II)

The sequence of fluorescent labelling of bone of dogs used in this thesis is outlined as follows:

(1) All dogs were held in appropriate cages, without external disturbances of any nature, for a period of two months after their arrival in the Animal Quarters of the Health Sciences Building of the University of Western Ontario. This two month period of rest was to conform to standard practice in methods investigating alterations of bone remodelling rates. As the remodelling activity may have been disturbed during transport (i.e. removal from previous environment, placing the dogs in smaller 'air freight' cages, handling and exposure to unknown individuals, foods, noises, etc.) it is necessary for a period equivalent to one sigma of bone remodelling activity to pass in order that one is assured that the measurements being carried out are on a steady state (Frost 1969).

(2) The first fluorescent label employed was tetracycline hydrochloride. This was administered in two separate intramuscular injections spaced fourteen days apart from each other. The dose of each injection was 50 mg/kg body weight dissolved in a sterile normal saline solution. This constituted the control fluorescent label.

(3) After the appropriate rib biopsy was taken, the dogs were exposed to the different substances under investigation for a period of at least two sigmas. The ages of the individual dogs at the time of biopsy is given in the description of the method for each substance employed.

(4) The second fluorescent label employed following the period

of exposure of the animals to the pollutant was DCAF. This fluorescent marker was prepared by diluting 120 mg with 5 ml of sterile 2% solution of dextrose and mixed with sodium bicarbonate. The DCAF was injected intramuscularly in injections spaced fourteen days from each other at a dose of 25 mg/kg body weight of the animal. This constituted the first experimental fluorescent label.

(5) After the second rib biopsy was taken, and where a further experimental exposure was carried out, the dogs were subjected to that extra exposure for a period of at least two sigmas. The ages at which the dogs underwent a second and third rib biopsy is stated in the individual methods.

(6) The third fluorescent marker to be used was oxytetracycline. This was injected into the dogs in two intramuscular injections spaced fourteen days between each injection. The dose employed was 20 mg/kg of body weight of the animal. This constituted the second fluorescent label. The second experimental rib biopsy was obtained five days after the last injection.

THE EXPERIMENTAL ANIMAL

Studies on bone turnover rates employing fluorescent markers have been carried out on various species of laboratory animals (mouse, rat, rabbit, dog, pig, monkey, etc). However, it has been stated that if experimental results obtained in laboratory animals are to be extrapolated to man, then a laboratory animal with a similar remodelling pattern to that of man should be employed (Frost 1973). Human bone remodelling activity, as has been stated by Frost (1969) takes place on the periosteum, Haversian system and endosteal areas (both cortical and cancellous components). The rat and mouse model

predominantly on the periosteal and endosteal surfaces (Frost 1973). The young rabbit remodels on the periosteal and endosteal surfaces but as it gets older it presents some degree of Haversian remodelling yet not in a sufficient quantity that it can be properly measured (Frost 1973). The dog is the smallest laboratory animal that presents an adequately measureable amount of periosteal, Haversian, cortical endosteal and cancellous endosteal remodelling activity (Frost 1973).

Although studies on bone turnover rates employing fluorescent markers can be applied successfully in the adult dog to all of the remodelling surfaces, measurements of Haversian bone turnover presents a distinctive advantage over the others. Haversian bone - in the appropriate species - turns over with a characteristic regularity which is very stable under normal conditions for each site although each site does present a variation with age. It is easier to obtain an adequate cross-section of Haversian bone as the majority of Haversian units in long bones are disposed along the long axis. The units of Haversian systems are easily quantitated per cross-sectional surface area and the features of physiological bone remodelling are easily recognizable at the histological level (Frost 1963).

Endosteal (cancellous) bone on the other hand is much more subject to variation in the same species, within the same site, as also with age. Calculations of distances between fluorescent markers is much more difficult due to the invariable obliquity of tissue section, and much more volume of cancellous bone than Haversian bone is required in order that one may state that the measurements are representative of the real state of the cancellous bone (Parfitt 1976).

As in the human, in experimental animals bone turnover rates vary

with age (Frost 1969) (Schock et al 1972). In the human being it is an accepted fact that remodelling activity is high during the early years of life and falls rapidly till the epiphyseal plates are closed. From then on, there is a more gradual age-related decline in bone remodelling activity. The course of these events in the dog is unknown.

Because of the nature of the substances being tested in this thesis (environmental pollutants) an experimental animal had to be chosen which would have been kept as relatively free as possible from the same environmental contaminants, and which at the same time would have been sufficiently controlled clinically and biochemically in such a manner that values for normality would be well established.

For all of the above reasons it was decided to use the skeletally mature purebred beagle as an experimental animal. All dogs were purchased from Laboratory Research Enterprises, Kalamazoo, Michigan, U.S.A. In an attempt to obviate possible chance variations in bone turnover rates attributable to individual genetic constitution decided to use male littermates in each experiment. It was also decided to study variations in bone remodelling rates on Haversian bone rather than cancellous bone. Where histological features of the latter show change which merit mentioning, this will be stated in the thesis. The rib was chosen as the long bone which contains sufficient quantity of Haversian units per cross sectional area of bone, and because it is the bone which is apparently least subjected to external anomalous mechanical stimuli, the muscles of respiration being these which on the whole are mainly responsible for mechanical impulses on that bone (Landeros and Frost 1966).

EXPERIMENTAL ANIMAL CARE AND CONTROL

All dogs were caged in one separate room in individual cages. The cages were separated into groups according to the substance investigated in order to eliminate contamination as much as possible. The dogs were routinely immunized and deparasitized according to standard practice. They were all fed stock chow (Purina Dog Chow), the diet being supplemented with a 13 ounce can of dog meat (Romar 90) per week.

At monthly intervals during their stay in the animal quarters, each dog was subjected to an S.M.A. biochemical profile (serum sodium, potassium, chloride, carbon dioxide, blood urea nitrogen, creatinine, total serum proteins, albumin, calcium, phosphate, glucose, uric acid, total bilirubin) as well as serum alkaline phosphatase determination. These tests were performed in the Clinical Biochemistry Department of the University Hospital. At the same intervals samples of whole blood were secured for haematological investigations. These were: red cell count, white cell count, haemoglobin and hematocrit. These determinations were performed on a Coulter Counter. The mean corpuscular volume, mean corpuscular haemoglobin, and the mean corpuscular haemoglobin concentration was subsequently calculated by the machine. The white cell differential count was performed by manual techniques. Urine and feces were routinely examined by the laboratory staff of the animal quarters as part of their function of general animal care.

In certain instances, blood samples were obtained for other supplementary investigations, such as metal concentration, the measurement of blood levels of canine immuno-reactive parathyroid hormone (iPTH) by the method of Hruska et al (1975) and blood levels of

1,25-$(OH)_2$ cholecalciferol according to the method of Eisman et al (1976). The methods employed for other random or unusual supplementary investigations will be quoted as the need arises.

Surgical Procedures

All surgical procedures were performed under aseptic conditions in the operating theatre of the animal quarters.

Five days following the second injection of a fluorescent time marker and under general anaesthetic using Nembutal (Abbott Laboratories, Montreal, Quebec, Canada) at a dosage of 1 ml/2.3 kg body weight, a 3 cm long fragment of the mid portion of the appropriate rib was removed. The anaesthetic was administered at a constant rate through the medial cubital vein. The hair within a 5 cm x 7 cm area overlying the operative site was removed with clippers. Skin antiseptic was applied to the prepared area. A drape was then placed over the animal which adequately exposed only the operative site.

An incision directly over the segment of rib to be removed was made which pierced the dermis and exposed the layer of fat. Hemostasis was adequately controlled. Blunt dissection through the fat layer and sharp dissection through the muscle layer resulted in exposure of the periosteum. Using a #15 scalpel blade a longitudinal incision was made along the length of the rib. The periosteum was separated from 4 cm of bone by using an Adson periosteal elevator (Zimmer, Brarpton, Ontario, Canada). The blunt end of a Gigli saw (Zimmer, Brampton, Ontario, Canada) was inserted between the periosteum and the bone cortex and two cuts were then made freeing a 3 cm fragment of rib.

In closing the wound four layers were sutured separately. The free edges of the periosteum were united using 4-0 catgut and a

running suture. Using 3-0 catgut with running sutures the free edges of the muscular and fat layers were carefully approximated separately.

The skin was closed with interrupted sutures approximately 4 mm apart using 2-0 silk. Following closure the surgical wound was washed with antiseptic solution.

PRELIMINARY DETERMINATIONS

Notwithstanding the fact that an apparently suitable animal had been chosen on which the experiments were to be carried out, for the purposes of the validity of data to be presented in a thesis like this, a number of questions had to be answered before this animal was exposed to the environmental agents.

In the first place it was necessary to know what the 'natural' age related decline of bone remodelling activity was in this animal. In order to find this out a normal curve had to be produced for all the bone remodelling parameters which were to be measured in the dog. This constituted preliminary determination 'A'.

In the second place it was necessary to find out if there was a 'natural' difference in the remodelling activity at different sites along the same rib. This appeared to be advisable since Amprino and Marotti (1969) found that bone remodelling varied from site to site on the fifth rib of the beagle dog. They reported that activity was minimal at the junction between the posterior and lateral thirds of the rib, and that this difference in remodelling activity along the rib disappeared when the animal was three years old. It was decided to sample the ribs at the mid posterior, mid lateral and mid anterior thirds (Fig. 11) and to find out whether there was a 'natural' statistically significant difference in bone remodelling activity between any of these sites. This constituted preliminary determination

'B'.

Thirdly, because it was necessary to carry out more than one biopsy of rib on the same side of the animal and also because Frost (1969) has remarked on the inadvisability of performing a biopsy on the same rib more than once, it was necessary to know if there were 'natural' statistically significant differences in bone remodelling activity between similar sites on alternate ribs of the same side of the animal. Alternate rather than contiguous ribs were chosen for biopsy sites because of the possible alteration produced on the contiguous rib by interruption of the fixation of the intercostal musculature and possible interference with the blood supply to that muscle distally during the previous surgical intervention. It was decided to compare the remodelling activities of the mid posterior, mid lateral and mid anterior portions of the eleventh, ninth, seventh, and fifth ribs ipsilaterally. This constituted preliminary determination 'C'.

In the fourth instance, it is also known that the cycle of remodelling of a Haversian unit is not uniform inasmuch as during the initial stages of bone formation (i.e. when the cavity to be replaced is larger) the bone formation rate is greater than in the later stages of development (i.e. when the osteon nears completion) (Lee 1964). It was then necessary to know the correlation coefficient 'r' of the relationship between the circumference of the outer fluorescent label (mm) and the distance between the fluorescent labels (μ) in order that the assessment of bone formation rate was gathered from situations proceeding at as uniform a speed as possible. This constituted preliminary determination 'D'.

PREPARATION OF MINERALIZED SECTIONS

Immediately following removal, the three cm fragment of rib was fixed in 70% ethyl alcohol and water. Ten sections, cut perpendicular to the long axis of the rib, each 200 µm thick, were obtained using a Gillings Thin Sectioning Machine (Hamco Machine Inc., Rochester, N.Y., U.S.A.). The sections were then stained for 48 h in Villanueva's osteochrome stain (Villanueva 1974) which was followed by hand grinding the bone with grade 400 sand paper, under running water to a thickness of 70 to 100 µm. The sections were then washed thoroughly, differentiated in 95% methyl alcohol, dehydrated with increasing concentrations of ethanol, cleared in xylol and mounted with Permount histological mounting medium (Fisher Scientific Company, Fair Lawn, N.J., U.S.A.) on glass slides. The sections were then stored in darkness until used.

Determinations of each parameter of Haversian bone remodelling, as described by Frost (1969) were carried out on each of the 10 sections of rib.

MEASUREMENTS IN HAVERSIAN BONE REMODELLING

The structures analysed when measuring rates of Haversian bone remodelling, as seen with the light microscope, are the osteoid seam, the resorption space, the 'waltzer', and the completed osteon. A typical representation of these structures is shown in Fig. (1). Other measurements (i.e. distance between labels) require the use of a fluorescent microscope. The same microscopic field as viewed with a light microscope in Fig. (1) is shown in Fig. (2) under fluorescent light.

Described below are the Haversian bone remodelling parameters, their numerical derivation as well as the number of measurements that were made on each and a description of those measurements for

FIGURE 1 A photomicrograph of a mineralized hand ground section showing the typical appearance of a portion of rib cortex. (Villanueva Osteochrome stain) 80X

FIGURE 2 A fluorescent photomicrograph of the same area as seen in FIGURE 1. Single and double tetracycline labels can be seen surrounding the osteoid seams. 80X

FIGURE 3 A representation of the Zeiss Intergrationsplatte III grid superimposed over a mineralized section of rib as seen through a low power objective of the microscope when the total cross-section area (A_t) and the cortical cross-section area (A_c) were measured.

FIGURE 4 A photomicrograph of one osteoid seam and a portion of another as visualized following staining with Villanueva Osteochrome stain. 300X

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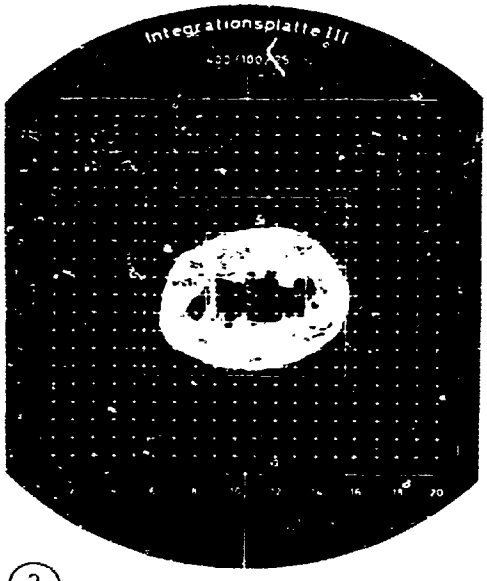

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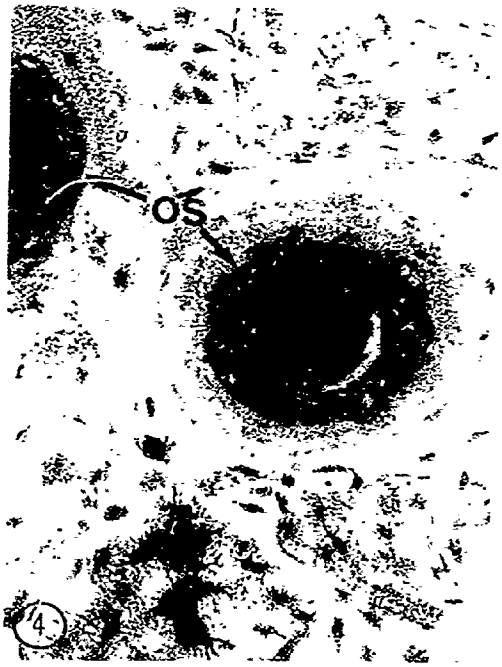

4

each of the parameters.

The cortical cross sectional area (A_c) was measured by the point-count technique, using a Zeiss 1x objective and by superimposing a calibrated square ruled grid (Zeiss Integrationsplatte III) over the histological section and by then counting the number of intersections which occur over cortical bone (Fig. 3). This procedure was carried out using a Leitz Ortholux microscope. In this thesis the mean cortical cross sectional area A_c was determined from five such counts on each mineralized bone section examined.

The total area (A_t) was measured by a similar technique in which the intersections which occurred over cortical bone, cancellous bone and medullary spaces are counted. The mean total area (A_t) was determined from five such counts on each mineralized section.

The cortical to total area ratio (C/T) is the ratio between the mean cortical area (A_c) and the mean total area (A_t).

The number of osteoid seams per mm^2 of cortical bone (A_f) was calculated by counting the total number of osteoid seams located within secondary Haversian systems and by then dividing by the cortical cross section area (A_c). The osteoid seams were identified using the Villanueva osteochrome stain (Villanueva 1974) as homogeneous green, blue or red structures lining the intima of Haversian canals (Fig. 4).

The number of resorption spaces per mm^2 of cortical bone (A_r) was determined by counting the number of regions which were void of bone, located between the periosteal and endosteal envelopes and which exhibited the scalloped borders characteristic of Howship's lucunae (Fig. 5), and then by dividing by the cortical cross sectional area (A_c). Due to the thickness of the sections, cell detail is

FIGURE 5 This photomicrograph shows a typical resorption cavity, Scalloped borders of Howship's lucanae line the majority of the perimeter of this cavity. 510X

FIGURE 6 A photomicrograph of a typical "Waltzer". These structures elicit both an osteoid seam and scalloped borders characteristic of a formation zone and a resorption space respectively. 400X

FIGURE 7 A representation of the Zeiss Intergrationsplatte II grid superimposed over an osteoid seam as seen through a high power objective when the circumference of osteoid seams were determined. The "H's" are located at the intersection of the grid line and the intima of the osteoid seam, each of these junctions is counted as one hit. 610X

FIGURE 8 This fluorescent photomicrograph represents a typical double labelled osteon used in determining the mean appositional rate. The distance between each of the four pairs of arrows was measured. (from the outer edge of the outer fluorescent label to the outer edge of the inner fluorescent label) 430X

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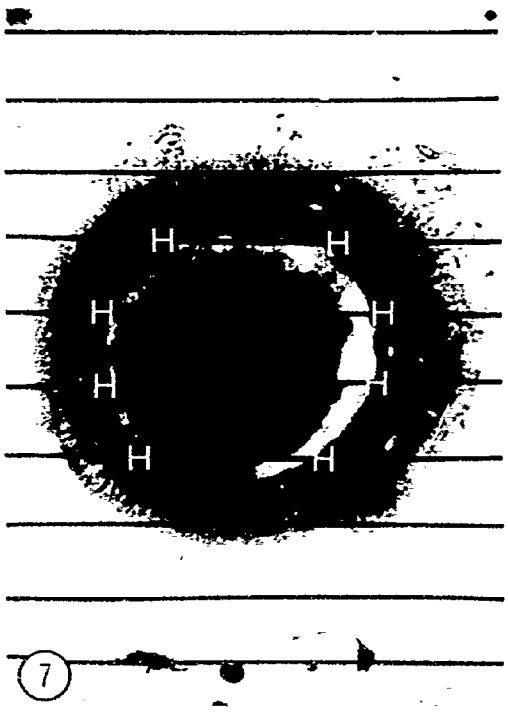

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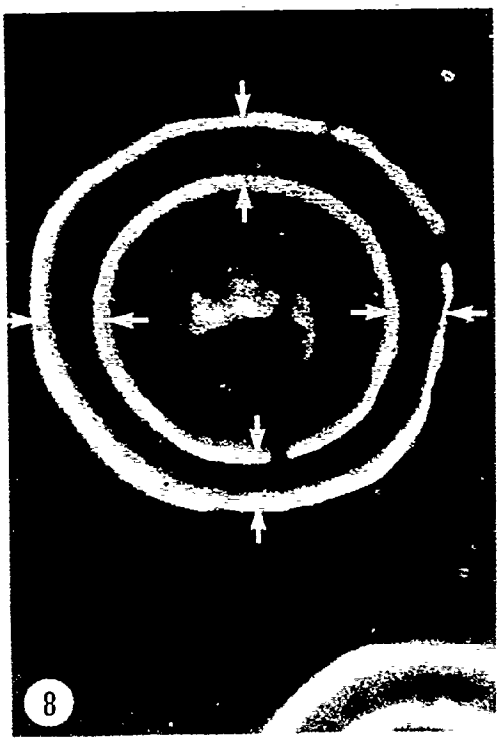


8

obscure, and although on occasion recognition of osteoclasts is feasible, their presence is not mandatory for a resorption space to be counted as such. Areas which exhibit both formative and resorptive phases are commonly seen. These areas designated as 'waltzers' (Fig. 6) (Villanueva, personal communication) allotted one credit to both the osteoid seam and the resorption space counts.

The circumference of the osteoid seams (S_f) was measured in mm by placing calibrated grid (Fig. 7) (Zeiss Integrationsplatte II) directly over osteoid seams that were chosen at random. This measurement was made using a 40x objective. The circumference of the osteoid seams (S_f) was determined by using the formula $S_f = \frac{n}{2} \times \frac{H}{T} \times a$, where H is the number of hits along the intima of the osteoid seam, T is the number of throws, and 'a' is the distance between the parallel lines in μ on the grid. The mean circumference of osteoid seams from each mineralized section was based on a minimum of twenty throws on seams chosen at random.

The mean appositional rate (M) is the average thickness of new bone produced per day in a typical bone forming centre and is expressed in μ/d. The appositional rate was determined in Haversian systems having two distinct labels and not less than half completed. The appositional rate was determined by measuring the distance between the two fluorescent labels at four equidistant radii around their circumference (Fig. 8) and by dividing by the number of days between the administration of the labels. Distances were measured with a linear calibrated grid located in the ocular of the microscope.

The mean appositional rate from each mineralized section was determined as the mean of 4 measurements from a minimum of 10 Haversian

systems bearing clear separate markers and which were less than half completed.

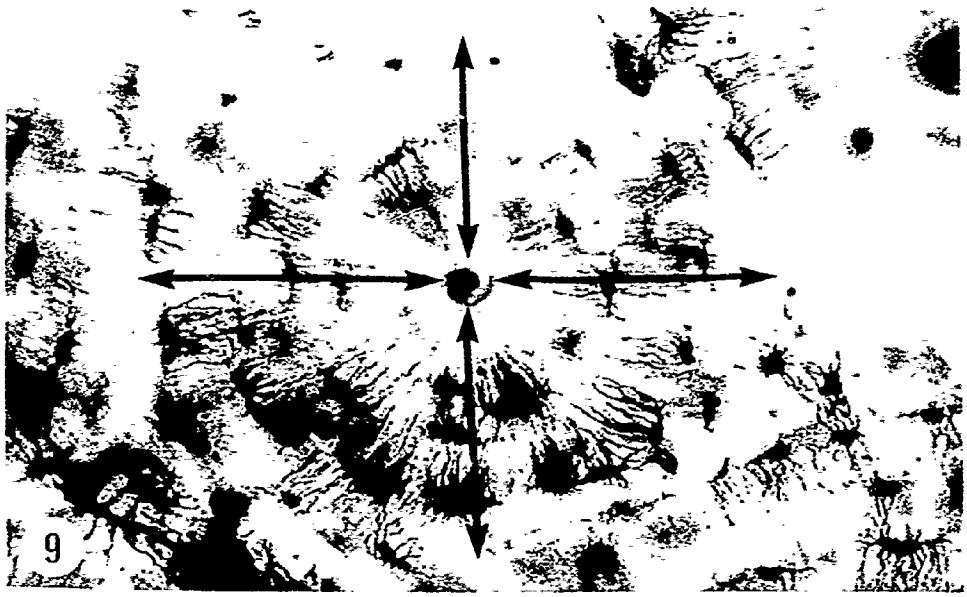
Not all osteoid seams are associated with fluorescent labels. In older individuals the mineralization process is thought to be intermittent. In any individual osteon the laying down of osteoid and its subsequent mineralization may occur after the last time marker has been administered. Therefore, in order to determine the appositional rate averaged over the total osteoid surface, the radial closure rate (M_f) which estimates bone formation at the level of the osteoblast (Frost 1969) is used. The radial closure rate is determined by using the formula $M_f = M \times \% \times \frac{365}{1000}$, where M is the mean apposition rate and '%' is the percent of osteoid seams taking a fluorescent label (see below). Multiplying by $\frac{365}{1000}$, converts μ/d into mm/y.

The wall thickness of completed osteons (WT) is determined by calculating the mean of four equidistant measurements from the edge of the Haversian canal to the point of intersection with the cement line (Fig. 9). A minimum of 20 completed Haversian systems without evidence of an osteoid seam were counted on each mineralized section, from which the mean wall thickness was calculated. Although it was previously thought that Haversian systems in a given bone were approximately the same size, Jaworski (1973) has recently stated that Haversian systems and their canals tend to increase in size towards the endosteal surface of the bone cortex.

The remodelling of bone occurs in discrete packets termed by Frost (1963) Basic Multicellular Units (BMU). The interval of time between activation of a BMU and its completion which includes both the resorptive and formative phases - has been termed 'sigma' by Frost

FIGURE 9 This photomicrograph illustrates the manner in which the wall thickness of completed osteons was calculated. For each osteon four equidistant measurements were made from the edge of the Haversian canal to the cement line at the periphery of that osteon. 710X

FIGURE 10 By measuring the distance between the four pairs of double arrows as is shown in the photomicrograph the width of the osteoid seam can be calculated. 745X



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(1969). Although inter-bone and intra-bone sigma values may differ, Frost (1973) has recently stated that this interval of time remains reasonably constant in each individual. Sigma, as defined by Frost (1973), is the minimal time following the onset of an experimental challenge, that is necessary for a new steady state to be reached which is representative of that new challenge. The sigma formation (σ_f) or the osteon formation time is that time period necessary to form (replace) the amount of bone removed during the resorptive phase of sigma. The osteon formation time was determined in each section by dividing the mean wall thickness (WT) by the mean appositional rate (M). It is acknowledged that since the upper limit of the appositional rate was determined and that the 'true' appositional rate varies between this maximum and zero, the estimations for osteon formation time (σ_f) are low.

The activation frequency (U_f) represents the birthrate of new bone formation centres per year per mm^2 bone. It is considered an index of mesenchymal cell activation. Activation frequency (U_f) was calculated by dividing the number of osteoid seams per mm^2 (A_f) by the osteon formation time (σ_f).

The ratio of resorption to formation (A_r/A_f) is the relationship between the number of resorption spaces per mm^2 of bone (A_r) and the number of formation centres, represented by the number of osteoid seams per mm^2 of bone (A_f). Although each resorption time interval (σ_r) is followed subsequently by the time required for bone formation (σ_f), the time required by the osteoclasts to resorb a quantum of bone is different from the time required by the osteoblasts to replace it.

The bone formation rate (V_f) represents the fraction of a volume

of bone cortex which is replaced each year by new bone. It is derived from existing determinations by the formula $V_f = A_f \times S_f \times M_f$, where A_f is the number of osteoid seams per mm^2 of bone cortex, S_f is the circumference of osteoid seams in mm, and M_f is the radial closure rate in mm/y.

The mean width of osteoid seams (W.O.S.) was determined by taking the average of four equidistant measurements through the thickness of the osteoid band as seen with the Villanueva osteochrome stain when measured by a linear calibrated grid located in the ocular of the microscope (Fig. 10). The mean width of the osteoid seams was determined from measurements on a minimum of 20 osteoid seams per mineralized section.

The percentage of Haversian systems taking a fluorescent label was calculated by determining the number of osteoid seams taking a fluorescent label and dividing by the total number of osteoid seams per mineralized section. Osteoid seams taking either one or more labels when viewed under fluorescent light were considered labelled.

PRELIMINARY DETERMINATION 'A'

Haversian bone remodelling rates were determined from the control rib biopsy from twenty two beagles. The age of the dogs at time of biopsy varied between 10 and 20 months. Statistical analysis to determine what the 'natural' age decline of bone remodelling activity was in this animal in this time interval was performed.

The data from all of the remodelling parameters from the twenty two control biopsies were punched onto computer cards. The statistical analysis was carried out by means of the computer program SPSS (Statistical Package for Social Sciences, Nie et al (1975). The

procedure REGRESSION of this program was used to calculate three regression lines for each bone remodelling parameter namely: the parameter regressed against age, the parameter regressed against $(age)^{-1}$, and the parameter regressed against $(age)^{-2}$. The regression line which maximized the ability to explain the age-related variation in the parameter was selected as the 'best fit' line.

For each parameter a computer program was written to calculate the 95% confidence bounds for the 'best fit' regression line and the 95% confidence bounds for the individual observations. The 'best fit' line, its 95% confidence bounds and the 95% confidence bounds for individual observation for each parameter are illustrated graphically in Appendices 1(i) to 1(xiii).

PRELIMINARY EXPERIMENT 'B'

Two fifteen month old beagle dogs were used in this experiment. These dogs came to our animal house when they were eight months old and until they were sacrificed they served as control animals in another experiment. At autopsy the 5th, 7th, 9th and 11th ribs on the right side of each dog was removed and freed of surrounding tissues. One cm samples from the posterior, lateral and anterior portions of each rib (Fig. 11) were removed and processed in the same manner as described (see PREPARATION OF MINERALIZED SECTIONS). Haversian bone remodelling rates based on four sections from each of the three fragments of bone from each rib were then determined (see MEASUREMENTS IN HAVERSIAN BONE REMODELLING).

The means and standard deviations of the Haversian bone remodelling parameters of the anterior, lateral and posterior segments when the values from rib 5, rib 7, rib 9 and rib 11 are pooled are shown in Appendix 2(i) A one way analysis of variance according to the method

FIGURE 11 The anterior, lateral and posterior fragments of bone as used in Preliminary Experiment "B" were removed from the same positions along the length of the rib as is shown in this drawing.

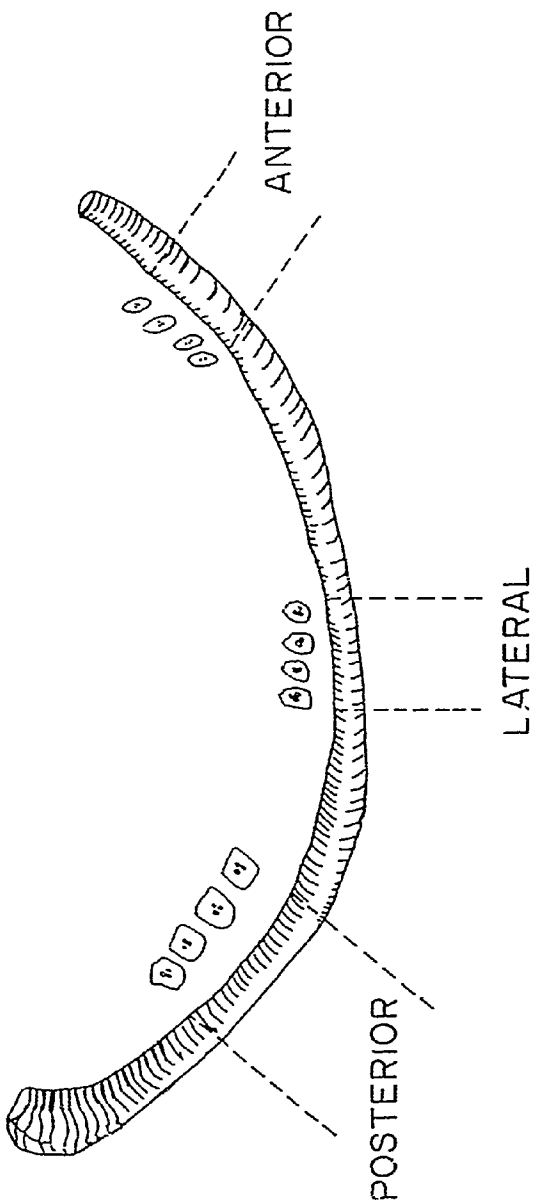


Figure II

described by Hickman and Hilton (1971) was performed to determine simultaneously if significant variation existed between any two of the three fragments. The calculated F ratios are listed in Appendix 2(i). In those parameters where the F ratio was large, and therefore significant at the .05 confidence interval (the between group differences are large in comparison to the within group variability), t-tests were used to increase precision and to localize significant differences between the two groups. In Appendix 2(ii) is illustrated the results of the t-tests used in order to locate if and where differences in bone remodelling parameters exist between: the anterior and lateral, lateral and posterior, and anterior and posterior samples of bone. The level of significance is indicated.

From the results it will be seen that differences in remodelling activity do exist along different sites of the same rib of the beagle at this age. The number of osteoid seams per mm^2 is highest anteriorly. There is a statistically significant difference ($P<.001$) between anterior and lateral, anterior and posterior, and lateral and posterior sites. Similar features characterize the activation frequency. Bone formation rate - though highest anteriorly - shows a statistically significant difference ($P<.001$) only between the anterior and posterior sites of the biopsy. In view of these results it was decided that rib biopsies from all dogs should be taken at a similar site along the length of the rib. The site of bone chosen for determinations of parameters of bone turnover rates was the mid portion of the middle third of the rib.

PRELIMINARY EXPERIMENT 'C'

In order to detect statistically significant differences between ribs, the data for the anterior, lateral and posterior fragments were

pooled together, and a one way analysis of variance was performed on all remodelling parameters. The means, standard deviations and the calculated 'F' ratios are shown in Appendix 3(i). In each case the 'F' ratio was small and not significant.

In order to detect statistically significant differences in bone remodelling activity between similar sites on alternate ribs the data from the mid anterior, mid lateral and mid posterior segments were separated. Statistical evaluation by one way analysis of variance again failed to illustrate statistically significant differences between similar sites on different ribs for any of the bone remodelling parameters (Appendix 3(ii), 3(iii) and 3(iv)). Since no significant difference was found between the ribs when either the data from anterior, lateral and posterior fragments were pooled together or when these values were analyzed separately, it appears justifiable to utilize the same site on alternate ribs as sites for biopsy before and after the experimental period.

PRELIMINARY DETERMINATION 'D'

Four hundred secondary osteons were examined from a mid axillary rib biopsy from control animals. The appositional rate (M) (as described in MEASUREMENTS IN HAVERSIAN BONE REMODELLING) and the circumference of the outer fluorescent label (obtained by the same method that was used for measuring the circumference of the osteoid seams (as described in MEASUREMENTS IN HAVERSIAN BONE REMODELLING) for each osteon was determined.

The correlation coefficient 'r' between the length of the circumference of the outer fluorescent label and the distance between the outer and inner fluorescent labels was found to be 0.75. Lee (1964)

performed similar measurements in the ribs of mongrel dogs and found a correlation coefficient of 0.77 when he measured the diameter of the outer fluorescent label and the distance between the inner and outer labels. In this thesis it was found that the distance between the inner and outer labels was inconstant if the circumference of the outer label was less than 0.35 mm, and that the distance between these two labels remained fairly constant when the circumference of the outer label was greater than 0.35 mm (Appendix 4i). I found that Haversian systems which were less than half completed were characterized by having two clear fluorescent markers, the outer label circumference measuring 0.35 mm or more. These were the Haversian systems in which apposition was preceeding at a constant rate, and were those on which appositional rates were determined.

CADMIUM EXPERIMENT

Two pairs of littermate beagles were used. During the months following the control biopsy all four dogs remained under similar conditions except that their ordinary drinking water was replaced by a solution containing $CdCl_2$ (Fisher Scientific Company, Fair Lawn, New Jersey, U.S.A.) at a concentration of 25 ppm. The average daily intake of solution was approximately 300 to 350 ml/d. The solution was available ad libitum. At the end of this experimental period the first experimental biopsy was taken.

After this biopsy the dose of Cd was reduced to 10 ppm in the drinking water. After completion of the second experimental period which lasted approximately 5 months the second experimental biopsy was taken.

Serum Fe, total Fe binding capacity, and the percent saturation of transferrin were determined at the department of haematology of University Hospital. The method used was similar to that described by Lauber (1965).

The ages of the dogs on the three biopsy dates are listed below in Table (1). For sequence of treatment and biopsy dates see Appendix to Methods II.

TABLE (1)

AGE OF CADMIUM TREATED DOGS ON BIOPSY DATES (in months)

DOG	FIRST BIOPSY	SECOND BIOPSY	THIRD BIOPSY
XN36	12.3	15.6	21.6
XO36	12.3	15.6	21.6
FU26	11.5	17.6	22.6
FV26	11.5	17.6	22.6

LEAD EXPERIMENT 'A'

Four 15-month-old littermate dogs were paired for control and experimental models. Radiographs of the knees of each showed that the epiphyseal plate scar had disappeared.

For a period lasting 201 days, the two experimental dogs were given a daily oral dose of Pb 1.3 mg/kg of body weight as lead acetate (Fisher Scientific Company, Fairlawn, New Jersey).

During the total experimental period, the dogs received 2.89 g of Pb. All dogs were exercised daily for a minimal period of 20 minutes under close supervision. At 31 and 17 days prior to sacrifice, all dogs were given a single intramuscular injection of a fluorescent label. Blood Pb levels were monitored monthly. The examination of peripheral red blood cells for basophilic stippling was also performed. At autopsy, weights of all organs were taken and samples from adjoining areas were secured for examination of lead concentration and histologic examination.

The 11th rib on the right side of each dog was freed of surrounding soft tissues and samples were then further processed so that Haversian bone remodelling rates could be measured.

LEAD EXPERIMENT 'B'

Two pairs of littermate beagles were used in this experiment. Two days following the control rib biopsy all four dogs were given an oral dose of 7 mg Pb/d. The Pb was administered as Pb acetate with dextrose filler all contained in a gelatin capsule. During the experimental period all dogs received approximately 1.7 g Pb. Following approximately six months of treatment the animals were biopsied again. This fragment of bone constituted the experimental biopsy.

The age of the dogs on the two biopsy dates are listed below in Table (2). For sequence of treatment and biopsy dates see Appendix to Methods II.

TABLE (2)

AGE OF DOGS TREATED WITH Pb ON BIOPSY DATES (in months)

DOG	FIRST BIOPSY	SECOND BIOPSY
CC16	18.3	24.0
CB16	18.3	24.0
JO16	14.6	23.6
JR16	14.6	23.6

Na_3NTA EXPERIMENT

One pair of skeletally mature beagles was used. For seven months following a control rib biopsy both dogs remained under similar conditions except that their ordinary drinking water was replaced by a solution containing Na_3NTA at a dose of 2.5 mg/kg/d. The average daily intake of water was 350 ml. The solution was available ad libitum. After completion of the experimental period, a 3 cm portion of the left 11th rib was removed. This fragment of bone constituted

the experimental biopsy.

The ages of the dogs on the two biopsy dates are listed below in Table (3). For sequence of treatment and biopsy dates see Appendix to Methods II.

TABLE (3)

AGE OF Na_3NTA TREATED DOGS ON BIOPSY DATES (in months)

DOG	FIRST BIOPSY	SECOND BIOPSY
TFY5	15.4	22.9
FEY5	15.4	22.9

ZINC EXPERIMENT

Two pairs of skeletally mature beagles were used in this experiment. Commencing two days after removal of a control rib biopsy, the drinking water was replaced by a solution containing 100 ppm Zn. This solution was prepared by dissolving ZnO in tap water - the tap water being mixed with small amounts of HCl - at pH5. After 9 months of treatment all dogs were operated on to remove a portion of the left 11th rib. This fragment of bone constituted the experimental biopsy.

At monthly intervals blood was secured for the determination of blood Zn values to insure that adequate Zn absorption was occuring.

The ages of the dogs on the two biopsy dates are listed below in Table (4). For sequence of treatment and biopsy dates see Appendix to Methods II.

TABLE (4)

AGE OF DOGS TREATED WITH ZINC ON BIOPSY DATES (in months)

DOG	FIRST BIOPSY	SECOND BIOPSY
RQX5	16.4	25.6
RRX5	16.4	25.6
FK16	14.9	24.1
FJ16	14.9	24.1

STATISTICAL ANALYSIS

The statistical evaluation of the results was performed with the assistance of the statistical laboratory, Department of Mathematics, University of Western Ontario by two methods.

The first involved the method for small samples with the significant difference between the means of each parameter for the control and experimental biopsy determined by a "t" test, using the pooled square deviations to compute the standard error of the difference (Hill, 1971). When more than two biopsies were performed a one-way analysis of variance was used to determine if any statistical significant differences existed between any two means (Hickman and Hillman, 1971). In those parameters where significance was indicated, similar "t" tests as to those described above were utilized to determine the location and magnitude of that significant difference (as was the case in the cadmium experiment).

As the results of the first preliminary experiment illustrated variations in bone remodelling rates with age, it was necessary to determine what quantity of the observed changes could be attributed to a normal physiological slowing down of bone remodelling and what amount could be ascribed to treatment. The following procedure was used.

For each treatment group of dogs, (a) to test whether the dogs in that group before treatment have the same regression line as age-matched control dogs and (b) to test whether the dogs in that group after treatment have the same regression line as untreated age-matched control dogs. The test statistic is similar to that used by Searle (1971). If, for a variable, some dogs before treatment have a different regression line from the age-matched controls, it may be feasible that observed differences in post-treatment results are due

to pre-treatment differences. In such instances caution must be exercised in conclusions about the post-treatment results. However, if all dogs before treatment have the same regression lines, then any post-treatment differences may be considered to be caused by the treatment alone.

DETERMINATION OF TISSUE CADMIUM, LEAD AND ZINC

The tissue samples were weighed and digested overnight in 0.5 ml of concentrated HCl. The following morning 0.5 ml of 30% hydrogen peroxide was added. The combined solution was heated to 95°C in a water bath for 10 minutes. After cooling the situation was transfered quantitatively along with 4 ml of distilled water to a 5 ml volumetric flask (Heisler, 1977).

DETERMINATION OF TISSUE LEAD

Lead was analyzed using a Jarrell-Ash Flameless Atomizer and an Atomic Absorbtion Spectrophotometer, Model 810 (Jarrell-Ash Division, Fisher Scientific Company, Waltham, Mass., U.S.A.) using the method given by the manufacturer. The absorbing wavelength used was 283.3 nm for lead while for background correction was 283.3 nm.

DETERMINATION OF TISSUE CADMIUM

Cadmium was analyzed using a Jarrell-Ash Flameless Atomizer and an Atomic Absorbtion Spectrophotometer, Model 810 (Jarrell-Ash Division, Fisher Scientific Company, Waltham, Mass., U.S.A.) using the method given by the manufacturer. The absorbing wavelength used for cadmium analysis was 228.8 nm while the background correction wavelength was 226.6 nm.

DETERMINATION OF TISSUE ZINC

Zinc was analyzed using flame atomic absorption with Model 810,

Atomic Absorbtion Spectrophotometer (Jarrell-Ash Division, Fisher Scientific Company, Waltham, Mass., U.S.A.) using the method given by the manufacturer. The absorbing wavelength for zinc was 213.9 nm while the wavelength for background correction was 210.0 nm.

PARATHYROID HORMONE MEASUREMENT

Serum levels of canine immunoreactive parathyroid hormone (iPTH) were determined by radioimmunoassay (RIA) according to the method described by Hruska et al (1975). Only C-terminal bindings sites were measured in the determination of iPTH. Measurements were performed in the Renal Division, Department of Medicine, Washington School of Medicine, St. Louis, Missouri, U.S.A.

Normal values of canine anti-bovine iPTH range from undetectable levels to 80 microlitre equivalents/ml (Slatopolsky 1978, personal communication).

1,25-Dihyroxyvitamin D Measurement

Serum levels of 1,25-dihyroxycholecalciferol 1,25-$(OH)_2D_3$ were determined by the competitive binding assay technique according to the method of Eisman et al (1976). These measurements were performed in the laboratory of Dr. H. DeLuca, Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin, Madison, Wisconsin, U.S.A.

At present the normal value for this vitamin D metabolite in the dog is not known. The mean value of 1,25-$(OH)_2D_3$ in humans is 29 ± 2 pg/ml of plasma. It is thought, however, that normal canine values for this metabolite are similar (DeLuca 1977, personal communication).

APPENDIX TO METHODS I

The author realizes that other investigators have warned that some tetracyclines may inhibit bone formation (Yen and Shaw, 1975) (Cruickshank 1978, personal communication). The methods employed in studies reaching these conclusions are not free of criticism, as (i) the vital dye used to measure bone formation was lead acetate and was administered intravenously in high doses, (Van Muellen and Stadhouders (1974) have stated the use of lead markers in bone formation studies should be discontinued due to their toxic effect on bone), (ii) the tetracyclines were also given in high doses on consecutive days, (iii) the areas that were measured to determine bone formation rates were random and not standardized, (iv) the results of studies of bone formation rates in organ culture and the extrapolation of those to in vivo situations without reservation is at present speculative.

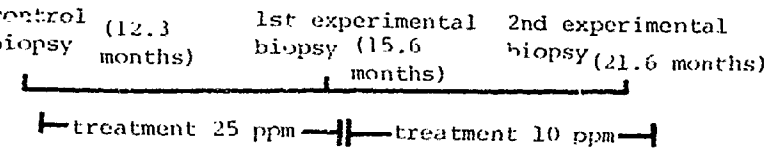
Other centres (Henry Ford Hospital, Detroit, Michigan) have developed standard in vivo methods to measure bone turnover on all the bone surfaces using the various fluorescent dyes (including the tetracyclines at dosages employed in this thesis).

Common to these procedures, is that a single dose of a fluorescent dye is given. this is followed by a suitable time interval which in turn is followed by administration of another label. Using this procedure, persistently high levels of tetracycline in the circulation are not seen.

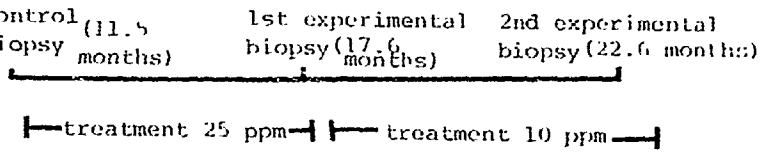
APPENDIX TO METHODS II

CADMIUM EXPERIMENT

DOG XN36, XO36

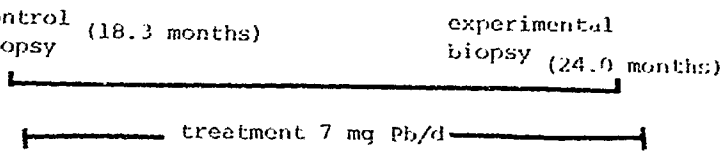


DOG FU26, FV26

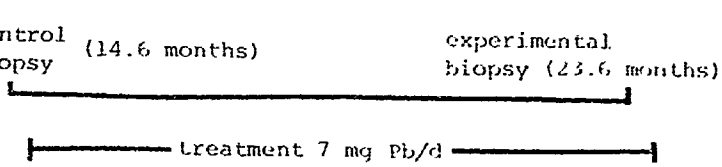


LEAD EXPERIMENT

DOG CC16, CB16

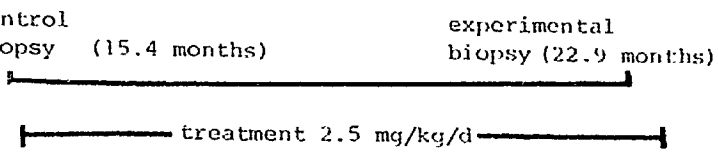


DOG JO16, JR16



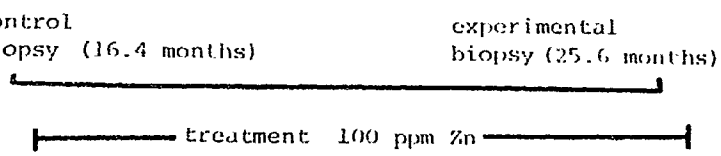
NaNTA EXPERIMENT

DOG TFY5, TEY5

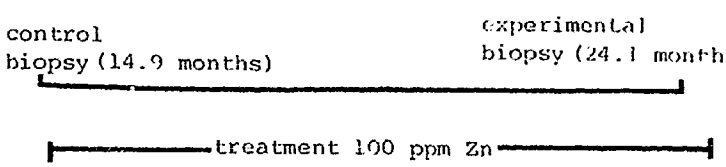


ZINC EXPERIMENT

DOG RQX5, RRX5



DOG FK16, FJ16



RESULTS

CADMIUM EXPERIMENT

The physical appearance of the four Cd treated dogs was normal for the duration of the experiment. Haematologic data collected during the treatment period are illustrated in Appendix 5(i). The results were always within normal limits. Serum iron, total Fe binding capacity and the percent saturation of transferin, determined using the method described by Lauber (1965) were found to be normal, Appendix 5(i). The means, standard deviations, minimums, maximums and ranges of the biochemical parameters collected at monthly intervals are shown in Appendix 5(ii). As may be seen from this table, the determinations showed normal results.

The circulating levels of 1,25-$(OH)_2D_3$ measured just prior to the three biopsy dates are listed in Appendix 5(iii). No significant variation in pre or post-treatment levels were found.

Serum levels of canine anti-bovine iPTH determined prior to the biopsy dates are shown in Appendix 5(iv). These are within normal limits for this animal.

The results of the Cd bone concentration found in the biopsied ribs are illustrated in Appendix 5(v). These were not significantly different from the control values.

Measurements of the Haversian bone remodelling parameters from the control and the two experimental biopsies for the Cd treated dogs are shown in Appendix 5(vi). Using a one-way analysis of variance, statistically significant differences were found at the 0.05 confidence level in the measurements of the following parameters: cortical area, ratio cortical/total area, number of osteoid seams, appositional rate, radial closure rate, activation frequency, osteon

formation time, ratio resorption to formation, bone formation rate and the percent of osteoid seams taking a label. The calculated F ratio for each parameter is listed in Appendix 5(vi). In those parameters where the F ratio was greater than 4.26 and therefore significant, "t" tests were used to increase precision by localizing significant differences between biopsies. Appendix 5(vii), 5(viii) and 5(ix) illustrates the results of the "t" tests. The level of significance is indicated.

When the regression lines from the second biopsy of the Cd treated dogs were tested against the regression lines of age-matched untreated controls, a statistical significance at the 0.05 confidence level was found in the circumference of osteoid seams. At the 0.01 level of confidence, statistical significant differences were found in: cortical/total area ratio, number of osteoid seams, appositional rate, radial closure rate, activation frequency, ratio of resorption to formation and bone formation rate. These results are illustrated graphically in Appendices 5(x) to 5(xvii).

When the regression lines from the third biopsy of the Cd treated dogs were tested against similar regression lines of age-matched untreated control dogs statistically significant differences were found at the 0.01 confidence interval for: osteon formation time, ratio of resorption/formation, radial closure rate and percentage of osteoid seams taking a fluorescent label. These results are illustrated in Appendices 5(xviii), 5(xvi), 5(xiv) and 5(xv) respectively.

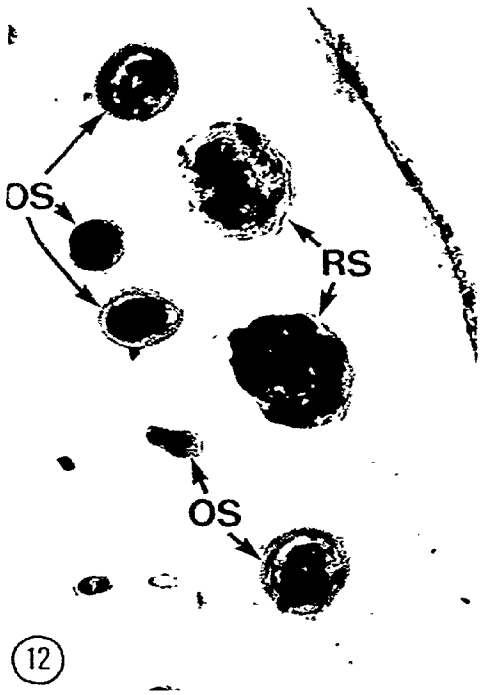
The morphological appearance of the cross section of the control rib biopsy shown in Figures 12 and 13. As may be appreciated from the examination of the light and fluorescent pictures of a random area of cortical bone there is evidence of active bone remodelling.

FIGURE 12 This photomicrograph identifies portions of five actively growing osteoid seams (OS) and two resorption spaces (RS) from the cortex of the middle portion of the right 11th rib removed prior to the administration of Cd. The osteoid seams are easily identified in mineralized hand ground sections by their coloured hyline appearance when stained with Villanueva Osteochrome Stain while resorption spaces are identified by their characteristic scalloped border. Villanueva Osteochrome Bone Stain. X150

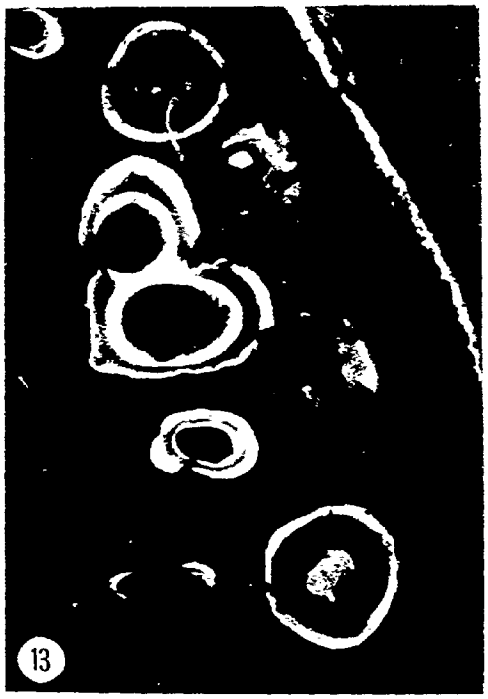
FIGURE 13 The same area shown in Figure 12, under fluorescent light reveals single and double tetracycline labels administered fourteen days apart which surround the osteoid seams, therefore confirming activity of the involved osteons. X150

FIGURE 14 This photomicrograph illustrates two osteoid seams (OS) from a portion of the cortex of the middle segment of the left 11th rib removed after Cd administration at a concentration of 25 ppm in drinking water for six months. X150

FIGURE 15 The same area shown in Figure 14, under fluorescent light reveals double fluorescein bands around the two osteoid seams. Located throughout this portion of rib cortex are numerous unresorbed segments of tetracycline labels which have persisted from the pre-treatment labelling. The lack of osteoid seams within these labels is proof that they are from the pre-treatment labelling. It is evident in this particular region, that following long term Cd administration there is a considerable decrease in the number of osteoid seams/unit area. X150



12



13

14

15

The appearance of the cross section of the first post-treatment rib biopsies is shown in Figures 14 and 15. These are light and fluorescent pictures taken of a random area of cortical bone in which numerous fluorescent labels are seen. Most of these fluorescent labels correspond to the tetracycline markers utilized as a pre-treatment indicator and are not associated with osteoid seams. It will be appreciated that there are only two DCAF labels (arrows) associated with osteoid seams. This, compared with Figures 1 and 2 allows a visual appreciation of the marked decrease in remodelling activity per surface area.

LEAD EXPERIMENT 'A'

The physical appearance of all the dogs throughout the experimental time period was unremarkable. The mean Pb levels in control and experimental dogs found during periodical monitoring during the administration of the Pb are outlined in Appendix 6(i). The average tissue Pb concentrations found at autopsy in both control dogs and experimental dogs are shown in Appendix 6(iii). The haematologic data collected are shown in Appendix 6(ii). These results were always found to be within the normal ranges for the Beagle. The smears of the peripheral red blood cells were examined for nucleated red blood cells and basophilic stippling but none were found. The mean and ranges of the results of the biochemical investigations carried out on control and experimental dogs during the period of the experiment are shown in Appendix 6(iv). These values fell within normal limits. Routine examination of histologic slides from tissues in Appendix 6(ii) showed no significant abnormality between control and experimental animals. Notwithstanding a careful search, no inclusion bodies were found in liver or kidneys.

The results of the measurements of bone remodelling parameters from the 11th rib of the right side of the control dogs and experimental dogs are shown in Appendix 6(v). Statistically significant difference was found at the 0.025 confidence level between appositional rates of the control and experimental dogs. Statistical significant differences were found at the 0.05 confidence level between control and experimental dogs in radial closure rates, activation frequencies, osteon formation times and bone formation rates.

LEAD EXPERIMENT 'B'

The physical appearance of the four dogs during the course of the experiment as testified by the Animal House veterinarian and his staff was unremarkable. The mean blood Pb levels taken at fifty day intervals throughout the experimental period are illustrated in Appendix 7(i). Haematologic data collected at monthly intervals are shown in Appendix 7(ii). These results fell within normal limits. The means, standard deviations, minimums, maximums and ranges of the biochemical data are shown in Appendix 7(iii). As can be seen from these results no biochemical abnormality was present.

Bone Pb concentrations from rib obtained at both the control and experimental biopsies are illustrated in Appendix 7(iv).

The circulating levels of iPTH obtained prior to control and experimental biopsies are shown in Appendix 7(v). These results fell within the normal range for the dog.

The results of the measurements of the bone remodelling parameters from the control and experimental biopsies are shown in Appendix 7(vi). Using one-tailed "t" tests statistically significant differences were found at the 0.005 level in the number of osteoid seams/mm^2, appositional rate and activation frequency. At the 0.01 confidence

level statistical significance was found between the radial closure rates and the bone formation rates of the control and experimental biopsies. Significant differences between the control and experimental biopsies in percentage of osteoid seams labelled and the wall thickness of completed osteons were observed at the 0.05 confidence level.

When the regression lines from the second biopsy of the Pb treated dogs were tested against the regression lines of age-matched untreated dogs statistical significance was found at the 0.01 confidence level between radial closure rates, osteon formation times, ratios resorption to formation and percentage of osteoid seams taking a fluorescent label. These results are illustrated graphically in Appendices 7(vii), 7(viii), 7(ix) and 7(x).

Na_3NTA EXPERIMENT

The physical appearance of both dogs during the experiment was normal. Haematologic data collected throughout the treatment period are shown in Appendix 8(i). All of these values fell within normal ranges. The biochemical outline of the dogs during the same time interval is shown in Appendix 8(ii). Similarly, these values were within normal limits.

Circulating levels of canine anti-bovine iPTH measured prior to the control and experimental rib biopsies are illustrated in Appendix 8(iii). Thes values were within the normal range for the dog.

Measurements of the bone remodelling parameters from the control and experimental biopsies are shown in Appendix 8(iv). Using a two-tailed student's "t" test statistical significant differences were found; at the 0.05 confidence level in the appositional rate, at the

0.025 confidence level in the radial closure rate and the ratio resorption to formation, at the 0.01 confidence level in the bone formation rate and at the 0.001 confidence level in the percentage of osteoid seams taking a fluorescent label, between the control and the experimental bone biopsies.

When the points from the second biopsy of the Na_3NTA treated dogs were tested against the regression lines of age-matched untreated dogs statistical significance was found at the .05 level between radial closure rates and the percentage of osteoid seams taking a fluorescent label. These results are presented graphically in Appendices 8(v) and 8(vi) respectively.

ZINC EXPERIMENT

The physical appearance of the Zn treated animals during the course of the experiment remained normal as testified by the Animal House Veterinarian and his staff. The haematologic data collected at monthly intervals is summarized in Appendix 9(i), and these results were observed to be normal. The biochemical parameters monitored at similar intervals during the treatment period are shown in Appendix 9(ii). No abnormality was detected.

The circulating levels of canine anti-bovine iPTH measured in blood samples removed prior to the control and experimental biopsies are shown in Appendix 9(iii). No changes were observed in these measurements.

The level of Zn in the control and experimental bone biopsies is shown in Appendix 9(iv).

Measurements of the bone remodelling parameters from the control and experimental biopsies for the Zn treated dogs are shown in Appendix 9(v). Using a two-tailed student's "t" test statistical

significant differences were found at the 0.05 confidence level in the number of osteoid seams/mm^2, and the activation frequency and at the 0.01 confidence level in the ratio resorption to formation, between the control and the experimental biopsies.

When the regression lines of the bone remodelling parameters were tested against regression lines from similar parameters in untreated age-matched controls only marginal significant difference was found in the percentage of osteoid seams taking a label at the 0.05 confidence level. This result is graphically illustrated in Appendix 9(vi).

DISCUSSION

Cadmium Experiment

There is no doubt that there exists enough epidemiological evidence to suggest that environmental Cd - either alone or combined with other substances or in certain nutritional conditions - is associated with lesions of bone after a prolonged exposure (Adams et al 1969, Tsuchiya 1969a, Tsuchiya 1969b, Murata et al 1970, Kobayashi 1971, Nordberg 1974). Nevertheless, obscurity surrounds this relationship.

In the first place it has not been established whether these bony lesions result from a primary toxic effect upon bone cells thereby directly interfering with bone remodelling patterns (Kimura et al 1974, Yoshiki et al 1975) or are they related to a secondary effect on bone cells moderated through parathyroid and renal involvement (Larsson and Piscator 1971). In other words, at what stage of renal insufficiency, if at all, do skeletal lesions become manifest.

The second point which merits a question is the reported association of increased levels of other environmental pollutants - such as Pb and Zn - along with elevated Cd levels in the geographical areas where "Itai-itai" disease is endemic. In other words could the bony lesions seen in humans in endemic areas be the result of a cumulative effect of various environmental pollutants on bone remodelling activity, rather than the solitary effect of Cd?

It is surprising that there is no mention of a condition similar to "Itai-itai" disease occuring in the animals in the endemic area in Japan. One may expect that if the condition is such a problem for human beings it would also have been seen in smaller house-hold or wild animals.

Possible explanations of these phenomena may include the following. In human Cd poisoning the bone lesions are consistent with osteomalacia and/or osteopenia, both of these diseases are characterized by abnormal bone remodelling rates (Frost 1973) and although Frost (1969) has stated that the canine bone remodelling system is "similar" to that of the human, small differences between the two remodelling systems may still exist. Secondly, if the cumulative effects of Cd on bone cells are increased with each sigma - therefore the severity of this condition may be related to the number of new osteons constructed during the intoxication period - the fewer number of units actively remodelling bone and the shorter life span of domestic animals may be of paramount importance in the protection of smaller animals from contracting clinical skeletal lesions.

Experiemental studies in animals have been useful in studying such things as absorption, (Ingersoll and Wasserman 1971, Sugawara et al 1976, Ando et al 1977, Hamilton and Smith 1977) distribution and tissue levels, (Itokawa et al 1974, Colucci et al 1975, Bawden and Hammarstrom 1975, Thind and Fischer 1975, Attramadal and Jansen 1976) and dose related damage (Yoshiki et al 1975). But all these investigative studies on experimental animals have used rather crude methods of assessment of bone abnormalities such as the variation in inorganic (ash) weight (Larsson and Piscator 1971, Cousins et al 1977), the radiological appearance of bones (Itokawa et al 1973, Nomiyama et al 1975), or the study of bone by histology using conventional decalcification techniques (Yoshiki et al 1975). Even so - as seen from the literature review - the conclusions have been far from categorical, and indeed have been controversial. One group of investigators state that Cd induces osteopenic changes (Yoshiki et al 1975) while another

group considered these lesions as osteomalacic ones (Itokawa et al 1974).

This controversy, stems directly from use of various models, which incorporate the administration of various compounds of Cd in all sorts of concentrations by different routes for different periods of time. With this in mind, on reviewing the literature one finds that in those experimental situations where bone lesions are described, the investigators created a rather artificial setting in that the high dosages employed by most investigators over short time periods created an acute or subacute situation (Yoshiki et al 1975) rather than a chronic one in which low doses administered over longer periods of time are demanded in order to simulate the human condition.

Many of these administered diets reflected the investigators' preconceived ideas that Cd and Fe counteracted Ca absorption and thus effected bone. Few investigators have subjected their animals to an excess of Cd in an otherwise normal diet containing the full requirements of Ca and vitamin D.

In the consideration of vitamin D it is necessary to point out that species differences in the metabolism of this "hormone" which have been described (Rasmussen and Bordier 1974) have been disregarded in some reports (Lorentzon and Larsson 1977). Many experiments investigating the toxicity of Cd employ tissue and organ culture techniques (Suda et al 1973, Sakai et al 1975), these studies serve to reflect the alterations of the metabolism of tissue explants only in the context of the tissue culture experiment, whereas the "in vivo" situation may be very different.

Results of the measurements of Haversian bone remodelling parameters in the Cd treated dogs reveal some interesting features.

In the first place it will be seen that the administration of either 25 ppm or 10 ppm during the experimental periods did not produce any statistically significant change in the circulating levels of either iPTH or 1,25-$(OH)_2D_3$. Since the demonstration of the immunological heterogeneity of PTH, it may be argued that as only the C-terminal fragments were measured and that the N-terminal fragments which are considered to be the biologically active fragments were not, the results of this measurement may not be as valid as intended. Nevertheless, Slatopolsky et al (1975) have stated that an antibody which has the ability to recognize the carboxyl terminal is a more sensitive measure since it is this terminal which represents the majority of the circulating fragments of PTH normally found in the circulation. Elevations if iPTH seen in hyperparathyroidism and in uremia are reflected by increases of mostly C-terminal fragments.

To date little work has been done in relation to the metabolism of vitamin D and its metabolically active by-products in the dog let alone in the Beagle. DeLuca (personal communication) has stated that the metabolism of vitamin D has not been investigated in the dog, nor are the normal levels of 25-$(OH)D_3$ and 1,25$(OH)_2D_3$ in the serum of the dog established. If the metabolism of this vitamin is similar in the dog as it is in the human (and there is so far no reason to doubt that it is not) then one would expect that interference with renal tubular cell mitochondria and/or endoplasmic reticulum by Cd over a prolonged period of time would have altered the pre-, para-, and post-experiemntal levels of the 1,25$(OH)_2D_3$ metabolite. A situation which DeLuca himself failed to demonstrate in the sera of the Cd treated dogs.

Also worthwhile noticing is the fact that neither during nor

after the experimental period were there detectable abnormalities in serum haematology or biochemistry. One would assume - according to reported experiments - that if the amount of Cd administered to the dogs was creating a situation which would be manifested by damage to the small intestinal villi (Valberg et al 1977) and therefore to its absorptive capacity, then one would have expected that - over the prolonged experimental period - there would have been some alteration in the circulating levels of Fe, or to the levels in circulation of Ca, PO_4, and/or alkaline phosphatase. In fact, serum Fe studies revealed no abnormalities. Serum Ca, serum PO_4 and serum levels of alkaline phosphatase were within normal limits and at no time allowed an assumption of decreased intake of Ca or excess loss of Ca via the kidneys. Serum BUN, uric acid and creatinine levels were within normal limits, this being in accordance with a previous report where renal damage was ruled out in an experiment using beagles and a higher dose of Cd (Loeser and Lorke, 1976). Serum levels of albumin and glucose were also within normal limits, thus ruling out the presence of abnormalities previously reported in experimental animals but in which higher doses of Cd over a shorter period of time had been used. If it is assumed that only 6% is absorbed through the gastrointestinal tract (Fassett 1975), the daily absorption approximates 0.5 mg of $CdCl_2$ when the dogs were receiving 25 ppm Cd.

Notwithstanding the apparent normality of the internal 'milieu', significant changes were observed in the measurements of some parameters of bone remodelling activity.

These differences withstood statistical analysis not only by the application of the "t" test, but also with respect to age-related

modifications in the normal animal. In other words, the assessment detected a true modification attributable only to the experimental situation. These changes reflect two types of alteration, the first in relation to the normal rate of activation of the mesenchymal cell which is responsible for triggering the mechanism of bone relacement, and the second in relation to the actual performance of the individual cells; osteoclasts and osteoblasts.

With respect to the former, the significant decrease in the circumference of osteoid seams may be correlated to impaired osteoclastic function. Although measurement of the cone's largest diameter as described by Jaworski et al (1975) was not determined, the reduced circumference of the osteoid seams may be related to a decrease in the maximum cone diameter which in turn is brought on by impaired osteoclastic activity.

If the figures obtained from the three biopsies are interpreted as manifestations of bone formation dynamics at the cellular, tissue, and organ levels, then the results suggest decreased bone formation rates at the osteoblastic level and even more potent inhibition of bone formation at the tissue and organ level of skeletal organization in the chronically low-level cadmium-intoxicated dog (Fig. 16).

Thus it would appear that the first order effect of cadmium on Haversian bone remodelling may be due to a toxic mechanism acting at the cellular level (Heath et al 1961, Springgate et al 1973, Mitra and Bernstein 1977, Hoffman and Niyogi, 1977).

The present results do not prove this, but from the review of the literature it will be appreciated that this is not at all impossible. This toxic effect is made more evident by the "rebound" in activity of bone remodelling which occured in the experimental animals when

TABLE 5

COMPARISON OF CELLULAR, TISSUE, AND ORGAN-LEVEL BONE FORMATION IN NORMAL AND CHRONICALLY LOW LEVEL CADMIUM INTOXICATED DOGS[a]

Level of Skeletal Organization of Bone Formation Activity	Control Biopsy	1st Experimental Biopsy	2nd Experimental Biopsy
Osteoblastic activity (Radial closure rate) % normal: (mm/yr)	.49 (100)	.37 (76)	.34 (70)
Tissue level (Haversian bone formation rate) % normal: (cu. mm/cu. mm/yr)	.63 (100)	.07 (11)	.16 (25)
Organ level (Bone formation rate x cortical cross-section area) % normal: (cu. mm/yr/mm - thick cross-section)	6.00 (100)	.86 (14)	1.86 (31)

[a] Values for percentage of normal are shown in parentheses.

the dose of cadmium was reduced from 25 ppm to 10 ppm.

In conclusion it may be said that exposure of dogs to low doses of cadmium over a prolonged period of time produces a reduction in Haversian bone remodelling rates which is apparently a dose-related phenomenon and which occurs as a primary effect on bone tissue without the intervention of either abnormalities of intestine, kidney, parathyroid hormone or vitamin D activity. This is different from the situation observed naturally in the human, but then in the latter there is often a variable degree of impairment of renal function by the time that the skeletal lesions become manifest.

Lead Experiment

The blood and tissue Pb levels in the control and experimental dogs in Lead Experiment "A" (Appendix 6(i) and 6(ii)) and post treatment blood and bone Pb levels observed in Lead Experiment "B" (Appendix 7(i) and 7(iv)) conform to those reported by Zook et al (1969) and Zook (1973) for normal healthy male mongreal dogs and lead-intoxicated dogs.

The haematological data obtained during the course of both experiments (Appendices 6(iii) and 7(ii)) and the biochemical data obtained during the same period (Appendices 6(iv) and 7(iii)) show no abnormality as far as the internal milieu of the Beagle is concerned (Andersen 1970, Appendix 10(i)). At no time was any basophilic stippling of red cells observed, nor was there any abnormality in the Ca/PO_4 figures to indicate metabolic bone disease by conventional methods. At no time during the experiments was there any biochemical evidence of liver or kidney impairment according to the results obtained.

The most sensitive indicator in measuring Pb absorption is the blood Pb level (Zook et al 1972). The blood Pb levels and the liver tissue levels found in the experimental group of Lead Experiment "A"

and the blood levels found in Lead Experiment "B" reflect adequate absorption of the administered Pb and also conform to the figures of liver Pb levels reflecting Pb intoxication as reported by Zook et al (1972) and Stowe et al (1973). The figures for bone Pb concentration (Appendices 6(ii) and 7(iv)) confirm that the ingested Pb was stored in this tissue.

In their review of the use of animal models for comparative studies in Pb poisoning, Scharding and Oehme (1973) state that animals with similar metabolic systems or disease modifications to humans should be used before experimental results may be stated to be similar to those which may occur in humans. Frost (1969) stated that the Haversian remodelling system in dogs is similar to that of man.

In Lead Experiment "A" statistically significant differences between control dogs and experimental dogs in the measurement of appositional rates, radial closure rates, activation frequencies, osteon formation times and bone formation rates were found. In Lead Experiment "B" statistically significant differences were observed between the radial closure rates, the osteon formation time, the ratio resorption to formation and the percentage of osteoid seams taking a label.

These results were to be expected when these experiments were started in view of the fact that Hass et al (1967) had previously described the inhibition of intercellular matrix synthesis in experimental animals during chronic ingestion of inorganic Pb.

If the figures are interpreted as manifestations of bone formation dynamics at the cellular, tissue and organ levels (Frost 1969), then the results suggest decreased bone formation rates at all three levels of skeletal organization in the chronically low-level Pb-intoxicated

dog (Fig. 17).

Lead toxicity is manifest by neuropathological. haematological, renal, reproductive, endocrine and immunopathological abnormalities (Goyer and Rhyne 1973). Although the highest concentration of body Pb are found in bony tissues, the effect of this metal on bone cell dynamics has previously not been investigated. At the cellular level Pb may cause interference with a number of mechanisms; phospholipid metabolism, Fe transport, mitochondrial function, ribosomal function and heme and globin synthesis. Interference of normal bone cell function could possibly result from any combination of these biochemical defects. The mechanisms underlying the bone lesions described in this thesis remain uncertain.

Cellular Pb inclusion bodies have been observed in osteoclasts following single injections of Pb (Van Mullen and Stadhouders 1974). The formation of these inclusion bodies are considered to be a cellular protective mechanism, of which the metabolic cost to the cell in the formation and maintenance is not known.

Normal levels of 25-(OH)D_3 have been reported in Pb-poisoned children (Rosen and Roginsky 1973). The increased incidence of Pb poisoning in the summer months was thought to be due to increased production of vitamin D_3 and 25-(OH)D_3. Recently it has been shown that vitamin D_3 and 25-(OH)D_3 are under the influence of a negative feedback system which regulates their concentration in plasma (Rasmussen and Bordier 1974).

Little is known concerning the status of the parathyroid glands in Pb poisoning. Mahaffey (1974) found histologically normal appearing parathyroids in Pb-intoxicated rats, but no measurement of circulating PTH was made. The levels of canine anti-bovine iPTH

TABLE 6

COMPARISON OF CELLULAR, TISSUE, AND ORGAN-LEVEL BONE FORMATION DYNAMICS IN NORMAL AND CHRONICALLY LOW LEVEL LEAD INTOXICATED DOGS[a]

Level of Skeletal Organization of Bone Formation Activity	Normal Control Dogs	Lead-Intoxicated Dogs	Before Pb Treatment	After Pb Treatment
Osteoblastic activity (Radial closure rate) % normal: (mm/yr)	0.41 (100)	0.37 (90)	.46 (100)	.32 (70)
Tissue level (Haversian bone formation rate) % normal: (cu. mm/cu. mm/yr)	0.40 (100)	0.34 (85)	.35 (100)	.12 (34)
Organ level (Bone formation rate x cortical cross-section area) % normal: (cu. mm/yr/mm - thick cross-section)	4.51 (100)	3.52 (78)	3.79 (100)	1.21 (32)

[a]Values for percentage of normal are shown in parentheses.

reported in this thesis confirmed the absence of this gland's involvement in Pb toxicity - at least - before the occurrence of hyperparathyroidism secondary to renal failure.

The effect of Pb on bone turnover rates observed in both experiments "A" and "B" occurred at levels of Pb exposure below those which produce other manifestations of Pb intoxication such as cellular inclusions, basophilic stippling of red blood cells, or anemia, the latter two being sensitive indicators of Pb toxicity on the hemopoietic system. Free erythrocytic porphyrins, aminolevulinic acid, and aminolevulinic acid dehydratase were not measured in this study.

These results may be of greater importance in view of the possible effects of the life time chronic accumulation of Pb from childhood onward (Barry 1975), and the variation in dietary content of Pb (Mahaffey 1974, Mahaffey et al 1974) on the total bone mass of the adult as well as the fact that bone Pb levels are greater in urban than in rural populations (Cohen et al 1973) and that complications arising from clinical states with decreased total bone mass in the elderly are greater in industrialized than in non-industrialized communities (Chalmers and Ho, 1970, and Doyle 1972).

Na_3NTA Experiment

Several reports that linked high doses of Na_3NTA to the development of various renal disturbances, including carcinoma in experimental animals (Nixon 1971, Mahaffey and Goyer 1972) have recently resulted in the government of the U.S.A. banning the usage of NTA compounds in that country's detergent industry.

In this experiment, the administration of Na_3NTA for more than seven months produced no clinical, haematological or biochemical abnormality.

As other people have reported that Na_3NTA is stored in kidney and bone tissue (Budny 1972, Michael 1971 and Budny and Arnold 1973) it was thought that the costly duplication of these experiments was unnecessary.

There has been some evidence to indicate that the levels of tissue fluid ionic Ca are interfered with following the intra-venous administration of Na_3NTA (Budny 1972) but there have been no reports to indicate that in the low doses - such as employed in this experiment - there is any evidence of chelation of ionic tissue fluid C· or interference with the availability of Ca for the purposes of bone matrix mineralization. But there is evidence (Michael 1971) that Na_3NTA may in fact increase the amount of Ca accretion in bone. If that increase in skeletal Ca were due to lowered ionic Ca levels in tissue fluids then one would expect that the serum levels of iPTH would be elevated. This experiment shows in fact that this was not so (Appendix 8(iii)).

Excessive administration of Na_3NTA to experimental animals with or without Zn supplements revealed increases in the bone Zn concentration (Michael 1971, Michael and Wakin 1972). No such increase in the bone Zn levels was found in this experiment.

The results of bone remodelling studies revealed a decrease in the appositional rate, radial closure rate, bone formation rate and the percentage of osteoid seams taking a fluorescent label. When these results were tested against regression lines from age-matched untreated dogs only a marginal downward trend was noted in the radial closure rate and the percentage of osteoid seams taking a label.

These results suggest an interference with the mineralization process. Since Na_3NTA is stored in bone, its presence in this tissue may disturb mineral homeostasis by binding to available ions and

making them unavailable to partake in the mineralization process.

It has been debated that the toxicity of NTA depends on the chemical form of the administered NTA. Although most of the experiments examining the biological effects of NTA incorporate Na_3NTA, it is thought that the Ca salt is the most likely NTA compound to be found in the environment (DHEW report 1975). CaNTA is also thought to be more toxic than Na_3NTA. Others in the same report argue that regardless of the chemical form of NTA, when ingested this ligand will dissociate in the stomach at a pH of 1 or 2 and will rebind to available ions in that environment.

It is not known whether the reported toxic effects of Na_3NTA on various tissues are due to a primary effect of this chelator on cells or a secondary effect by binding to various ions in the extracellular compartment and making them unavailable for intracellular use (DHEW report 1975).

Accepting the fact that the treatment population in this experiment was small and that the observed changes in two bone remodelling parameters, although statistically significant, were minimal, it cannot be discounted that the administration of Na_3NTA in low doses over long periods of time, may have an adverse effect on cells in Haversian bone.

Zinc Experiment

The importance of maintaining adequate dietary Zn is illustrated by the development of skeletal lesions in experiments where both Zn deficiency (Hegstead 1976) and Zn toxicity (Sadavisian 1951) have been investigated. Unlike the Cd and Pb experiments the threat of including renal failure during excess Zn administration - at least at concentrations used in this thesis - is considerably lower. Nevertheless, serum biochemistry, haematology was monitored and were found to be

normal, no clinical abnormality was seen in the Zn treated dogs.

The administration of the Zn resulted in a moderate elevation in the bone Zn concentration. This increase coincided with slight elevations in plasma and blood zinc levels. Therefore, absorption of Zn was taking place. The clinical and metabolic outline of members of a family described as having familial hyperzincemia (Smith et al 1976) showed no manifestations of the increased circulating Zn - with the exception of a raised bone Zn concentration. This profile is similar to that of the Zn treated dogs in this thesis. Normal level of iPTH illustrated non-involvement of the parathyroid glands.

When measurements of bone remodelling in the Zn treated dogs is compared with controls, no change in both formation at the cellular, tissue and organ levels were observed. In fact, considering the age related decrease in appositional rate that is normally found in dogs of this age, the Zn treated dogs elicited a small but nevertheless relative upward trend.

Zinc supplementation at the levels administered in this experiment has little effect on Haversian bone remodelling in the rib of the Beagle. However, there is a tendency that Zn supplementation may postpone or minimize the natural decrease in appositional rate. If so - zinc excess would not only be of benefit to the healing process in soft tissues (Pories 1966) but possibly to this process in mineralized tissues as well. A possible explanation of this phenomenon is that under normal conditions the amount of available Zn in tissue fluids is less than optimum for the upper rate of the mineralization process to occur. Therefore, Zn supplementation would slightly increase Zn in the tissue fluid - thereby making more Zn ions available - hence the apposition rate would approach nearer to the upper limit. Although this reasoning

is only speculative, Zn ion concentration in the tissue fluid may exert a positive effect on mineralization of matrix.

The influence of the excess Zn and Na_3NTA - at least at the concentrations used during the treatment interval in this thesis - with the exception of the radial closure in the Na_3NTA treated dogs were not significant, nevertheless a tendency toward interference with Haversian bone remodelling did exist. This is not to deny that either of these environmental pollutants - in combinations, in higher doses or longer treatment times - may produce a more dramatic effect in the Haversian bone turnover process.

On the other hand administration of low doses of Pb and Cd (especially the latter) over long time intervals resulted in a potent inhibition of cortical bone turnover.

Recently, using ultrastructural methods Luk et al (1973) classified osteocytes from cortical bone in the young adult rabbit into three categories; the formative osteocytes, the resorptive osteocytes and the degenerative osteocytes. The latter group appear relegated to the periphery of secondary osteons and to interstitial bone. A greater degree of hypoxia due to the greater distance that these cells are from a blood supply may in part explain their morphological appearance. It is the prime function of remodelling in cortical bone to remove regions containing such degenerative cells - in fact there is some evidence which suggests that interstitial bone, which contains a greater than normal percentage of degenerated cells, may in some way produce a "suicidal" stimulus, which activates a Basic Multicellular Unit (Frost 1969) and hence signals that start of that packet of bone destruction by osteoclasts.

The significance of depressed bone remodelling activity is dependent upon the consequences of interruption of its normal function. The first is the normal replacement of bone in which a series of microscopical foci of damage occur as a result of daily physical activity. A lowered mesenchymal activation frequency would permit these foci of damage to persist for long periods of time. These unreplaced packets of damaged bone could accumulate and coalesce until the point when physiological stress may produce symptomatic complications.

The second factor in a lowered turnover rate is that the "mean skeletal age" of the average osteon is allowed to increase (Frost 1973). These osteons then become hypermineralized, and as a consequence the normal mechanical properties of bone are altered leaving these individuals increasingly susceptible to suffer skeletal complications under normal mechanical conditions.

Therefore in the light of the results obtained in the Cd and Pb experiments a possible mechanism can be provided to correlate the lowered bone remodelling rates found in these dogs with many of the skeletal symptoms of the Cd intoxicated human, although - as mentioned earlier on - renal insufficiency may complicate matters further.

The results of this thesis confirm the role of Pb and Cd in evoking lowered bone remodelling rates in the Haversian bone from rib of Beagles and also potentially decreasing total skeletal mass. Although no clinical fractures were observed in this experiment, it is postulated that with time (i.e., years) these could have occurred. The proportion of the human population under a similar influence of environmental pollutants is not known, but it is interesting to note that Frost (1973) described a group of patients whose bone cell

dynamics revealed a negligible mesenchymal cell activation - about 3% of normal in the absence of an obvious attributable cause - manifest multiple spontaneous fractures. The role of environmental pollutants in the pathogenesis of such events should be investigated.

CONCLUSIONS

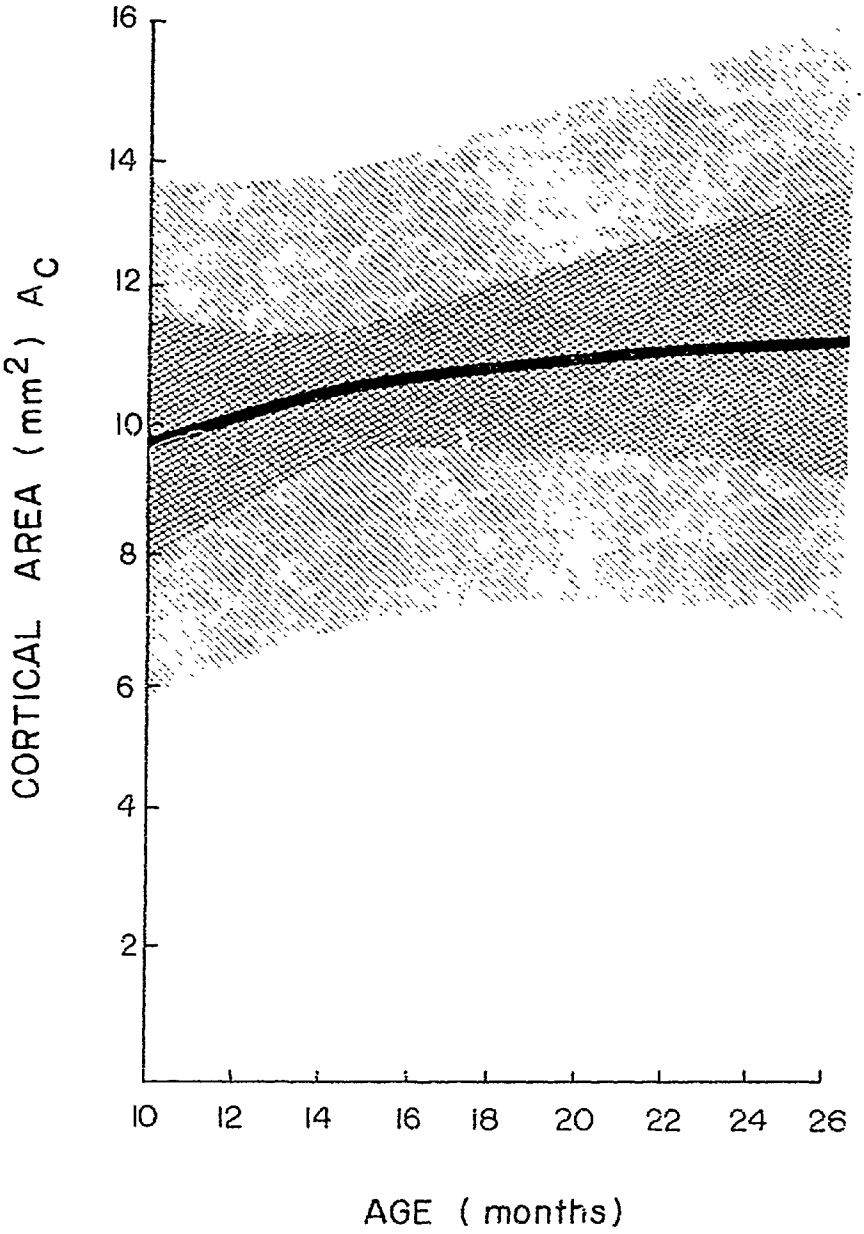
1. In the Beagle between the ages of one and two there are age-related changes in cortical bone remodelling in the rib.
2. Significant variation in bone remodelling activity exists at different sites along the same rib in young adult Beagles.
3. No significant difference in bone remodelling activity exists when similar sites on alternate ribs are compared in the Beagle.
4. Initially the upper limit of appositional rate at the level of the osteon is constant and is subsequently followed by a gradual decline till bone formation eventually reaches zero.
5. Cadmium is a potent inhibitor of cortical bone turnover and does so initially without influencing other organ systems.
6. Lead inhibits cortical bone turnover directly without involving other organs.
7. Although the results of this thesis fail to demonstrate a primary first order effect of Na_3NTA and zinc directly on bone that is not to deny that these environmental pollutants in combinations of either higher doses or longer treatment times may produce a more dramatic effect in the Haversian bone turnover process, than seen here.

APPENDIX 1(i)

PRELIMINARY EXPERIMENT "A"

CORTICAL AREA/mm^2 (A_C)

This graph represents normal age-related changes in cortical cross sectional area observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

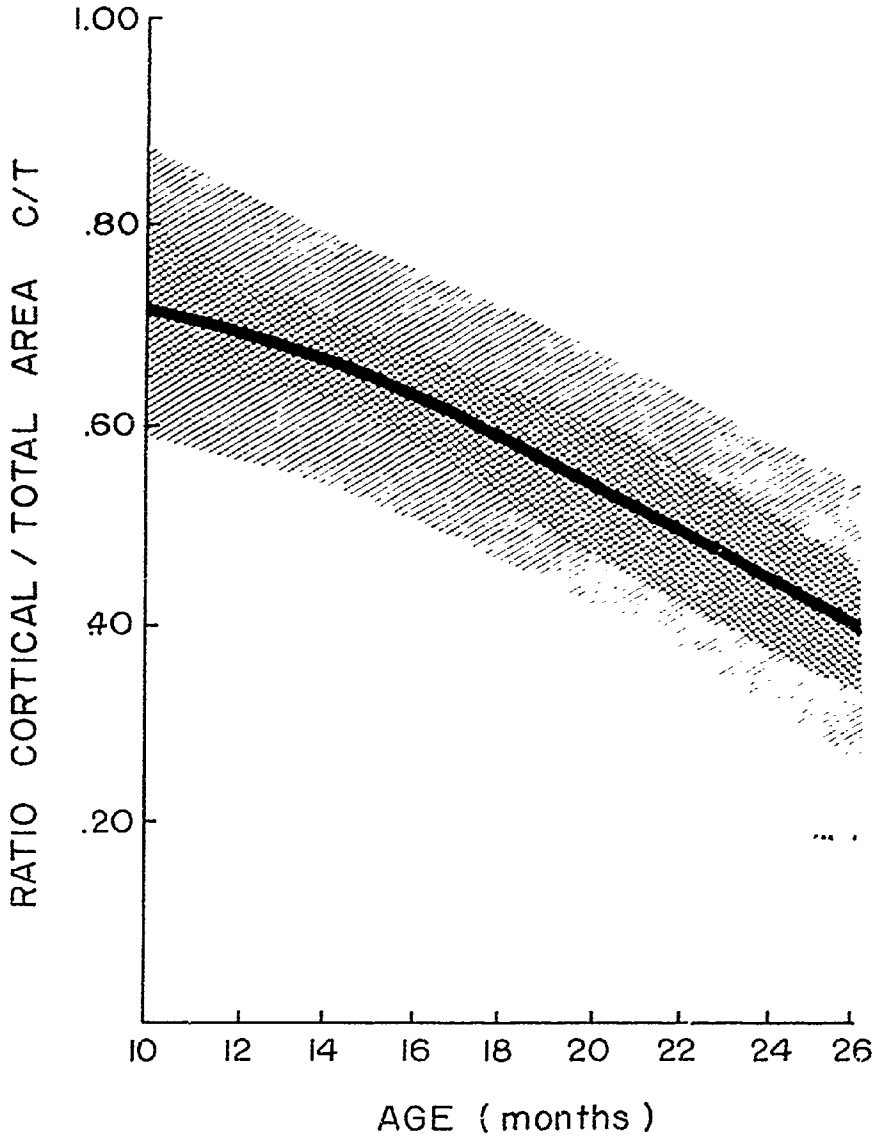

16
14
12
10
8
6
4
2
CORTICAL AREA (mm2) AC
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(ii)

PRELIMINARY EXPERIMENT "A"

RATIO CORTICAL/TOTAL AREA (C/T)

This graph represents normal age-related changes in the ratio cortical/total area observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

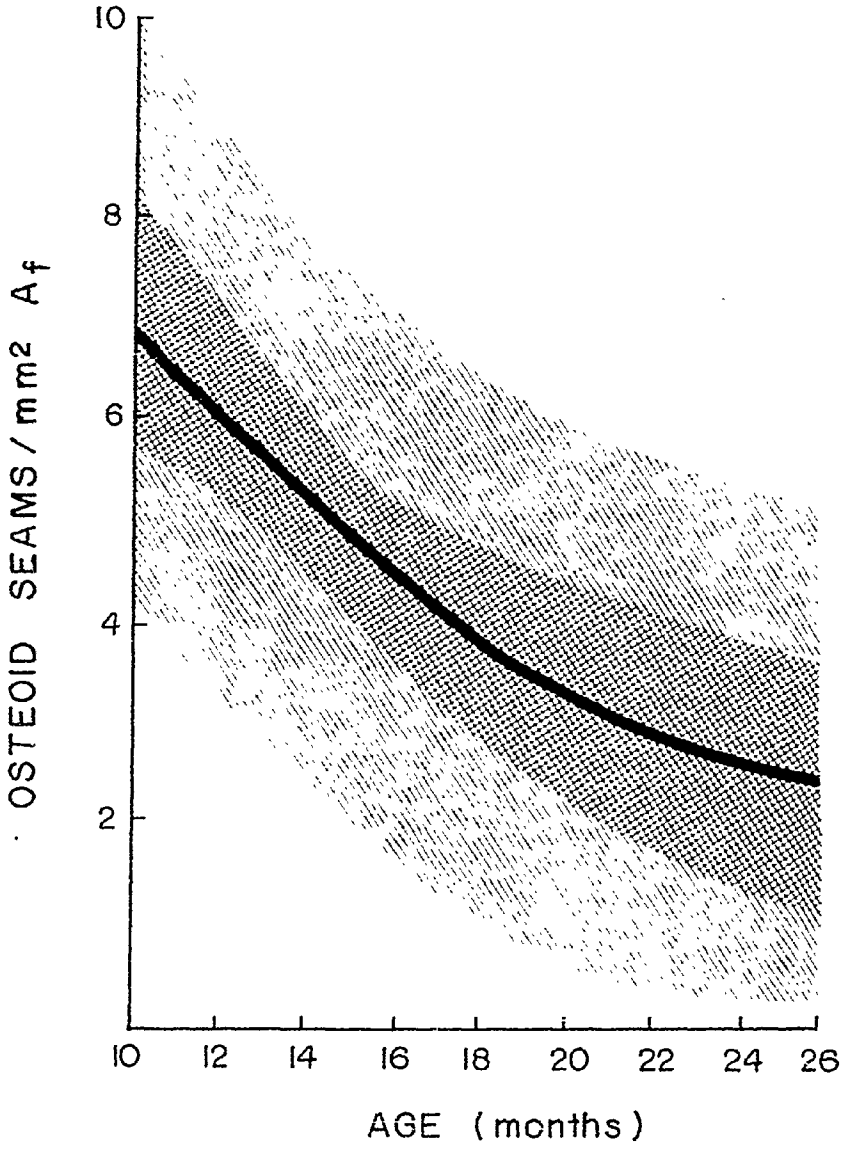

1.00
.80
.60
.40
.20
RATIO CORTICAL / TOTAL AREA C/T
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(iii)

PRELIMINARY EXPERIMENT "A"

NUMBER OF OSTEOID SEAMS/mm^2 (A_f)

This graph represents normal age-related changes in the number of osteoid seams/mm^2 observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

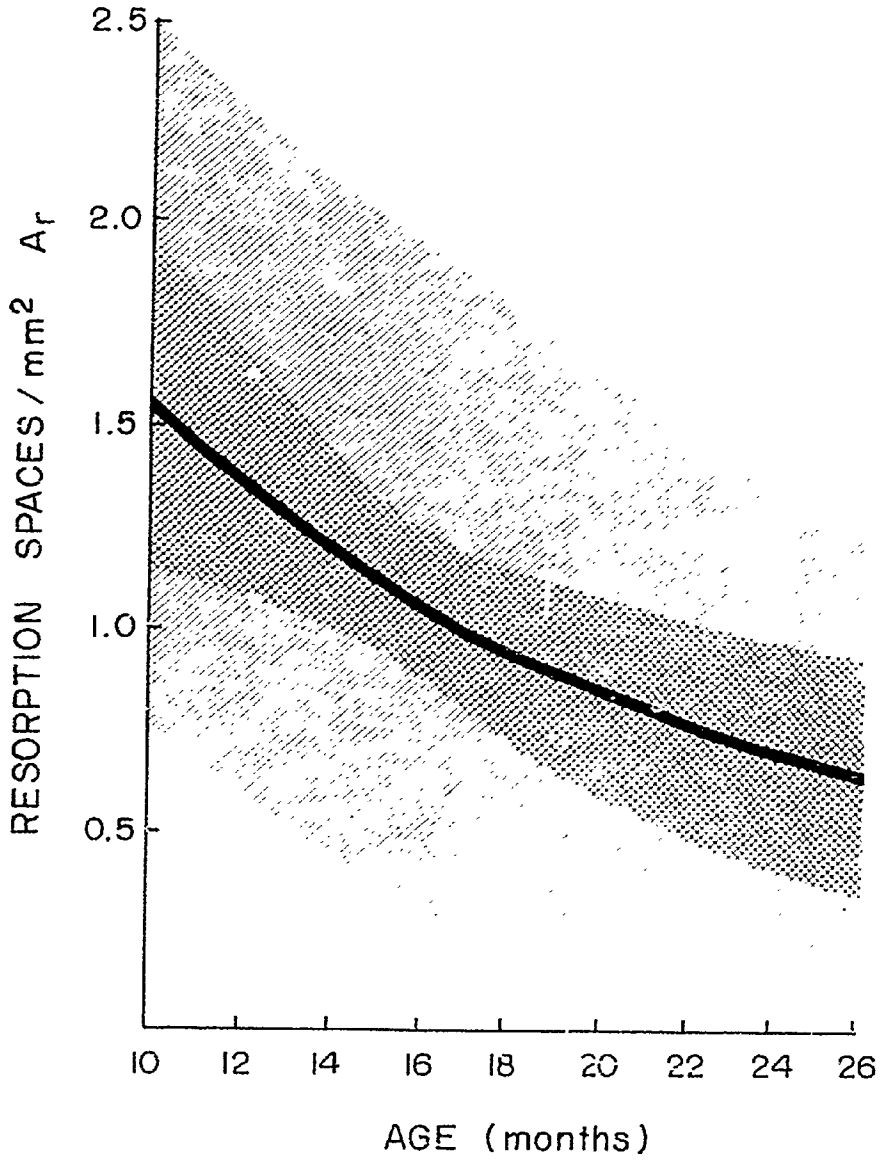

10
8
6
4
2
OSTEOID SEAMS / mm2 Af
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(iv)

PRELIMINARY EXPERIMENT "A"

NUMBER OF RESORPTION SPACES/mm^2 (A_r)

This graph represents normal age-related changes in the number of resorption spaces/mm^2 observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

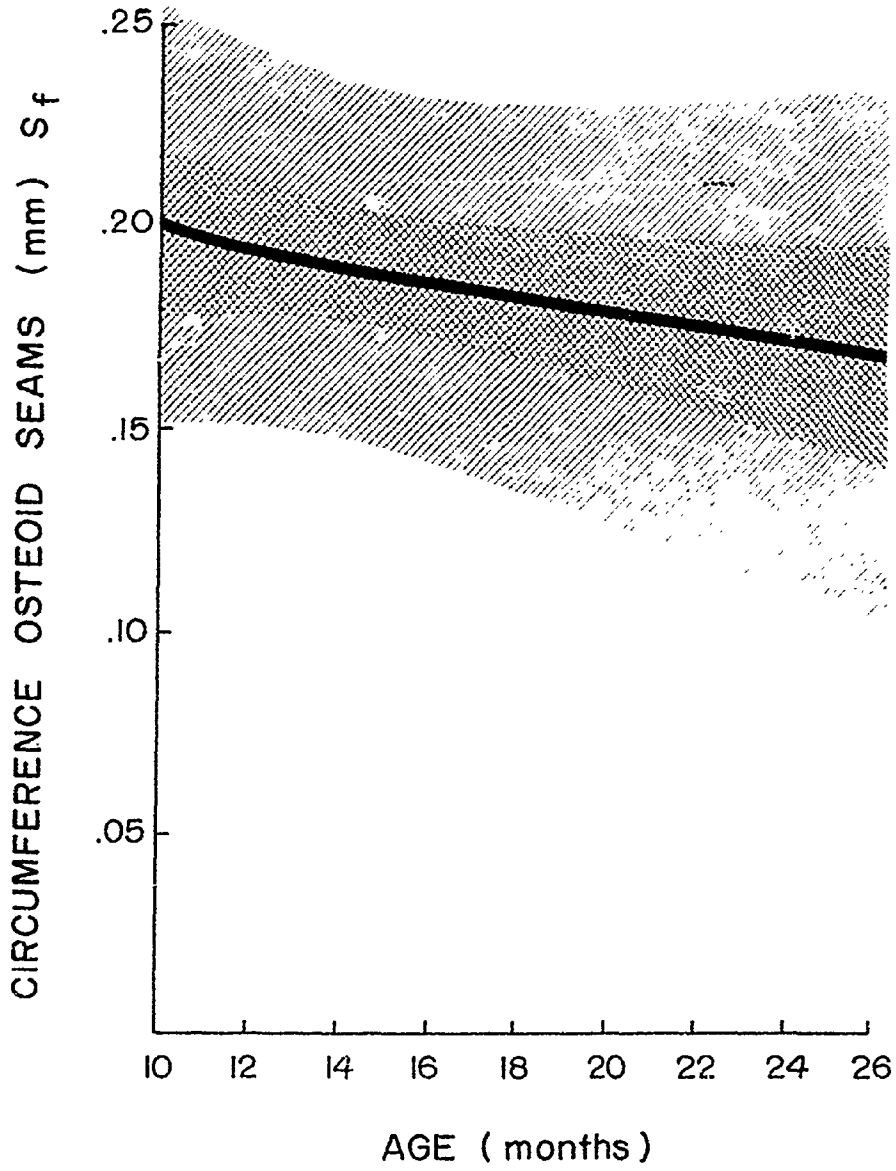

2.5
2.0
1.5
1.0
0.5
RESORPTION SPACES / mm² A_r
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(v)

PRELIMINARY EXPERIMENT "A"

CIRCUMFERENCE OSTEOID SEAMS, mm (S_f)

This graph represents normal age-related changes in the circumference of osteoid seams observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single line area).

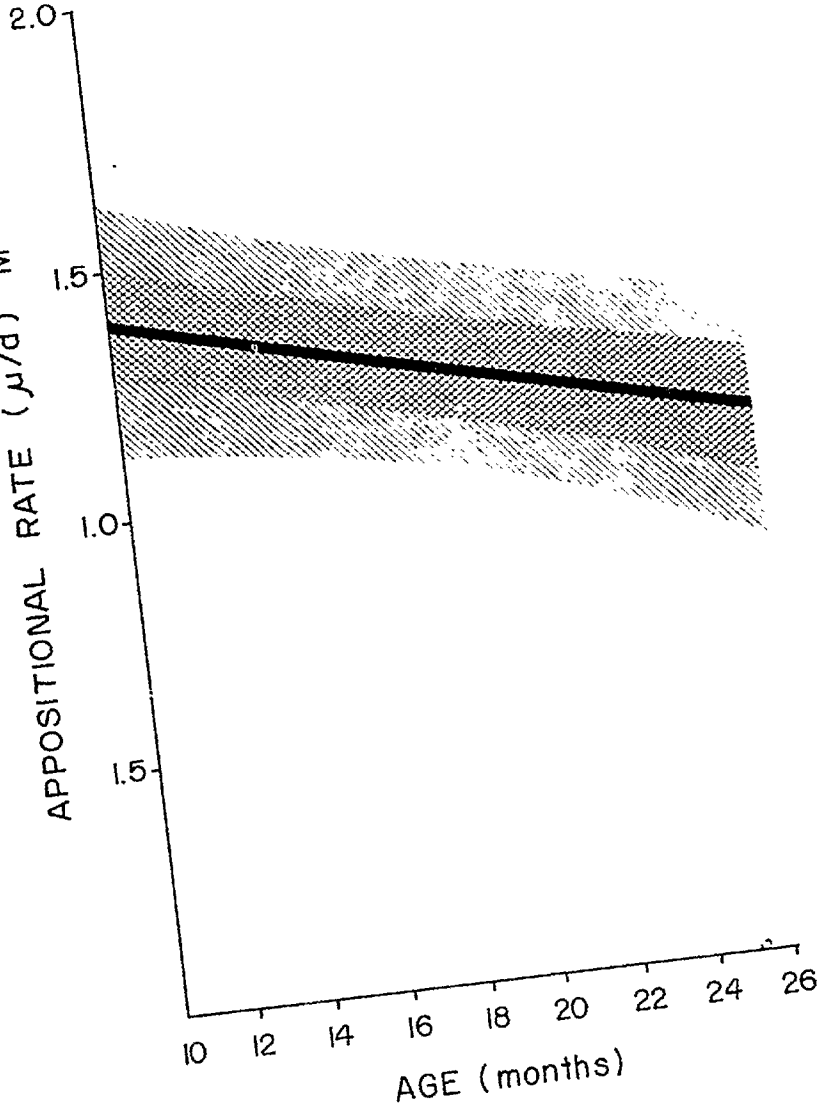

.25
.20
.15
.10
.05
CIRCUMFERENCE OSTEOID SEAMS (mm) S_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(vi)

PRELIMINARY EXPERIMENT "A"

APPOSITIONAL RATE, microns/day (M)

This graph represents normal age-related changes in the appositional rate observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

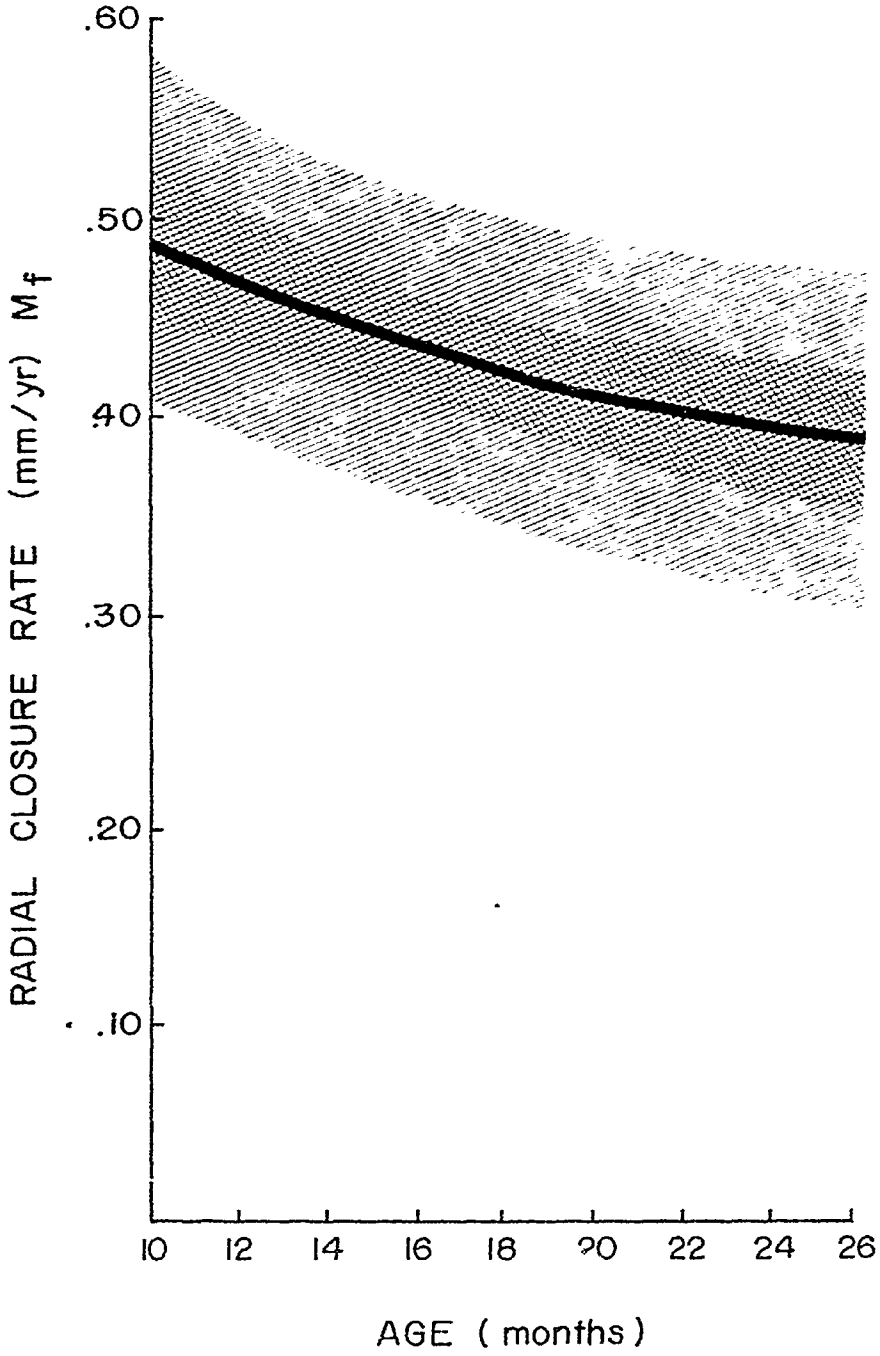

2.0
1.5
1.0
1.5
APPOSITIONAL RATE (μ/d) M
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(vii)

PRELIMINARY EXPERIMENT "A"

RADIAL CLOSURE RATE, mm/yr (M_f)

This graph represents normal age-related changes in the radial closure rate observed with age. Illustrated are the best-fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

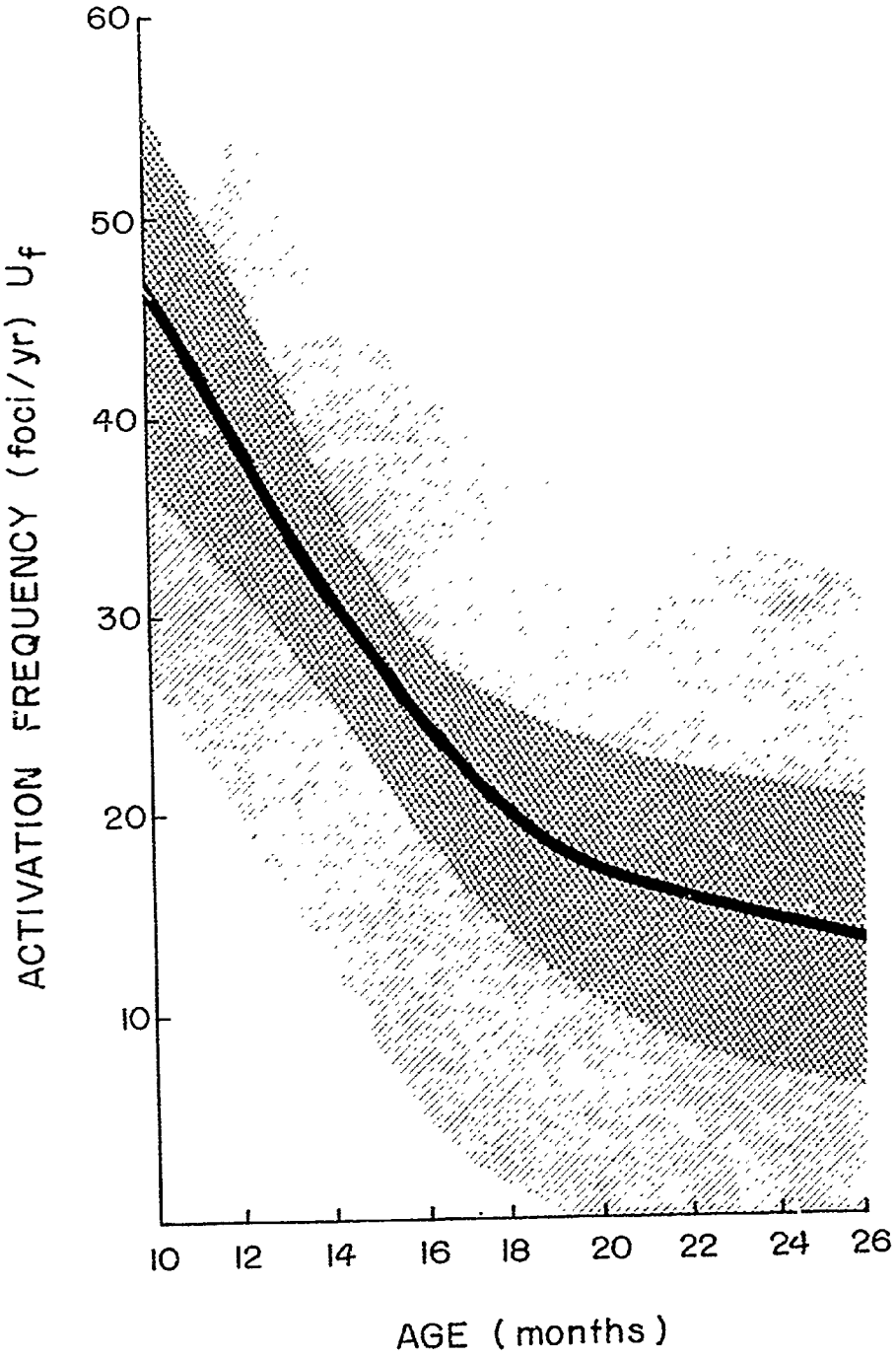

.60
.50
.40
.30
.20
.10
RADIAL CLOSURE RATE (mm/yr) M_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(viii)

PRELIMINARY EXPERIMENT "A"

ACTIVATION FREQUENCY, foci/yr (U_f)

This graph represents normal age-related changes in activation frequency observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

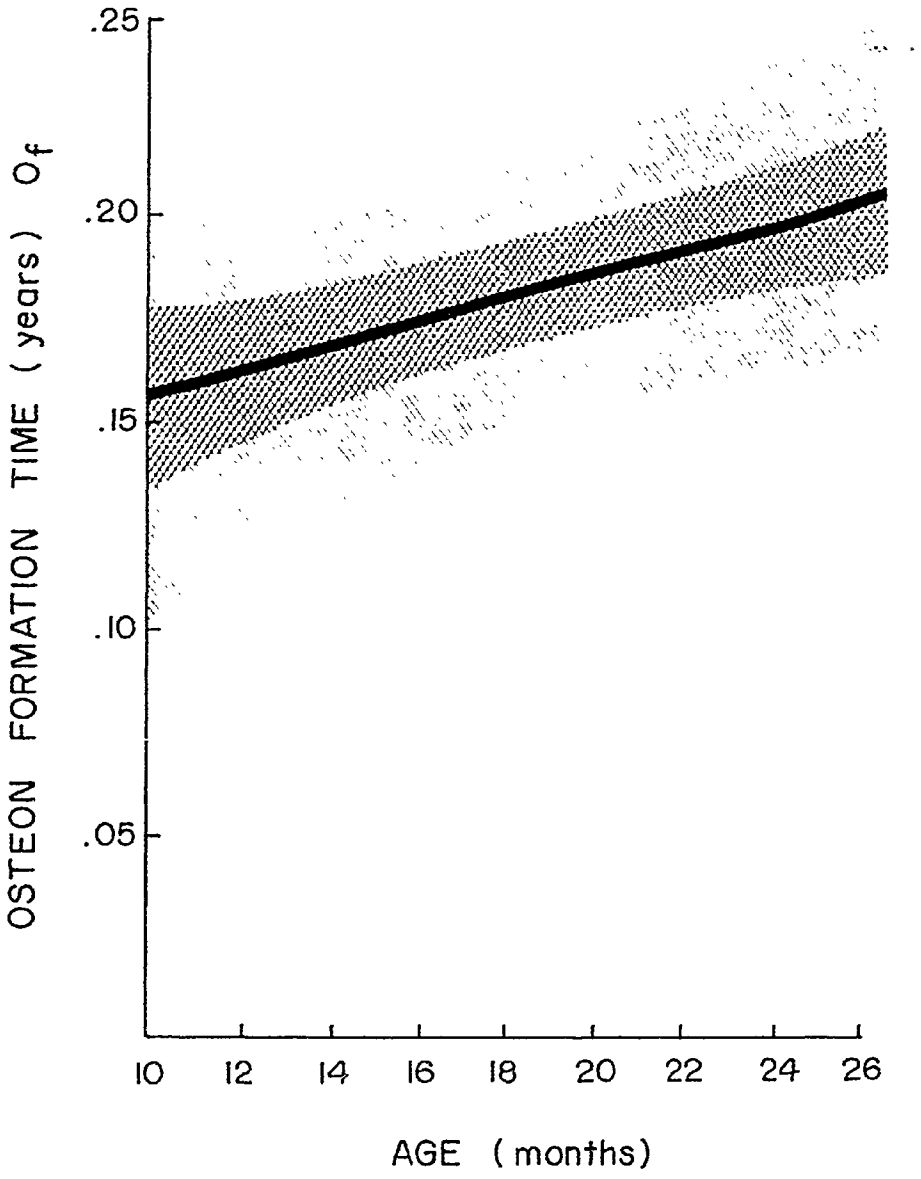

60
50
40
30
20
10
ACTIVATION FREQUENCY (foci/yr) U_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(ix)

PRELIMINARY EXPERIMENT "A"

OSTEON FORMATION TIME, yrs (O_f)

This graph represents normal age-related changes in the osteon formation time observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

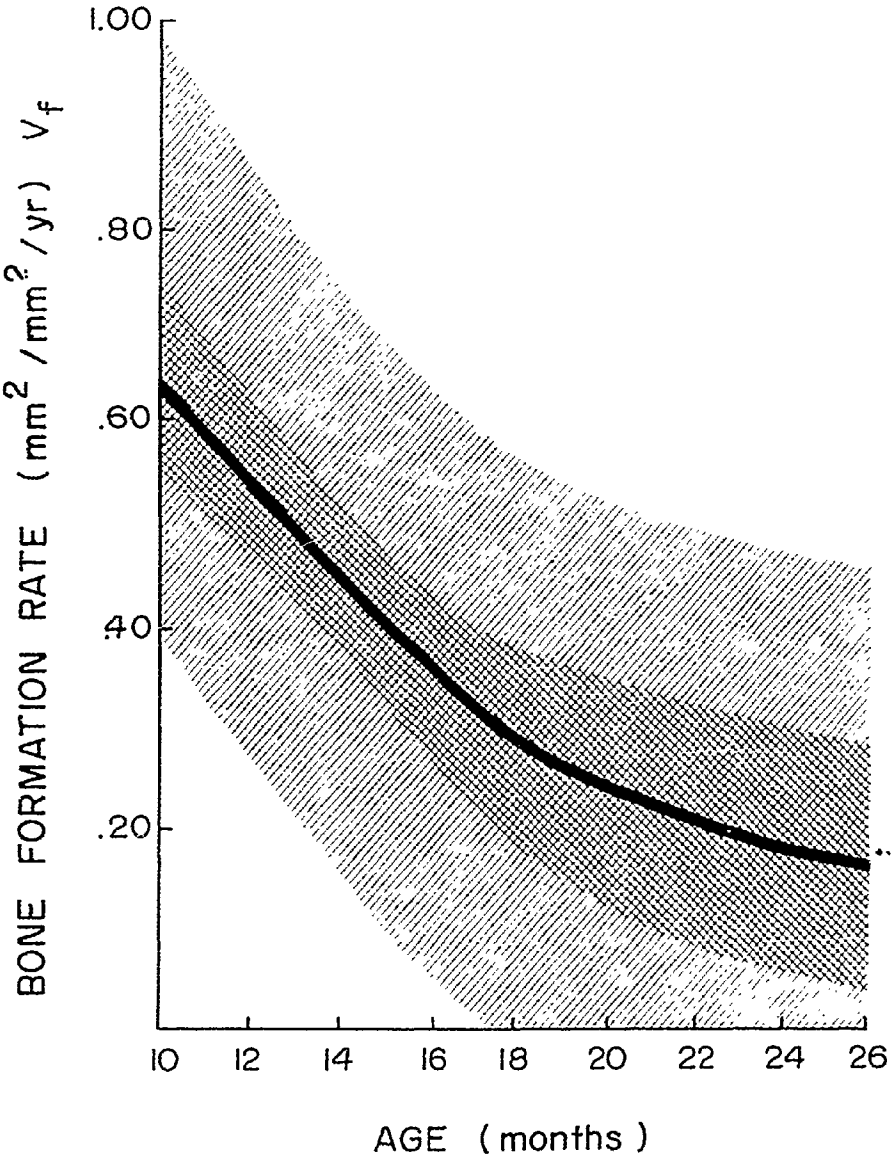

.25
.20
.15
.10
.05
OSTEON FORMATION TIME (years) O_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(x)

PRELIMINARY EXPERIMENT "A"

BONE FORMATION RATE, $mm^2/mm^2/yr$ (V_f)

This graph represents normal age-related changes in bone formation rate observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

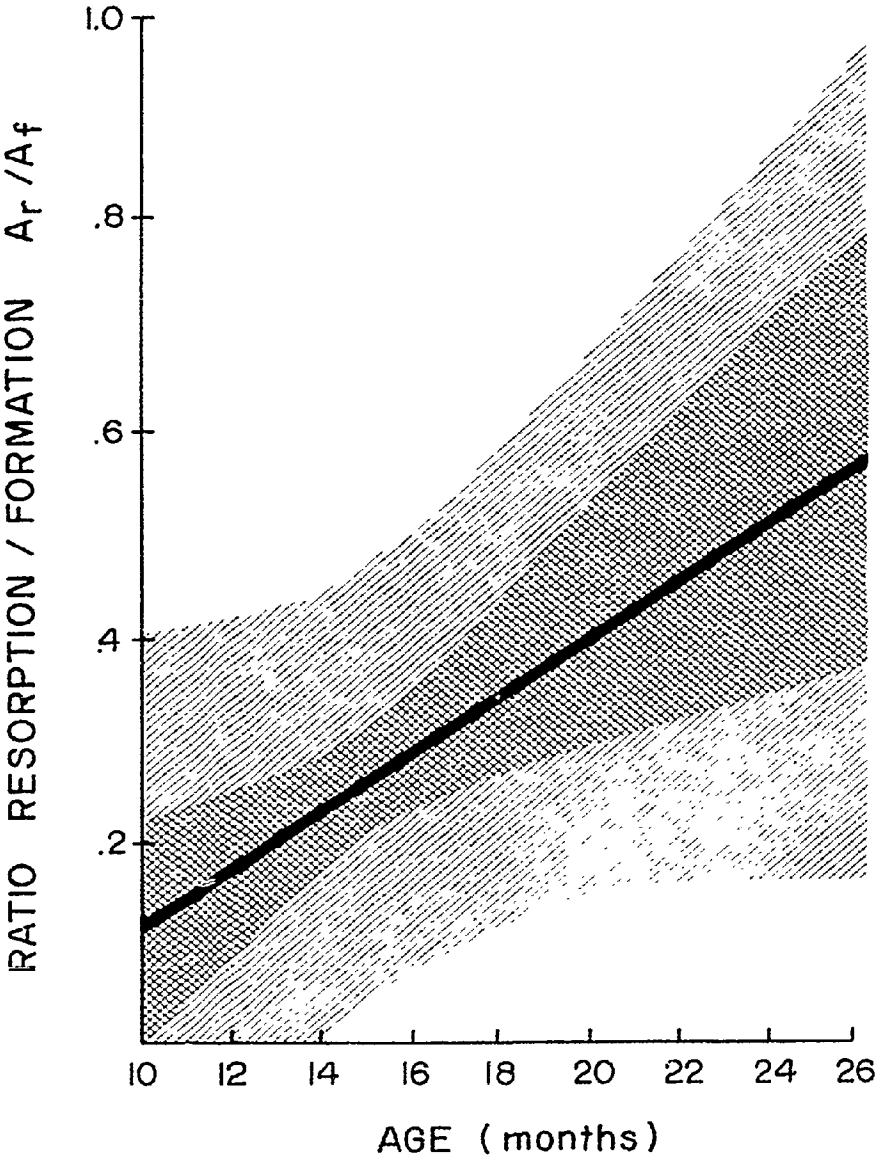

1.00
.80
.60
.40
.20
BONE FORMATION RATE (mm² / mm² / yr) V_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(xi)

PRELIMINARY EXPERIMENT "A"

RATIO RESORPTION TO FORMATION (A_r/A_f)

This graph represents normal age-related changes in the ratio resorption/formation observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

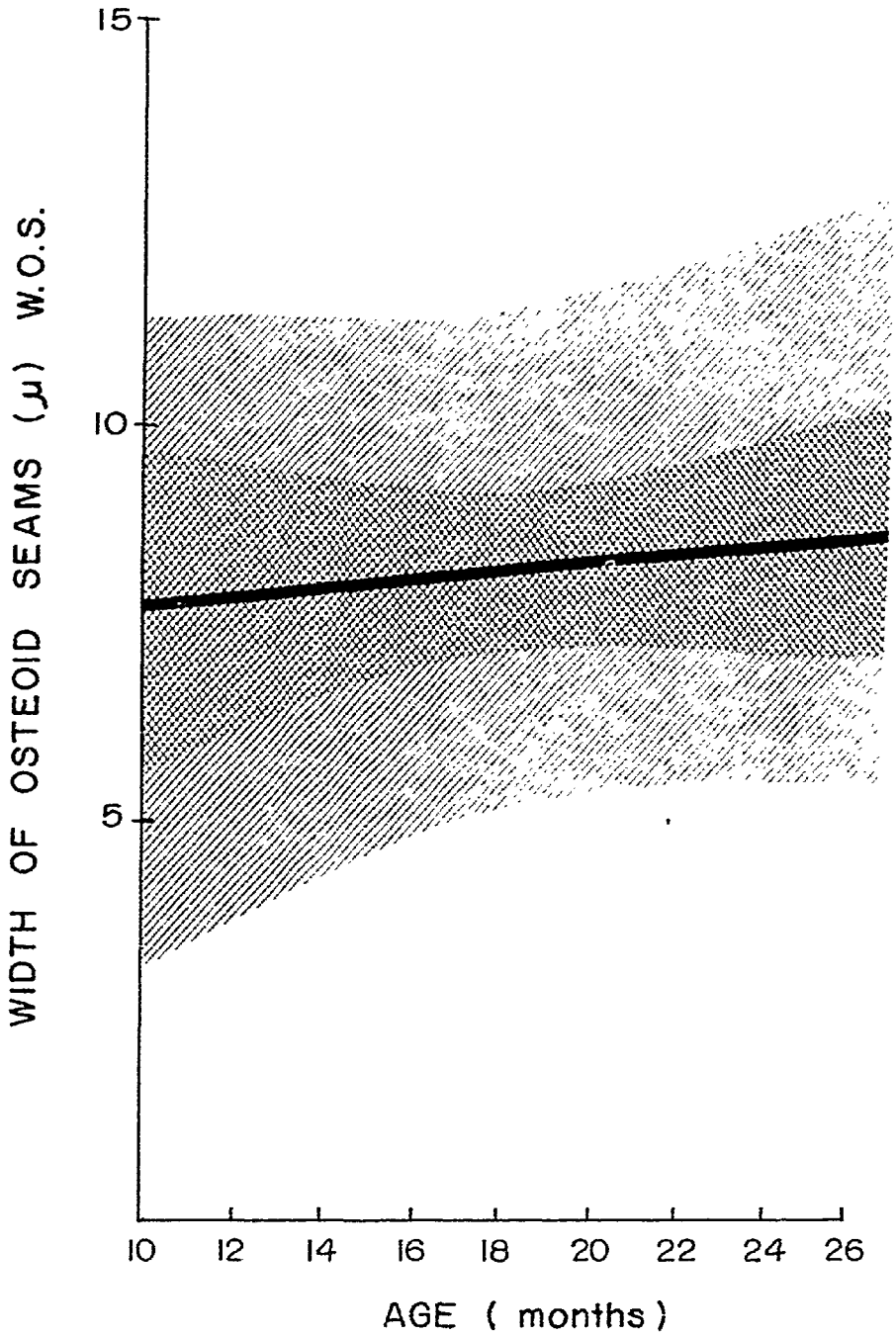

1.0
.8
.6
.4
.2
RATIO RESORPTION / FORMATION A_r / A_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(xii)

PRELIMINARY EXPERIMENT "A"

WIDTH OSTEOID SEAMS, microns (W.O.S.)

This graph represents normal age-related changes in the width of osteoid seams observed with age. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).

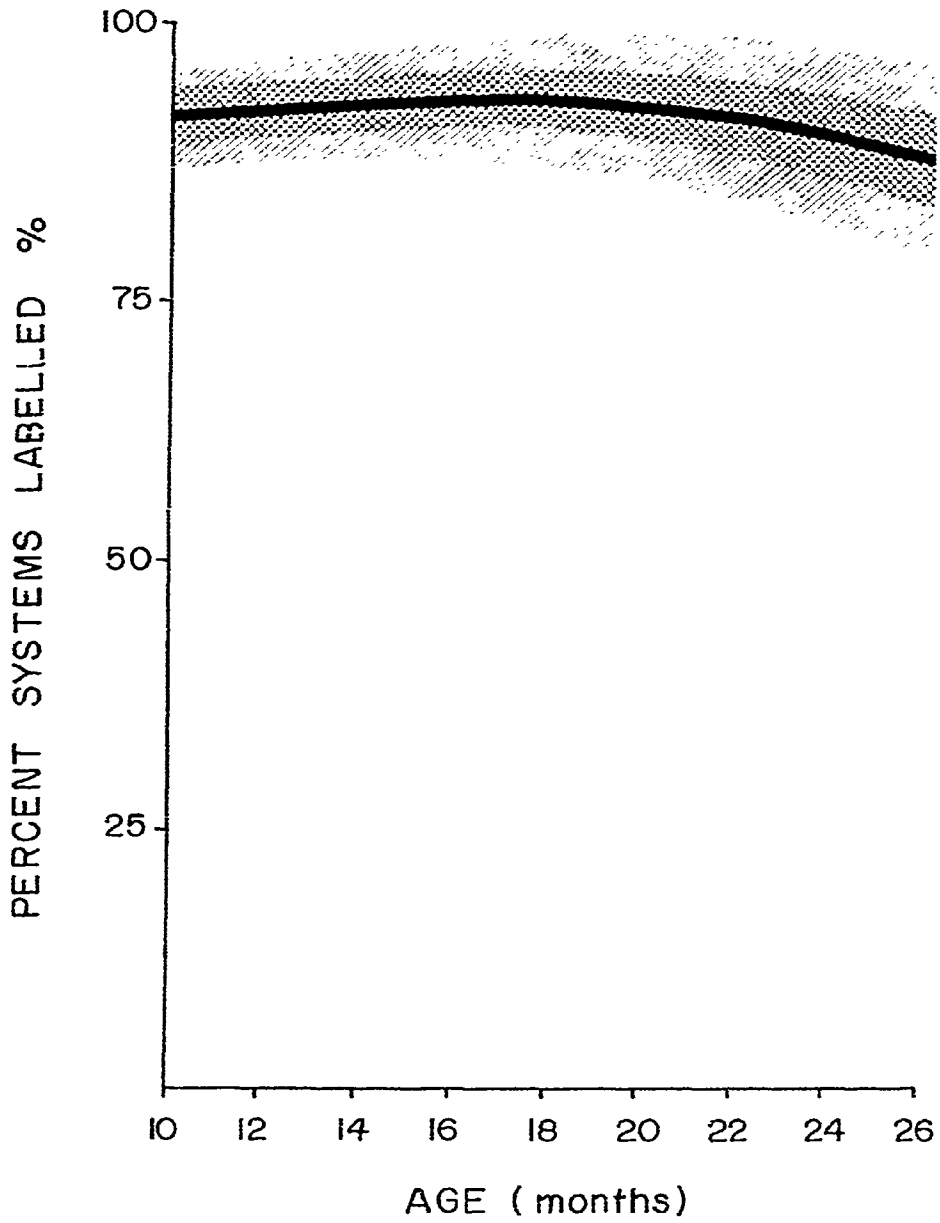

15
10
5
WIDTH OF OSTEOID SEAMS (μ) W.O.S.
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 1(xiii)

PRELIMINARY EXPERIMENT "A"

PERCENT LABELLED SYSTEMS (%)

This graph represents normal age-related changes in the percent of Haversian systems labelled with a fluorescent marker observed as a function of time. Illustrated are the best fit line, the 95% confidence bounds for that line (cross lined area) and the 95% confidence bounds for all individual observations (single lined area).


100
75
50
25
PERCENT SYSTEMS LABELLED %
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 2(i)

QUANTITATIVE HAVERSIAN HISTOLOGICAL MEASUREMENTS ON COMBINED RIBS 5,7,9,11

FOR ANTERIOR, LATERAL, AND POSTERIOR FRAGMENTS

	Anterior		Lateral		Posterior		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	F ratio
A_c (Cortical area/mm^2)	10.99	1.01	10.28	1.41	10.22	.61	1.40
C/T (Ratio cortical-total area)	.67	.05	.66	.04	.76	.03	17.85
A_f (Number of osteoid seams/mm^2)	6.55	.59	4.69	.94	4.00	.90	22.54
A_r (Number of resorption spaces/mm^2)	2.18	.26	1.30	.34	.90	.21	46.85
S_f (Circumference osteoid seams, mm)	.21	.01	.21	.02	.21	.03	.01
M (Appositional rate, microns/day)	1.37	.10	1.40	.16	1.36	.16	.09
M_f (Radial closure rate, mm/yr)	.44	.06	.44	.05	.42	.04	.50
U_f (Activation frequency, foci/yr)	38.33	4.99	27.14	5.59	22.22	5.40	20.64
O_f (Osteon formation time, yrs)	.17	.02	.17	.02	.18	.02	1.00
A_r/A_f (Ratio resorption to formation)	.33	.04	.29	.10	.24	.07	3.27
V_f (Bone formation rate, $mm^2/mm^2/yr$)	.61	.10	.43	.11	.35	.11	12.01
% (Percent labelled systems)	89.63	3.25	86.13	4.09	85.38	4.90	1.84

Significant differences between any two of the means of anterior, lateral and posterior fragment as determined by a one-way analysis of variance. The F ratio with 2 degrees of freedom in the numerator and 21 degrees of freedom in the denominator at the 0.05 confidence level is 3.47.

Significant differences at this confidence level may be seen in the ratio cortical-total area, the number of osteoid seams/mm^2, number of resorption spaces/mm^2, activation frequency and bone formation rate. In order to determine the location and magnitude of these differences these parameters were further examined using the t-test. These results are shown in Appendix 2(ii).

APPENDIX 2(ii)

QUANTITATIVE HAVERSIAN HISTOLOGICAL MEASUREMENTS OF ANTERIOR-LATERAL, LATERAL-POSTERIOR AND ANTERIOR-POSTERIOR FRAGMENTS

	ANTERIOR		LATERAL				
	Mean	S.D.	Mean	S.D.	t	S.E.	P
C/T (Ratio cortical-total area)	.67	.05	.66	.04	.82	.01	N.S.
A_f (Number of osteoid seams/mm^2)	6.55	.59	4.69	.94	4.74	.23	<.001
A_r (Number of resorption spaces/mm^2)	2.18	.26	1.30	.34	5.83	.10	<.001
U_f (Activation frequency, foci/yr)	38.33	4.99	27.14	5.59	4.22	1.45	<.001
V_f (Bone formation rate, mm^2/mm^2/yr)	.61	.10	.43	.11	3.48	.03	<.005

	LATERAL		POSTERIOR				
	Mean	S.D.	Mean	S.D.	t	S.E.	p
C/T (Ratio cortical-total area)	.66	.04	.76	.03	6.24	.01	<.001
A_f (Number of osteoid seams/mm^2)	4.69	.94	4.00	.80	1.59	.17	N.S.
A_r (Number of resorption spaces/mm^2)	1.30	.34	.90	.21	2.84	.17	N.S.
U_f (Activation frequency, foci/yr)	27.14	5.59	22.22	5.40	1.79	1.09	N.S.
V_f (Bone formation rate, mm^2/mm^2/yr)	.43	.11	.35	.11	1.41	.02	N.S.

	ANTERIOR		POSTERIOR				
	Mean	S.D.	Mean	S.D.	t	S.E.	p
C/T (Ratio cortical-total area)	.67	.05	.76	.03	4.57	.01	<.001
A_f (Number of osteoid seams/mm^2)	6.55	.59	4.00	.80	7.26	.27	<.001
A_r (Number of resorption spaces/mm^2)	2.18	.26	.98	.21	10.98	.13	<.001
U_f (Activation frequency, foci/yr)	38.33	4.99	22.22	5.40	6.19	1.77	<.001
V_f (Bone formation rate, mm^2/mm^2/yr)	.61	.10	.36	.11	4.94	.03	<.001

[a]Significant differences between the means of the anterior and lateral, lateral and posterior and anterior and posterior fragments as determined by two-tailed "t" tests, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with 14 degrees of freedom at the 0.05 level is 2.145; the "t" value with 14 degrees of freedom at the 0.005 level is 3.326; and the "t" value with 14 degrees of freedom at the 0.001 level is 4.140.

[b]NS, Not significant

APPENDIX 3(i)

QUANTITATIVE HAVERSIAN HISTOLOGICAL MEASUREMENTS ON COMBINED ANTERIOR, LATERAL, AND POSTERIOR FRAGMENTS FOR RIBS 5,7,9, and 11

	5th		7th		9th		11th		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	F ratio
A_c (Cortical area/mm^2)	9.98	.66	10.07	.85	10.53	.96	11.33	1.17	.15
C/T (Ratio cortical-total area)	.70	.06	.71	.07	.69	.06	.69	.07	1.24
A_f (Number of osteoid seams/mm^2)	4.86	1.24	5.64	1.80	4.88	1.23	4.95	1.18	.51
A_r (Number of resorption spaces/mm^2)	1.27	.56	1.52	.65	1.37	.69	1.68	.61	.32
S_f (Circumference osteoid seams, mm)	.23	.02	.22	.01	.20	.02	.20	.01	1.66
M (Appositional rate, microns/day)	1.52	.15	1.41	.09	1.31	.15	1.26	.14	2.89
M_f (Radial closure rate, mm/yr)	.47	.06	.46	.03	.41	.05	.41	.04	2.90
U_f (Activation frequency, foci/yr)	29.79	10.20	33.45	10.46	27.13	8.02	26.55	4.92	.53
O_f (Osteon formation time, yrs)	.17	.02	.17	.01	.18	.02	.19	.02	.72
A_r/A_f (Ratio resorption to formation)	.25	.04	.27	.07	.28	.09	.35	.07	1.71
V_f (Bone formation rate, $mm^2/mm^2/yr$)	.51	.13	.56	.19	.40	.14	.40	.09	2.70
W.O.S. (Width osteoid seams, microns)	11.15	.75	11.57	1.56	10.84	1.06	11.25	.85	.33
% (Percent labelled systems)	84.67	4.59	89.33	2.80	85.00	5.18	89.17	2.99	2.53

No significant difference between any two means of the 5th, 7th, 9th and 11th rib determined by a one-way analysis of variance was found. The F ratio with 3 degrees of freedom in the numerator and 20 degrees of freedom in the denominator at the 0.05 confidence level is 3.10.

APPENDIX 3(ii)

QUANTITATIVE HAVERSIAN HISTOLOGICAL MEASUREMENTS

OF THE ANTERIOR FRAGMENT OF RIB 5,7,9, AND 11

	5th		7th		9th		11th		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	F ratio
A_c (Cortical area/mm^2)	9.71	.41	10.95	.92	11.71	.29	11.59	1.00	4.43
C/T (Ratio cortical-total area)	.67	.04	.73	.04	.66	.04	.64	.01	1.00
A_f (Number of osteoid seams/mm^2)	6.27	.20	7.18	1.01	6.38	.48	6.39	.35	1.13
A_r (Number of resorption spaces/mm^2)	1.97	.25	2.29	.22	2.12	.35	2.35	.24	1.00
S_f (Circumference osteoid seams, mm)	.21	0	.22	.01	.22	.01	.21	.02	.90
M (Appositional rate, microns/day)	1.58	.21	1.40	.07	1.28	.21	1.21	.03	4.17
M_f (Radial closure rate, mm/yr)	.50	.07	.47	.04	.42	.08	.39	.02	1.59
U_f (Activation frequency, foci/yr)	40.63	4.45	43.11	2.79	36.93	4.71	46.34	16.19	.38
O_f (Osteon formation time, yrs)	.16	.02	.17	.01	.18	.04	.20	.01	.35
A_r/A_f (Ratio resorption to formation)	.30	.20	.31	.01	.34	.04	.38	.02	4.47
V_f (Bone formation rate, $mm^2/mm^2/yr$)	.65	.06	.73	.08	.57	.06	.51	.05	1.02
W.O.S. (Width osteoid seams, microns)	11.37	.37	10.70	.50	11.18	.70	11.13	.93	.81
% (Percent labelled systems)	89.50	4.95	91.50	2.13	89.50	4.95	88.00	2.83	.56

No significant difference between any two means of the anterior fragment of the 5th, 7th, 9th and 11th rib determined by a one-way analysis of variance was found. The F ratio with 3 degrees of freedom in the numerator and 4 degrees of freedom in the denominator at the 0.05 confidence level is 6.59.

APPENDIX 3(iii)

QUANTITATIVE HAVERSIAN HISTOLOGICAL MEASUREMENTS OF THE LATERAL FRAGMENT OF RIB 5,7,9, AND 11

	5th		7th		9th		11th		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	F ratio
A_c (Cortical area/mm^2)	9.92	.77	9.46	.12	9.84	.12	11.68	2.09	1.25
C/T (Ratio cortical-total area)	.68	.03	.63	.06	.66	.04	.65	.04	.46
A_f (Number of osteoid seams/mm^2)	4.74	.21	5.55	1.91	4.22	.18	4.25	.37	.72
A_r (Number of resorption spaces/mm^2)	.95	.06	1.37	.22	1.23	.47	1.66	.06	3.30
S_f (Circumference osteoid seams, mm)	.23	.02	.21	.01	.22	.02	.20	.02	1.30
M (Appositional rate, microns/day)	1.59	.12	1.39	.06	1.24	.13	1.37	.16	.69
M_f (Radial closure rate, mm/yr)	.49	.05	.45	.04	.38	.05	.44	.03	2.03
U_f (Activation frequency, foci/yr)	29.83	4.22	31.85	8.56	21.89	2.10	24.99	.12	1.67
O_f (Osteon formation time, yrs)	.16	.01	.17	.01	.20	.02	.17	.01	.50
A_r/A_f (Ratio resorption to formation)	.21	.02	.26	.05	.31	.13	.40	.06	2.01
V_f (Bone formation rate, mm^2/mm^2/yr)	.52	.04	.51	.15	.34	.05	.37	.05	2.37
W.O.S. (Width osteoid seams, microns	10.93	.36	11.56	.49	10.40	.23	11.64	.36	1.89
% (Percent labelled systems)	83.50	2.12	88.50	3.54	83.00	2.83	89.50	4.95	1.77

No significant difference between any two means of the lateral fragment of the 5th, 7th, 9th and 11th rib determined by a one-way analysis of variance was found. The F ratio with 3 degrees of freedom in the numerator and 4 degrees of freedom in the denominator at the 0.05 confidence level is 6.59.

APPENDIX 3(iv)

QUANTITATIVE HAVERSIAN HISTOLOGICAL MEASUREMENTS

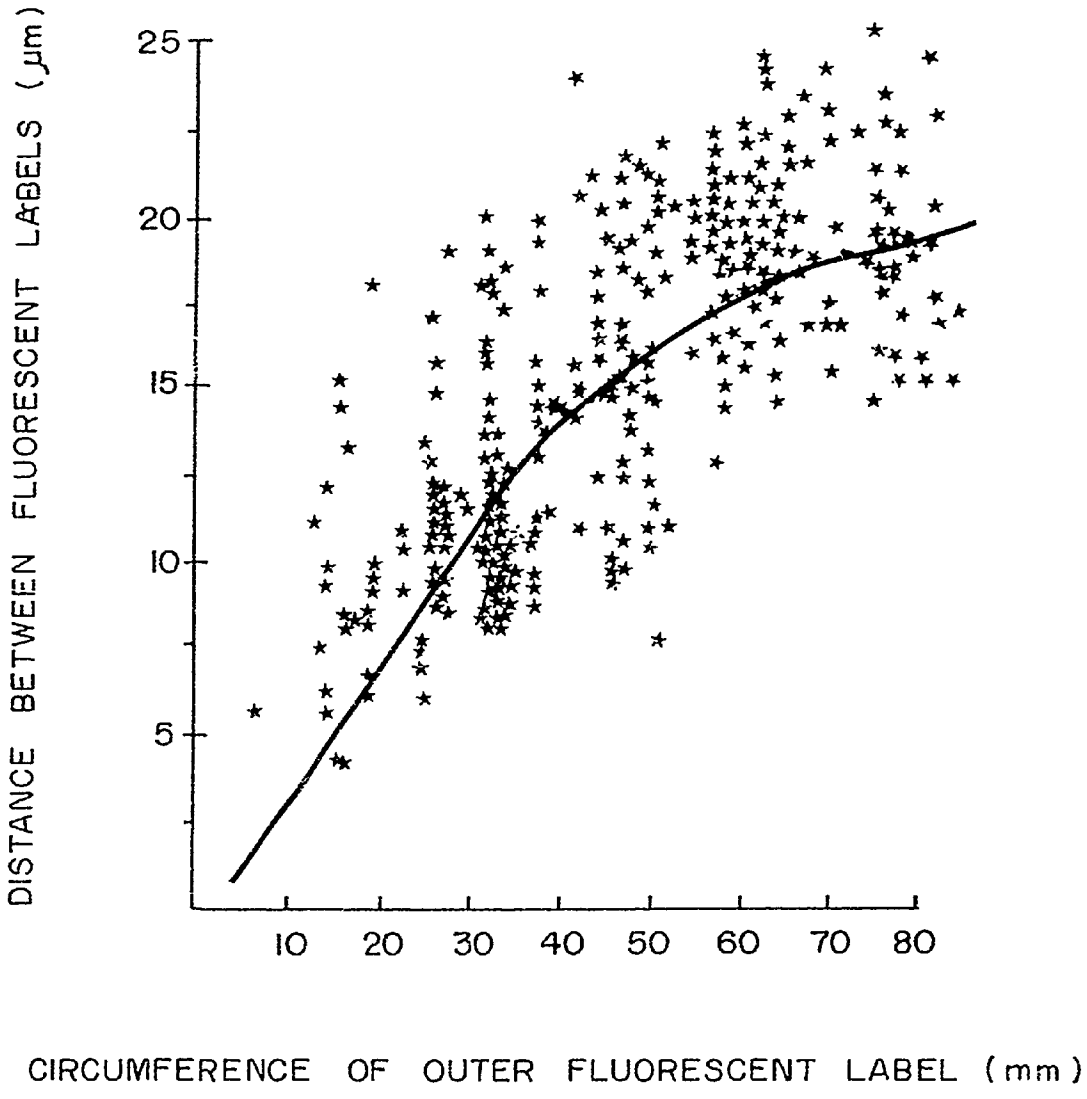
OF THE POSTERIOR FRAGMENT OF RIB 5,7,9, AND 11

	5th		7th		9th		11th		
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	F ratio
A_c (Cortical area/mm^2)	10.32	1.01	9.80	.54	10.06	.24	10.72	.57	1.97
C/T (Ratio cortical-total area)	.75	.01	.76	.01	.76	.01	.78	.03	.50
A_f (Number of osteoid seams/mm^2)	3.56	.47	4.18	1.61	4.05	.74	4.21	.74	.75
A_r (Number of resorption spaces/mm^2)	.83	.11	.90	.16	.78	.36	1.03	.23	1.19
S_f (Circumference osteoid seams, mm)	.23	.01	.22	.01	.18	.00	.20	.01	4.33
M (Appositional rate, microns/day)	1.39	.05	1.45	.18	1.42	.16	1.21	.21	1.07
M_f (Radial closure rate, mm/yr)	.41	.01	.46	.04	.42	.01	.40	.05	1.45
U_f (Activation frequency, foci/yr)	18.92	3.30	25.38	12.02	22.57	2.56	22.02	.48	.29
O_f (Osteon formation time, yrs)	.19	.01	.17	.02	.18	.01	.19	.03	.50
A_r/A_f (Ratio resorption to formation)	.25	.01	.25	.13	.19	.04	.28	.08	.43
V_f (Bone formation rate, $mm^2/mm^2/yr$)	.37	.08	.44	.22	.31	.05	.32	.04	.92
W.O.S. (Width osteoid seams, microns)	11.16	1.54	12.47	2.92	11.56	1.27	10.99	1.47	.71
% (Percent labelled systems)	81.50	.71	88.00	2.83	82.50	6.36	90.00	2.83	1.65

No significant difference between any two means of the posterior fragment of the 5th, 7th, 9th and 11th rib determined by a one-way analysis of variance was found. The F ratio was 3 degrees of freedom in the numerator and 4 degrees of freedom in the denominator at the 0.05 confidence level is 6.59.

APPENDIX 4(i)

The relationship between the outer circumference of the fluorescent label and the distance between the two fluorescent labels is illustrated in this scattergram. The distance between the inner and outer labels remained fairly constant when the circumference of the outer label was greater than 0.35 mm.


DISTANCE BETWEEN FLUORESCENT LABELS (μm)
25
20
15
10
5
10
20
30
40
50
60
70
80
CIRCUMFERENCE OF OUTER FLUORESCENT LABEL (mm)

APPENDIX 5(i)

HAEMATOLOGICAL DATA FOR CADMIUM DOGS

Dog	FU26	FV26	XN36	XO36	Normal Values*
White Blood Cell x 10^9/l	7.3	8.5	12.1	16.0	11.6±3.4
Red Blood Cell x 10^{12}/l	6.9	6.3	6.6	7.1	7.0±0.5
Haemoglobin g/dl	16.3	15.2	15.3	16.1	14.8±2.4
Hematocrit %	45.1	41.8	44.6	43.4	49.9±4.1
Mean Corpuscular Volume Fl	64.5	66.0	65.0	61.7	67.0±5.5
Mean Corpuscular Haemoglobin pg	23.5	23.8	23.1	22.6	23.0±1.5
Mean Corpuscular Haemoglobin Concentration g/dl	37.7	35.9	35.4	36.8	35.0±2.5
Differential					
Neutropnil	59.5	64.0	73.0	67.0	
Lymphocyte	25.0	26.3	21.3	23.3	
Monocyte	11.5	7.0	3.0	4.7	
Eosinophil	4.0	3.0	2.7	5.0	
Serum Iron ug/100ml	207	170	198	205	65-175
Total Iron Binding Capacity ug/100ml	403	414	394	388	250-410
Percent Saturation of Transferrin %	51%	41%	50%	53%	20-55%

This table shows the averages of the haematological results obtained on peripheral blood examination of each dog during the control and experimental period.

*The normal haematological values for the Beagle dog were obtained from Andersen (1970).

APPENDIX 5(ii)

BIOCHEMICAL DATA FOR CADMIUM DOGS

	XN36					XO36					FU26					FV26				
	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range
Sodium	147	2.30	144.0	150.0	6.0	147	2.14	145.0	151.0	6.0	147	2.18	145.0	151.0	6.0	146	2.36	145.0	152.0	7.0
Potassium	4.2	0.19	4.0	4.5	0.5	4.2	0.08	3.9	4.5	0.6	4.4	0.38	4.0	5.2	1.2	4.5	0.47	3.9	5.3	1.4
Chloride	108	1.60	106.0	110.0	4.0	108	3.60	102.0	114.0	12.0	109	1.66	106.0	112.0	6.0	108	3.25	104.0	113.0	9.0
Carbon dioxide	24.1	2.92	20.0	27.5	7.5	24.2	2.07	22.0	28.0	6.0	24.0	0.98	22.0	25.0	3.0	23.6	2.63	20.0	28.8	8.8
Blood urea nitrogen	14	4.12	12.0	23.0	11.0	21	8	15.0	39.0	24.0	12	4.61	10.0	24.5	14.5	12.	4.08	9.0	20.0	11.0
Creatinine	0.8	0.11	0.7	1.0	0.3	0.9	0.09	0.8	1.0	0.2	0.8	0.17	0.5	1.0	0.5	0.8	0.31	0.0	1.2	1.2
Total protein	6.0	0.60	4.7	6.4	1.7	6.0	0.38	5.6	6.5	0.9	6.2	0.24	6.0	6.8	0.8	5.6	0.40	5.2	6.5	1.3
Albumin	3.0	0.21	2.6	3.2	0.6	3.3	0.92	2.6	4.9	2.3	2.8	0.07	2.7	2.9	0.2	2.9	0.30	2.6	3.6	1.0
Calcium	10.3	0.45	9.8	11.0	1.2	27.9	41.72	9.8	113.0	103.2	10.9	1.57	9.2	13.8	4.6	10.4	0.66	9.7	12.0	2.3
Phosphate	4.2	0.89	3.4	5.8	2.4	3.6	0.56	3.1	4.6	1.5	4.2	1.07	3.3	6.7	3.4	4.4	0.97	2.8	5.9	3.1
Glucose	86	17.97	51.0	109.0	58.0	87	13.58	61.0	100.0	39.0	93	13.40	68.0	110.0	42.0	96	15.44	73.0	129.0	56.0
Uric acid	0.7	0.37	0.3	1.3	1.0	0.3	0.14	0.1	0.4	0.4	0.6	0.31	0.3	1.2	0.9	0.6	0.61	0.1	1.8	1.7
Total bilirubin	0.4	0.57	0.1	1.4	1.3	0.2	0.24	0.0	0.6	0.6	0.2	0.20	0.1	0.7	0.6	0.2	0.21	0.0	0.6	0.6
Alkaline phosphatase	41	11.12	27.0	53.0	26.0	43	5.90	37.0	55.0	18.0	35	20.01	22.0	86.0	64.0	42.	22.44	29.0	100.0	71.0

The units and the normal biochemical data for the Beagle is listed in Appendix 10(i).

APPENDIX 5(iii)

CIRCULATING LEVELS OF $1,25(OH)_2D_3$ IN CADMIUM TREATED DOGS IN pg/ml PLASMA

DOG	CONTROL MEASUREMENT	1st EXPERIMENTAL MEASUREMENT	2nd EXPERIMENTAL MEASUREMENT
	pg $1,25(OH)_2D_3$/ ml plasma	pg $1,25(OH)_2D_3$/ ml plasma	pg $1,25(OH)_2D_3$/ ml plasma
FU26	9	13	27
FV26	22	21	24
XN36	17	20	17
XO36	10	17	19

Plasma levels of $1,25(OH)_2D_3$ were determined by the competitive binding assay technique according to the method of Eisman et al (1976).

APPENDIX 5(iv)

CIRCULATING LEVELS OF CANINE ANTI-BOVINE PARATHYROID HORMONE IN CADMIUM TREATED DOGS

(micro liter equivalents/ml)

DOG	1st BIOPSY	2nd BIOPSY	3rd BIOPSY
XO36	less than 20	less than 20	37
XN36	less than 20	18	28
FU26	less than 20	17	*
FV26	less than 20	20	*

Circulating levels of canine anti-bovine iPTH were determined using the method described by Hruska et al (1975).

* These samples were lost during iPTH measurement.

APPENDIX 5(v)

CADMIUM EXPERIMENT

BONE CADMIUM CONCENTRATION IN THE CADMIUM TREATED DOGS (µg/g)

DOG	CONTROL BIOPSY	1st EXPERIMENTAL BIOPSY	2nd EXPERIMENTAL BIOPSY
FV26	1.20	1.57	1.62
FU26	1.42	1.85	1.58
XN36	4.69	2.27	1.32
XO36	0.70	0.49	2.41

Bone cadmium concentration measured from ribs of 18 untreated dogs was found to be 1.96 $\pm$ 2.30 µg/g.

APPENDIX 5(vi)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM CADMIUM TREATED DOGS

	Control Biopsy		1st Experimental Biopsy		2nd Experimental Biopsy		F Ratio
A_c (Cortical area/mm^2)	9.53	1.15	12.25	.79	11.68	1.67	5.2095*
C/T (Ratio cortical-total area)	.68	.02	.73	.04	.63	.05	7.9713*
A_f (Number of osteoid seams/mm^2)	6.45	1.06	1.25	.24	2.11	.76	52.53*
A_r (Number of resorption spaces/mm^2)	1.57	.21	.62	.12	.88	.30	19.5470*
S_f (Circumference osteoid seams, mm)	.20	.01	.15	.01	.22	.02	39.9783*
M (Appositional rate, microns/day)	1.41	.13	1.04	.07	1.09	.05	18.9872*
M_f (Radial closure rate, mm/yr)	.49	.04	.37	.02	.34	.02	27.1770*
U_f (Activation frequency, foci/yr)	43.67	7.76	6.83	1.49	10.81	2.72	70.2919*
O_f (Osteon formation time, yrs)	.15	.01	.19	0.15	.20	.02	7.6525*
A_r/A_f (Ratio resorption to formation)	.25	.05	.53	.03	.48	.27	3.3592
V_f (Bone formation rate, mm^2/mm^2/yr)	.63	.15	.07	.02	.16	.06	37.9991*
W.O.S. (Width osteoid seams, microns)	7.29	.48	8.41	.87	8.77	1.47	2.1125
% (Percent labelled systems)	93.53	.68	99.47	.75	86.67	2.36	32.9485*
W.T. (Wall thickness, µm)	70.64	94.23	77.37	3.39	67.90	8.19	2.9467

Significant differences between any two of the means of the control biopsy, first experimental biopsy and the second experimental biopsy as determined by a one-way analysis of variance. The F ratio the 2 degrees of freedom in the numerator and 9 degrees of freedom in the denominator at the 0.05 confidence level is 4.26.

*Statistical significant at 0.05 confidence interval between any two of the means.

APPENDIX 5(vii)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM CADMIUM TREATED DOGS

	Control		1st Experimental				
	Mean	S.D.	Mean	S.D.	S.E.[a]	t	p
A_c (Cortical area/mm^2)	9.53	1.15	12.25	0.80	0.29	9.49	<.005
C/T (Ratio cortical-total area)	0.68	0.02	0.73	0.04	0.02	2.82	N.S.[b]
A_f (Number of osteoid seams/mm^2)	6.45	1.06	1.25	0.24	0.60	8.74	<.005
A_r (Number of resorption spaces/mm^2)	1.57	.21	0.62	0.12	0.14	6.81	<.01
Sf (Circumference osteoid seams, mm)	.20	.01	.15	.01	.01	6.23	<.01
M (Appositional rate, microns/day)	1.42	.13	1.04	.08	.08	4.99	<.02
M_f (Radial closure rate, mm/yr)	0.49	.04	.37	.02	.03	4.23	<.025
U_f (Activation frequency, foci/yr)	43.67	7.76	6.83	1.49	4.12	8.95	<.005
O_f (Osteon formation time, yrs)	.15	.01	.19	.02	.01	4.39	<.025
A_r/A_f (Ratio resorption to formation)	.63	.15	.07	.02	.08	6.88	<.01
V_f (Bone formation rate, $mm^2/mm^2/yr$)	93.53	.68	94.75	1.26	.86	1.42	N.S.
% (Percent labelled systems)	.25	.05	.53	.03	.01	26.42	<.001

[a]The significant difference between the means of the control biopsy and the first experimental biopsy as determined by a two-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with three degrees of freedom at the 0.025 confidence level is 4.1765, the "t" value with three degrees of freedom at the 0.02 confidence level is 4.541, the "t" value with three degrees of freedom at the 0.01 confidence level is 5.8409, the "t" value with three degrees of freedom at the 0.005 confidence level is 7.4533, and the "t" value with three degrees of freedom at the 0.001 confidence level is 12.924.

[b]NS, Not significant.

APPENDIX 5(viii)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM CADMIUM TREATED DOGS

	1st Experimental		2nd Experimental				
	Mean	S.D.	Mean	S.D.	S.E.[a]	t	p
A_c (Cortical area/mm^2)	12.25	.80	11.66	1.62	0.72	.81	N.S.[b]
C/T (Ratio cortical-total area)	.73	.04	.63	.05	.02	6.51	<0.1
A_f (Number of osteoid seams/mm^2)	1.25	.24	2.11	.77	.47	1.83	N.S.
A_r (Number of resorption spaces/mm^2)	.62	.12	.88	.30	.13	2.01	N.S.
S_f (Circumference osteoid seams, mm)	.15	.01	.22	.02	.01	6.48	<0.1
M (Appositional rate, microns/day)	1.04	.08	1.09	.05	.06	.84	N.S.
M_f (Radial closure rate, mm/yr)	.36	.02	.34	.02	.02	1.36	N.S.
U_f (Activation frequency, foci/yr)	6.83	1.49	10.81	2.72	1.92	2.06	N.S.
O_f (Osteon formation time, yrs)	.19	.02	.20	.02	.02	0.58	N.S.
A_r/A_f (Ratio resorption to formation)	.53	.03	.48	.27	.15	.32	N.S.
V_f (Bone formation rate, mm^2/mm^2/yr)	.07	.02	.16	.06	.03	3.40	<.05
% (Percent labelled systems)	94.47	.75	85.67	2.16	1.45	6.07	<.01

[a]The significant difference between the means of the first experimental biopsy and the second experimental biopsy as determined by a two-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with three degrees of freedom at the 0.05 confidence level is 3.1825, and the "t" value with three degrees of freedom at the 0.01 confidence level is 5.8409.

[b]NS, Not significant.

APPENDIX 5(ix)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM CADMIUM TREATED DOGS

	Control Biopsy		2nd Experimental				
	Mean	S.D.	Mean	S.D.	S.E.[a]	t	p
A_c (Cortical area/mm^2)	9.53	1.15	11.68	1.67	1.01	2.13	N.S.[b]
C/T (Ratio cortical-total area)	.68	.02	.63	.05	.02	2.30	N.S.
A_f (Number of osteoid seams/mm^2)	6.45	1.06	2.11	.77	.76	5.74	<.02
A_r (Number of resorption spaces/mm^2)	1.57	.21	.88	.30	.24	2.80	N.S.
S_f (Circumference osteoid seams, mm)	.20	.01	.22	.02	.01	2.18	N.S.
M (Appositional rate, microns/day)	1.42	.13	1.09	.05	.07	4.65	<.02
M_f (Radial closure rate, mm/yr)	.49	.04	.34	.02	.02	6.16	<.01
U_f (Activation frequency, foci/yr)	43.67	7.76	10.81	2.72	4.77	6.89	<.01
O_f (Osteon formation time, yrs)	.15	.01	.20	.02	.01	4.28	<.025
A_r/A_f (Ratio resorption to formation)	.25	.05	.48	.27	.15	1.47	N.S.
V_f (Bone formation rate, mm^2/mm^2/yr	.63	.15	.16	.06	.10	4.55	<.02
% (Percent labelled systems)	93.53	.68	85.67	2.16	.99	7.94	<.005

[a]The significant difference between the means of the control biopsy and the second experimental biopsy as determined by a two-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with three degrees of freedom at the 0.025 confidence level is 4.1765, the "t" value with three degrees of freedom at the 0.02 confidence level is 4.541, the "t" value with three degrees of freedom at the 0.01 confidence level is 5.8409 and the "t" value with three degrees of freedom at the 0.005 confidence level is 7.4533.

[b]NS, Not significant

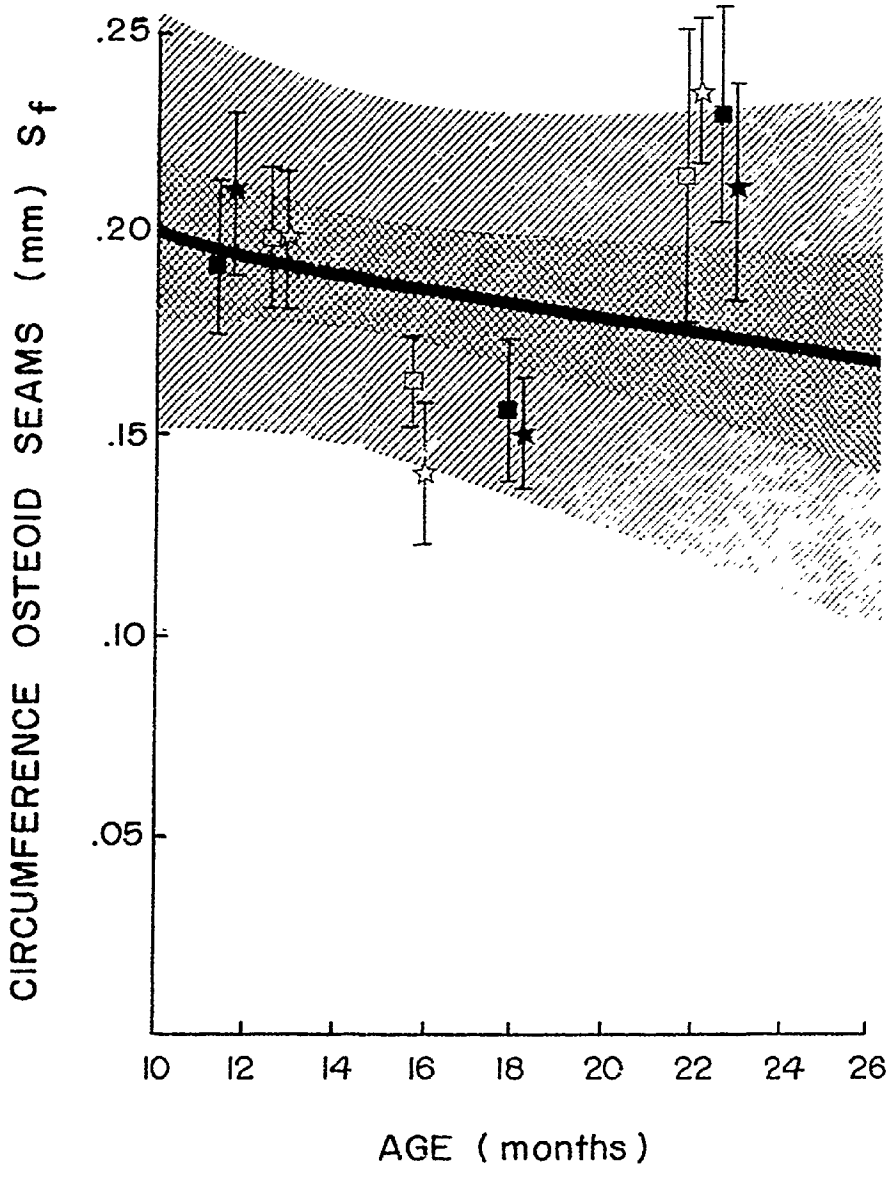
APPENDIX S(x)

CADMIUM EXPERIMENT

CIRCUMFERENCE OSTEOID SEAMS, mm (S_f)

■ Dog FV26
★ Dog FU26
□ Dog XO36
☆ Dog X!36

There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


.25
.20
.15
.10
.05
CIRCUMFERENCE OSTEOID SEAMS (mm) S_f
10
12
14
16
18
20
22
24
26
AGE (months)

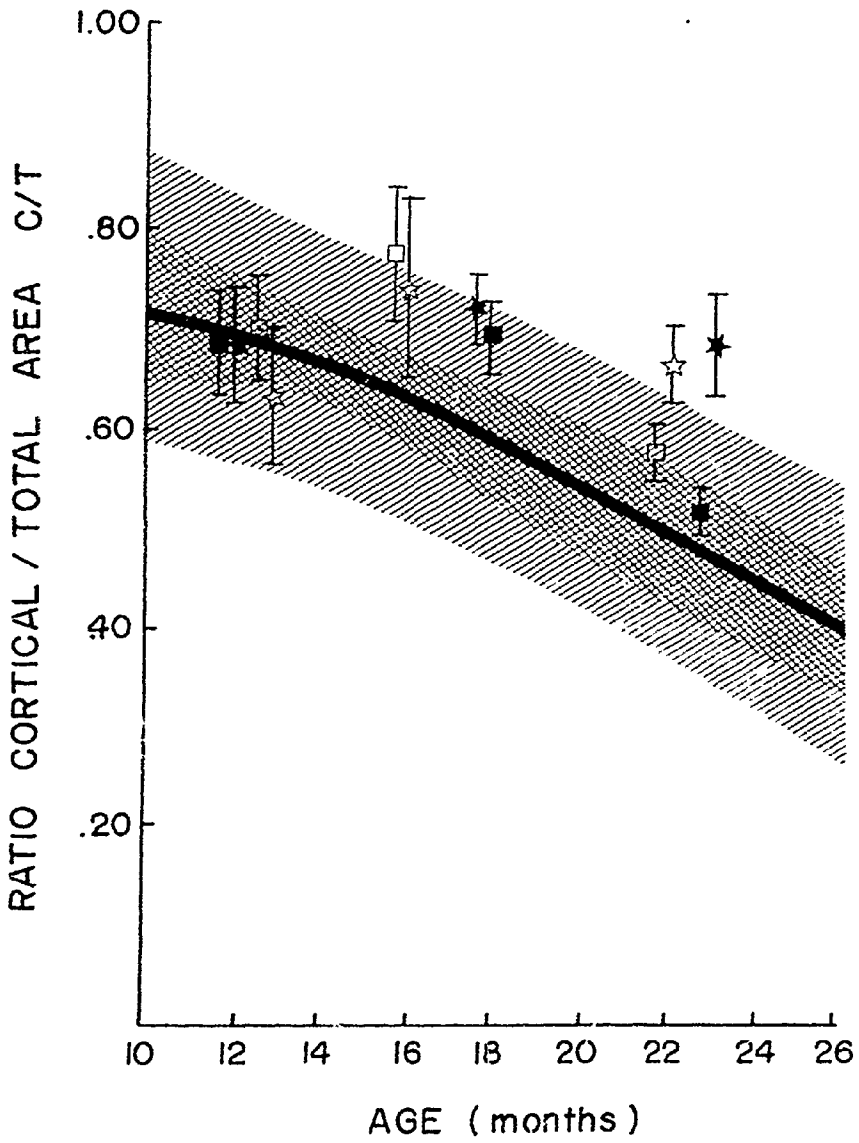
APPENDIX 5(xi)

CADMIUM EXPERIMENT

RATIO CORTICAL/TOTAL AREA (C/T)

■ Dog FV26
★ Dog FU26
□ Dog XO36
☆ Dog XN36

There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


1.00
.80
.60
.40
.20
RATIO CORTICAL / TOTAL AREA C/T
10
12
14
16
18
20
22
24
26
AGE (months)

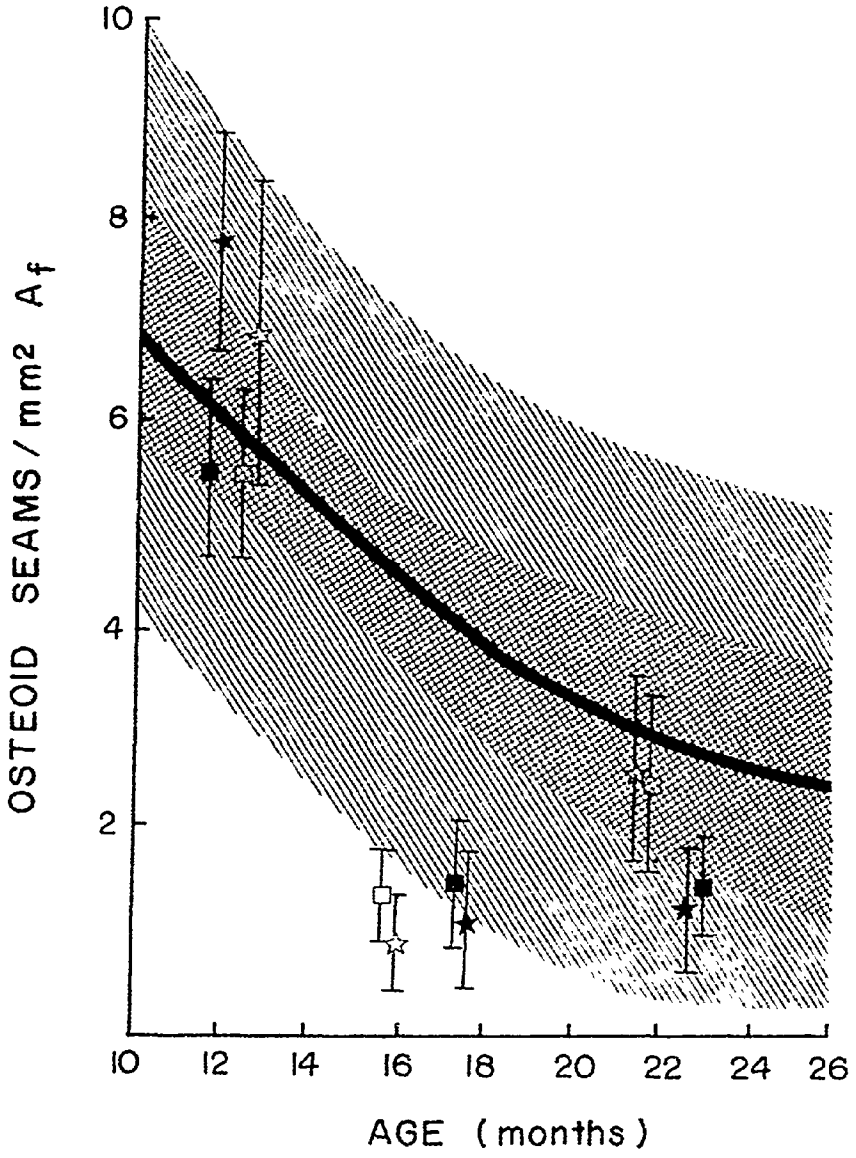
APPENDIX 5(xii)

CADMIUM EXPERIMENT

NUMBER OF OSTEOID SEAMS/mm^2 (A_f)

■ Dog FV26
★ Dog FU26
□ Dog XO36
☆ Dog XN36

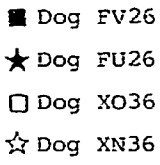
There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


OSTEOID SEAMS / mm2 Af
10
8
6
4
2
10
12
14
16
18
20
22
24
26
AGE (months)

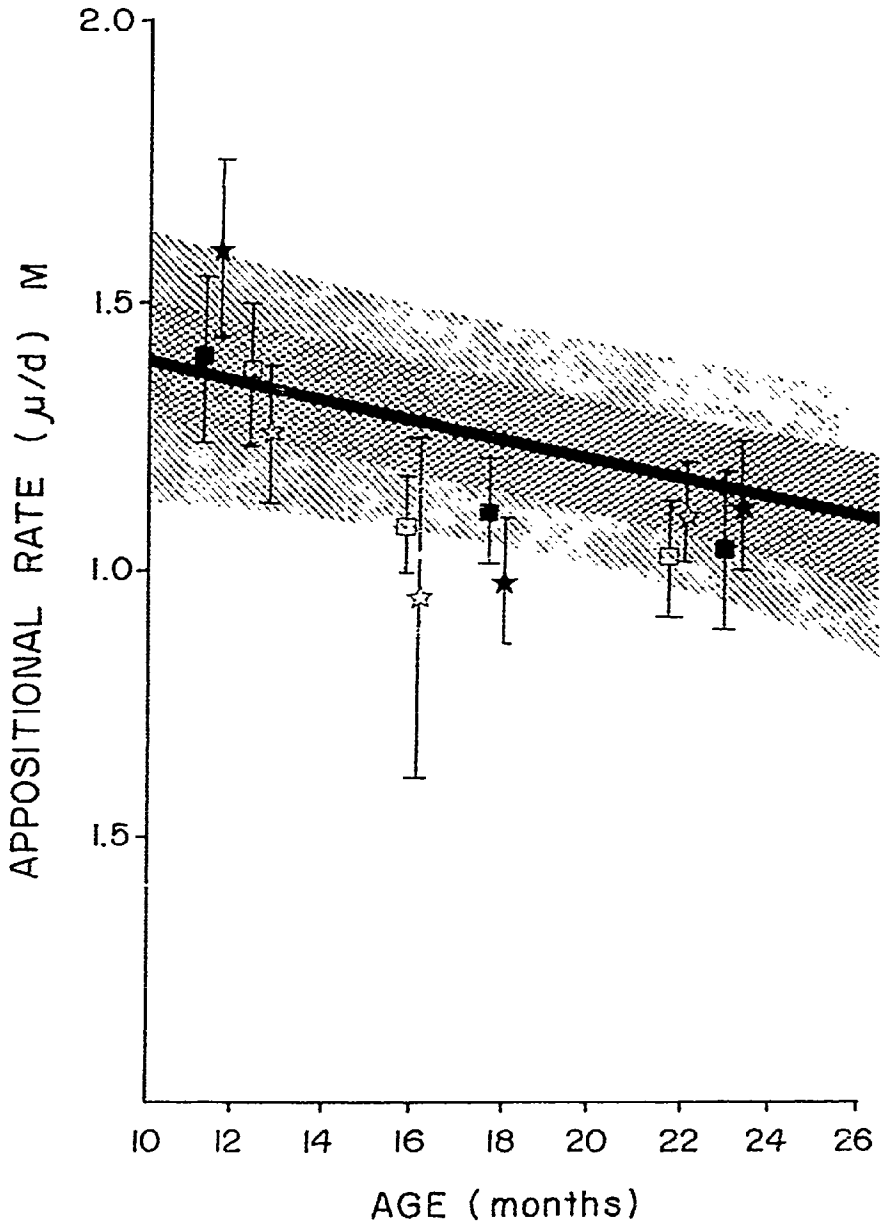
APPENDIX 5 (xiii)

CADMIUM EXPERIMENT

APPOSITIONAL RATE, microns/day (M)



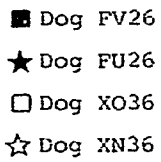
There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


2.0
1.5
1.0
1.5
APPOSITIONAL RATE (μ/d) M
10
12
14
16
18
20
22
24
26
AGE (months)

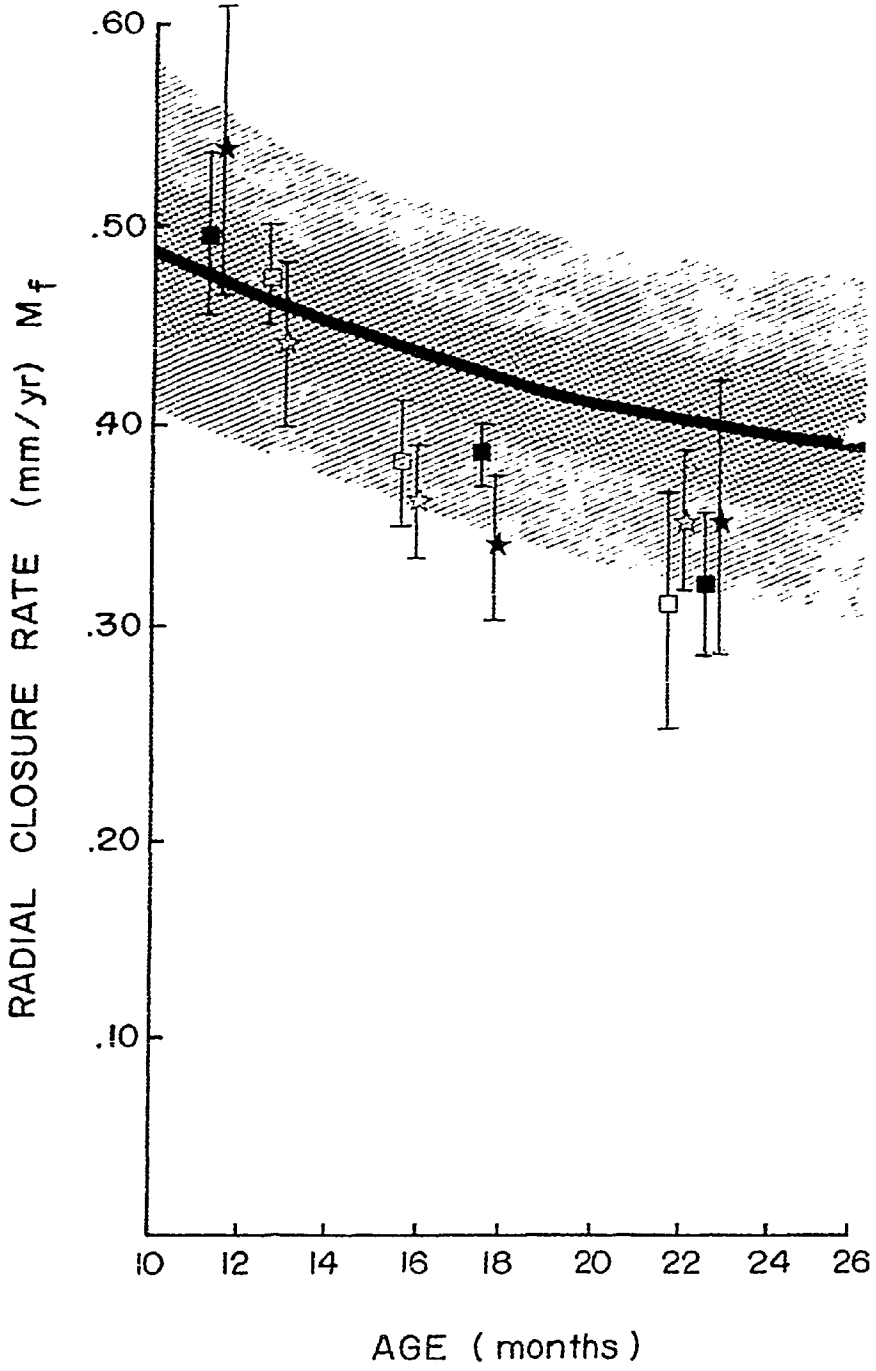
APPENDIX 5(xiv)

CADMIUM EXPERIMENT

RADIAL CLOSURE RATE, mm/yr (M_f)



There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


.60
.50
.40
.30
.20
.10
RADIAL CLOSURE RATE (mm/yr) M_f
10
12
14
16
18
20
22
24
26
AGE (months)

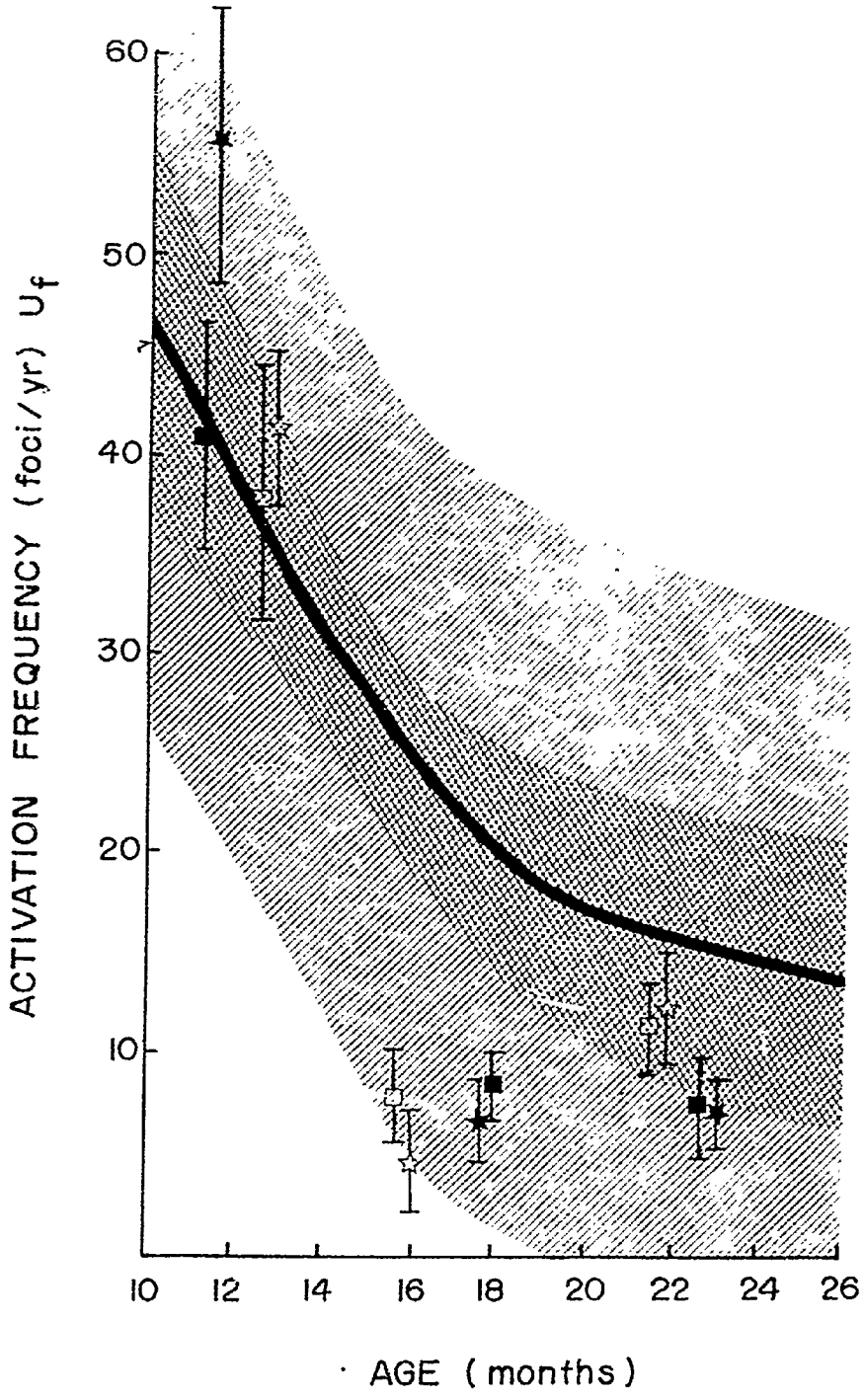
APPENDIX 5(xv)

CADMIUM EXPERIMENT

ACTIVATION FREQUENCY, foci/yr (U_f)

■ Dog FV26
★ Dog FU26
☆ Dog XO36
□ Dog XN36

There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


ACTIVATION FREQUENCY (foci/yr) U_f
60
50
40
30
20
10
10
12
14
16
18
20
22
24
26
AGE (months)

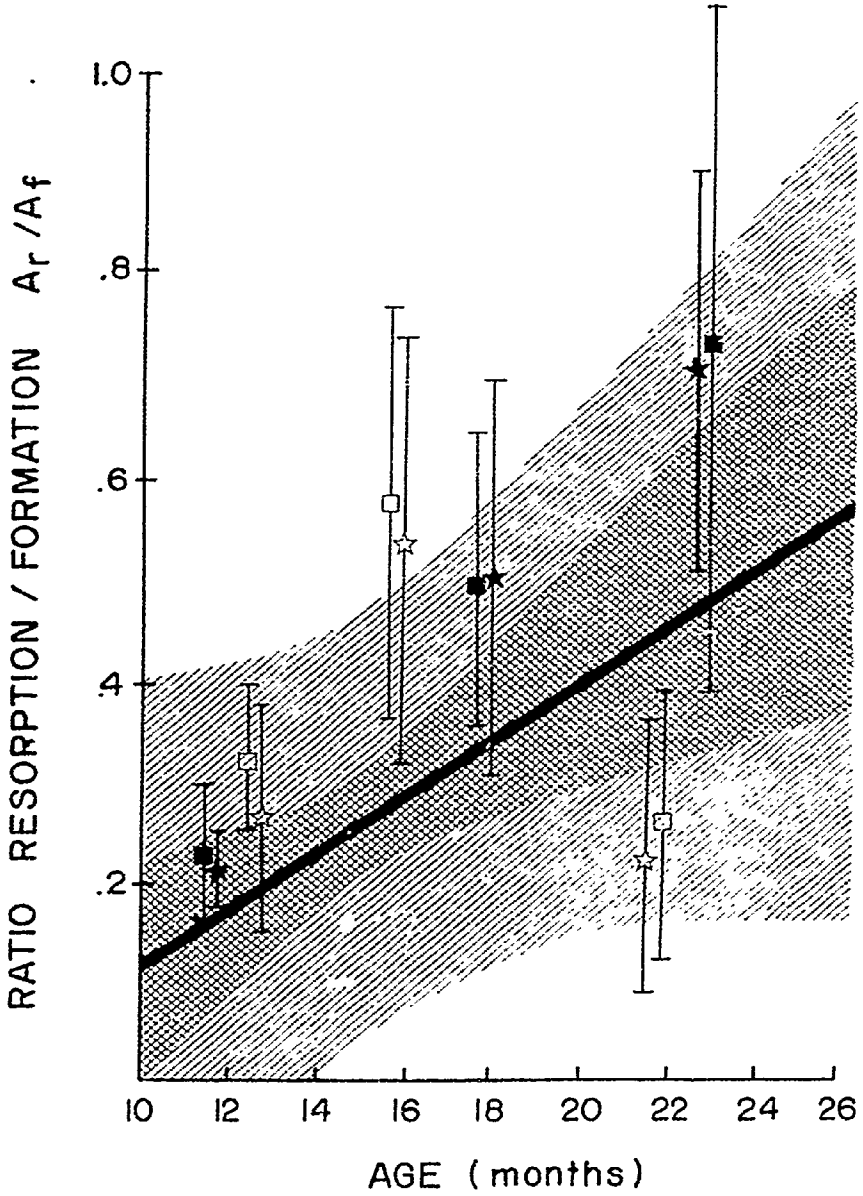
APPENDIX 5(xvi)

CADMIUM EXPERIMENT

RATIO RESORPTION TO FORMATION (A_r/A_f)

■ Dog FV26
★ Dog FU26
□ Dog XO36
☆ Dog XN36

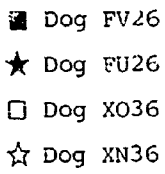
There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


RATIO RESORPTION / FORMATION A_r/A_f
1.0
.8
.6
.4
.2
10
12
14
16
18
20
22
24
26
AGE (months)

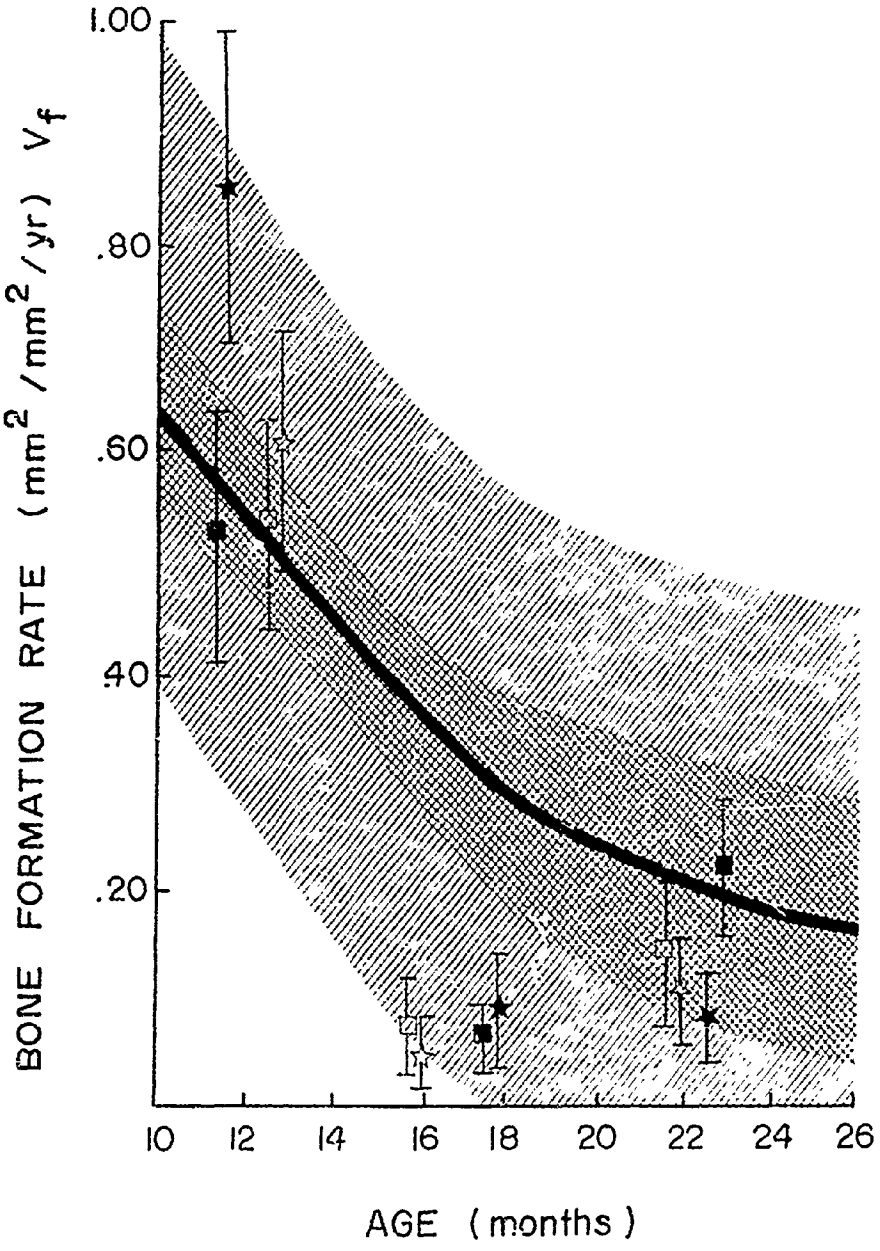
APPENDIX 5(xvii)

CADMIUM EXPERIMENT

BONE FORMATION RATE, $mm^2/mm^2/yr$ (V_f)



There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


1.00
.80
.60
.40
.20
BONE FORMATION RATE (mm²/mm²/yr) Vf
10
12
14
16
18
20
22
24
26
AGE (months)

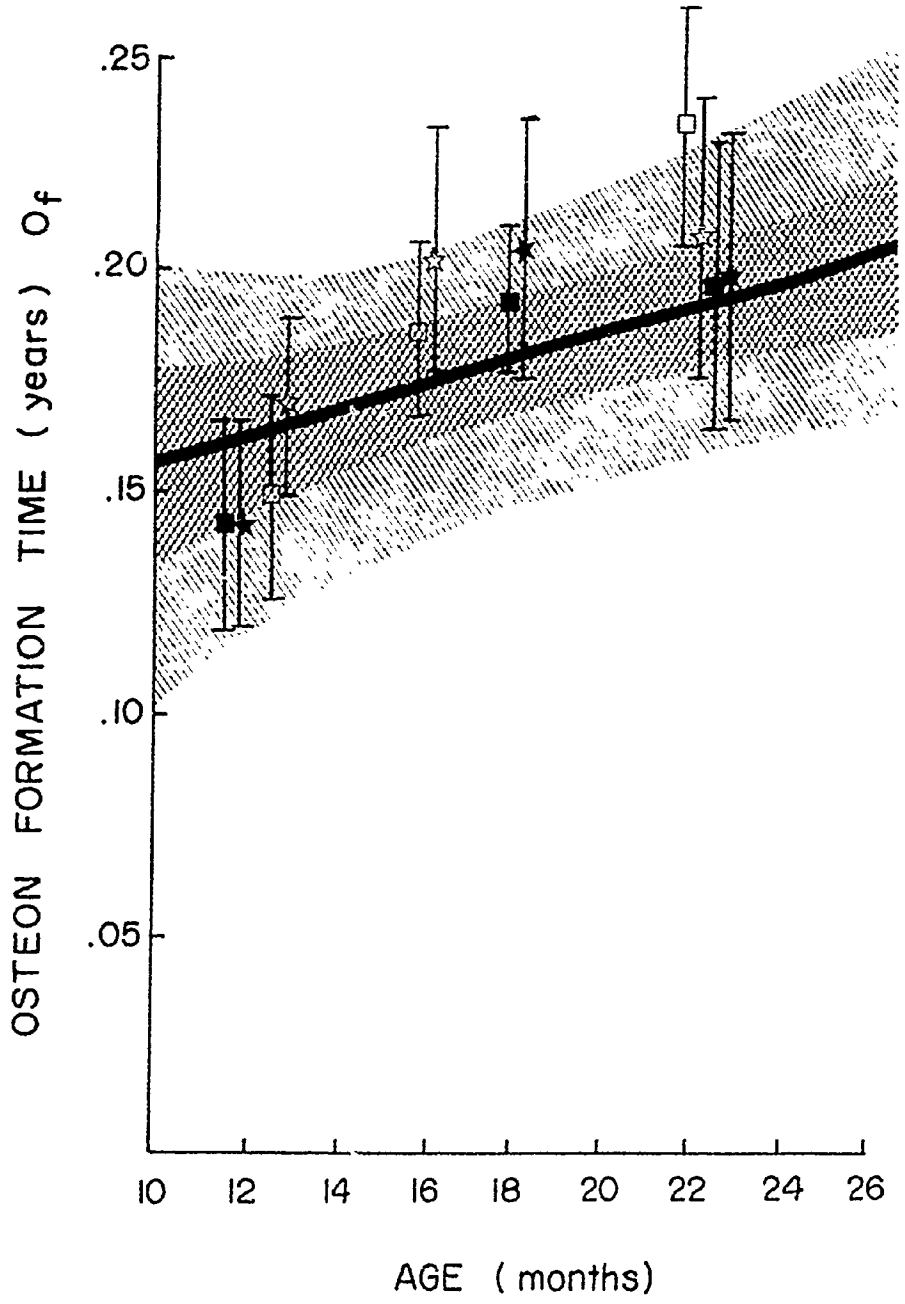
APPENDIX 5(xviii)

CADMIUM EXPERIMENT

OSTEON FORMATION TIME, yrs (O_f)

■ Dog FV26
★ Dog FU26
□ Dog XO36
☆ Dog XN36

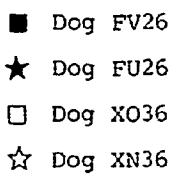
There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


OSTEON FORMATION TIME (years) O_f
.25
.20
.15
.10
.05
10
12
14
16
18
20
22
24
26
AGE (months)

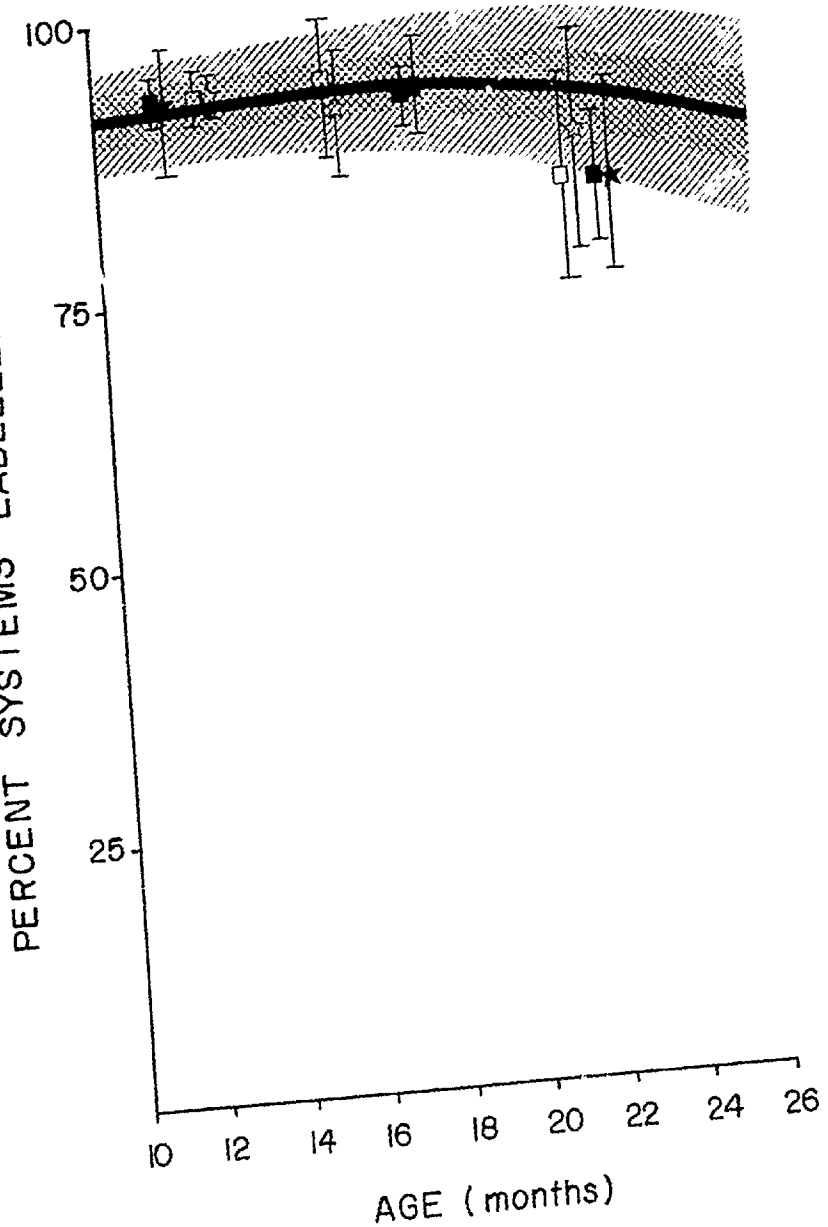
APPENDIX 5(xix)

CADMIUM EXPERIMENT

PERCENT LABELLED SYSTEMS (%)



There are three points in this graph for each dog. During the period between the first and second points all dogs were exposed to 25 ppm $CdCl_2$. During the period between the second and third points all dogs were exposed to 10 ppm $CdCl_2$.


100
75
50
25
PERCENT SYSTEMS LABELLED %
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 6(i)

LEAD EXPERIMENT 'A'

BLOOD LEAD CONCENTRATIONS

Blood Lead Levels (µg/100 ml)	Control Dogs	Experimental Dogs
Day 30	10	17
Day 50	20	65
Day 70	20	72
Day 90	10	70
Day 110	15	82
Day 130	15	65
Day 150	15	60
Day 170	15	70
Day 200	12	55

This table illustrates the average blood lead levels obtained from control and experimental dogs during the course of the experiment.

APPENDIX 6(ii)

LEAD EXPERIMENT 'A'

HAEMATOLOGICAL DATA FOR LEAD DOGS

	White Blood Cell x 10^9/l	Red Blood Cell x 10^{12}/l	Haemoglobin g/dl	Sedimentation Rate
Control Dog (1)	14,500	6.6 x 10^{12}	15.3	Normal
Control Dog (2)	11,000	7.1 x 10^{12}	16.0	Normal
Experimental Dog (1)	14,500	6.0 x 10^{12}	16.6	Normal
Experimental Dog (2)	12,300	6.1 x 10^{12}	15.17	Normal
Normal Values	11,600 $\pm$ 3,400	7.0 x 10^{12} $\pm$ .05 x 10^{12}	14.8 $\pm$ 2.4	

This table shows the haematological parameters of both control and experimental dogs. The figures are averaged for the whole duration of the experimental period.

The normal haematological values for the Beagle dog were obtained from Andersen (1970).

APPENDIX 6(iii)

LEAD EXPERIMENT 'A'

TISSUE LEAD CONCENTRATIONS

Tissues (µg/gm)	Control	Experimental
Kidney (medulla)	0.175	0.623
Kidney (cortex)	0.274	2.545
Brain (white matter)	0.178	0.525
Brain (grey matter)	0.278	0.755
Liver	0.209	8.429
Spleen	0.111	0.564
Pancreas	0.185	0.684
Thyroid	0.068	0.332
Urinary Bladder Wall	0.124	1.200
Prostate	0.090	0.153
Testes	0.065	0.389
Striated Muscle	0.072	0.093
Adrenal	0.091	0.111
Salivary Gland	0.086	0.192
Lymph Node	0.071	0.411
Lung	0.076	0.366
Peripheral Nerve	0.168	0.426
Myocardium	0.083	1.475
Tibia (cancellous bone)	0.933	59.306
11th Rib Right Side (cortical bone)	1.632	272.333
Urine	0.007	0.017
Bile	0.063	0.082

This table shows average tissue concentration of lead in µg/gm at time of autopsy in control and experimental dogs.

APPENDIX 6(iv)

LEAD EXPERIMENT 'A'

BIOCHEMICAL DATA FOR LEAD DOGS

	CONTROL		EXPERIMENTAL	
	Mean	Range	Mean	Range
Sodium	146	144-150	146	142-150
Potassium	4.4	4.1-5.1	4.4	4.1-5.0
Chloride	107	104-112	108	106-111
Carbon Dioxide	23.0	19-27	23.0	18-26
BUN	9	7-13	9	5-14
Creatine	.7	.6-.9	.7	.4-19
Total Protein	5.8	5.6-6.2	5.6	5.4-6.2
Albumin	3.1	2.8-3.4	2.8	2.6-3.5
Calcium	10.6	10.2-11.1	10.5	9.8-11.1
Phosphate	5.2	3.8-6.4	5.5	4.3-6.7
Glucose	107	84-127	104	82-123
Uric Acid	.5	.3-.7	.5	.3-.7
Total Bilirubin	.2	.1-.3	.1	.1-.2
Alkaline Phosphatase	100	61-142	91	53-130

This table shows the means and ranges of biochemical data in control and experimental dogs during the experimental period.

The units and the normal biochemical values for the Beagle can be found in Appendix 10(i).

APPENDIX 6(v)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM CONTROL AND LEAD TREATED DOGS

	Control Dogs	Experimental Dogs	S.E.[a]	"t"	p
A_c (Cortical area/mm^2)	11.34	10.57	1.14	0.67	N.S.[b]
C/T (Ratio cortical-total area)	0.68	0.64	0.04	1.21	N.S.
A_f (Number of osteoid seams/mm^2)	4.93	4.77	0.19	0.86	N.S.
A_r (Number of resorption spaces/mm^2)	1.68	1.58	0.35	0.27	N.S.
S_f (Circumference osteoid seams, mm)	0.20	0.19	0.01	1.00	N.S.
M (Appositional rate, microns/day)	1.26	1.15	0.02	5.50	<0.025
M_f (Radial closure rate, mm/yr)	0.41	0.37	0.01	4.02	<0.05
U_f (Activation frequency, foci/yr)	26.69	23.66	0.91	3.33	<0.05
O_f (Osteon formation time, yrs)	0.19	0.20	0.01	3.00	<0.05
A_r/A_f (Ratio resorption to formation)	0.35	0.35	0.09	0.06	N.S.
V_f (Bone formation rate, mm^2/mm^2/yr)	0.40	0.34	0.02	3.05	<0.05
W.O.S. (Width osteoid seams, microns)	11.25	11.00	1.14	0.65	N.S.
% (Percent labelled systems)	89.22	88.00	1.49	0.82	N.S.
W.T. (Wall thickness, μm)	69.92	80.20	6.46	1.59	N.S.

[a]Significant difference between the means of the two control and the two experimental animals as determined by a one-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with two degrees of freedom at 0.05 level is 2.920; the "t" value with two degrees of freedom at 0.025 level is 4.303.

[b]NS, Not significant.

APPENDIX 7(i)

LEAD EXPERIMENT 'B'

BLOOD LEAD CONCENTRATIONS (µg/dl)

DOG	DAY 50	DAY 100	DAY 150
CC16	47	39	30
CB16	69	53	52
JA16	24	22	58
JO16	37	37	40
	$\bar{x}$ = 49.3	$\bar{x}$ = 40.3	$\bar{x}$ = 45.0

This table illustrates the average blood lead levels (µg/dl) obtained from the lead treated dogs during the course of the experiment.

APPENDIX 7(ii)

LEAD EXPERIMENT "B"

HAEMATOLOGICAL DATA FOR LEAD TREATED DOGS

Dog	CC16	CB16	JO16	JR16	Normal Values*
White Blood Cell x 10^9/l	12.9	13.4	13.2	14.0	11.6 ± 3.4
Red Blood Cell x 10^{12}/l	7.1	7.1	7.2	7.3	7.0 ± 0.5
Haemoglobin g/dl	13.9	15.8	16.1	15.5	14.8 ± 2.4
Hematocrit %	42.5	44.5	43.6	39.6	49.9 ± 4.1
Mean Corpuscular Volume Fl	65.1	66.3	70.1	64.2	67.0 ± 5.5
Mean Corpuscular Haemoglobin pg	22.7	22.7	23.1	22.4	23.0 ± 1.5
Mean Corpuscular Haemoglobin Concentration g/dl	33.9	36.7	36.6	37.8	35.0 ± 2.5
Differential					
Neutrophil	75.0	76.0	75.0	62.0	
Lymphocyte	28.0	21.0	17.0	19.0	
Monocyte	4.0	3.0	3.0	4.0	
Eosinophil	4.0	3.1	5.9	2.8	

This table shows the averages of the haematological results obtained on peripheral blood examination of each dog during the control and experimental period.

*The normal haematological values for the Beagle dog were obtained from Andersen (1970).

APPENDIX 7(iii)

BIOCHEMICAL DATA FOR LEAD DOGS (EXPERIMENT B)

	CB16					CC16					J016					JR16				
	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range
Sodium	145	.71	145.0	146.0	1.0	146	.70	145.0	146.0	1.0	145	1.15	145.0	147.0	2.0	145	0.58	145.0	146.0	1.0
Potassium	4.0	.07	3.9	4.0	.1	4.1	.14	4.0	4.2	.2	4.3	0.25	4.0	4.5	0.5	4.5	0.30	4.2	4.8	0.6
Chloride	108	.71	108.0	109.0	1.0	110	0.00	110.0	110.0	0.0	108	2.08	107.0	111.0	4.0	111	0.58	111.0	112.0	1.0
Carbon dioxide	19.5	2.12	18.0	21.0	3.0	20.4	1.91	19.0	21.7	2.7	22.4	0.98	21.3	23.0	1.7	20.0	1.73	18.0	21.0	3.0
Blood urea nitrogen	11	1.41	10.0	12.0	2.0	9	.71	9.0	10.0	1.0	16	3.46	12.0	18.0	6.0	13	1.53	12.0	15.0	3.0
Creatinine	.8	.07	.7	.8	.1	.7	0.00	.7	.7	0.0	0.8	0.06	0.8	0.9	0.1	0.8	0.10	0.7	0.9	0.2
Total protein	6.7	.42	6.4	7.0	.6	6.1	.70	6.0	6.2	.2	6.0	0.15	5.9	6.2	0.3	5.9	0.57	5.4	6.5	1.1
Albumin	2.9	.14	2.8	3.0	.2	2.9	.21	2.7	3.0	.3	2.7	0.17	2.6	2.9	0.3	2.7	0.15	2.5	2.8	0.3
Calcium	10.4	.07	10.3	10.4	.1	10.2	.28	9.9	10.2	.3	10.0	0.36	9.7	10.4	0.7	10.3	0.23	10.2	10.6	0.4
Phosphate	4.0	.35	3.7	4.2	.5	3.4	0.00	3.4	3.4	0.0	3.8	0.26	3.5	4.0	0.5	4.3	0.23	4.2	4.6	0.4
Glucose	93	10.61	86.0	101.0	15.0	90	5.66	86.0	94.0	8.0	92	4.58	88.0	97.0	9.0	91	7.57	83.0	97.0	14.0
Uric acid	.5	.14	.4	.6	.2	.4	0.00	.4	.4	0.0	0.4	0.00	0.4	0.4	0.0	0.5	0.26	0.3	0.8	0.5
Total bilirubin	.2	.07	.1	.2	.1	.3	.28	.1	.5	.4	0.1	0.00	0.1	0.1	0.0	0.6	0.70	0.1	1.4	1.3
Alkaline phosphatase	60	16.26	49.0	72.0	23.0	33	.70	33.0	34.0	1.0	61	9.24	56.0	72.0	16.0	43	2.08	42.0	46.0	4.0

APPENDIX 7(iv)

LEAD EXPERIMENT "B"

BONE LEAD CONCENTRATION IN THE LEAD TREATED DOGS (µg/g)

DOG	CONTROL BIOPSY	EXPERIMENTAL BIOPSY
CC16	6.02	55.82
CB16	2.45	54.79
JR16	1.88	22.39
JO16	2.81	63.53

The bone lead concentration obtained from ribs of 18 untreated dogs was found to be 3.76 $\pm$ 2.69 µg/g. (Standard Deviation).

APPENDIX 7(v)

CIRCULATING LEVELS OF CANINE ANTI-BOVINE IMMUNOREACTIVE PARATHYROID HORMONE IN LEAD TREATED DOGS

(micro liter equivalents/ml)

DOG	1st BIOPSY	2nd BIOPSY
CC16	82	54
CB16	27	less than 20
JO16	less than 20	less than 20
JR16	75	70

Circulating levels of canine anti-bovine iPTH were determined using the method described by Hruska et al (1975).

Normal levels of canine iPTH range from undetectable levels to 80 micro liter equivalents/ml (Slatopolsky, personal communication 1978).

APPENDIX 7(vi)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM LEAD TREATED DOGS

	Control		Experimental		S.E.(x_1-x_2)[a]	"t"	p
A_c (Cortical area/mm^2)	10.82	2.32	10.05	1.65	0.86	0.89	N.S.[b]
C/T (Ratio cortical-total area)	0.64	0.12	0.61	0.07	0.03	1.04	N.S.
A_f (Number of osteoid seams/mm^2)	3.69	1.51	1.53	0.80	0.45	4.81	0.005
A_r (Number of resorption spaces/mm^2)	1.12	0.17	0.84	0.39	0.25	1.17	N.S.
S_f (Circumference osteoid seams, mm)	0.20	0.03	0.22	0.02	0.01	1.89	N.S.
M (Appositional rate, microns/day)	1.34	0.08	1.10	0.11	0.05	5.37	<0.005
M_f (Radial closure rate, mm/yr)	0.46	0.02	0.32	0.05	0.03	4.57	<0.01
U_f (Activation frequency, foci/yr)	22.15	8.78	8.26	5.46	3.00	4.63	<0.005
O_f (Osteon formation time, yrs)	0.17	0.01	0.22	0.05	0.03	1.51	N.S.
A_r/A_f (Ratio resorption to formation)	0.44	0.18	0.60	0.11	0.08	1.93	N.S.
V_f (Bone formation rate, mm^2/mm^2/yr)	0.35	0.15	0.12	0.08	0.05	4.17	<0.01
W.O.S. (Width osteoid seams, microns)	9.10	1.31	8.90	8.12	0.74	0.27	N.S.
% (Percent labelled systems)	94.36	3.06	80.50	8.12	5.51	2.52	<0.05
W.T. (Wall thickness, μm)	77.46	3.31	69.99	4.47	2.69	2.77	<0.05

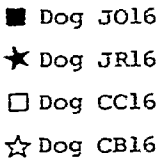
[a] Significant difference between the means of the four control and the four experimental biopsies as determined by a one-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with four degrees of freedom at 0.05 level is 2.1318; the "t" value with four degrees of freedom at the 0.01 level is 3.747; the "t" value with four degrees of freedom at 0.005 level is 4.6041.

[b] NS, Not significant.

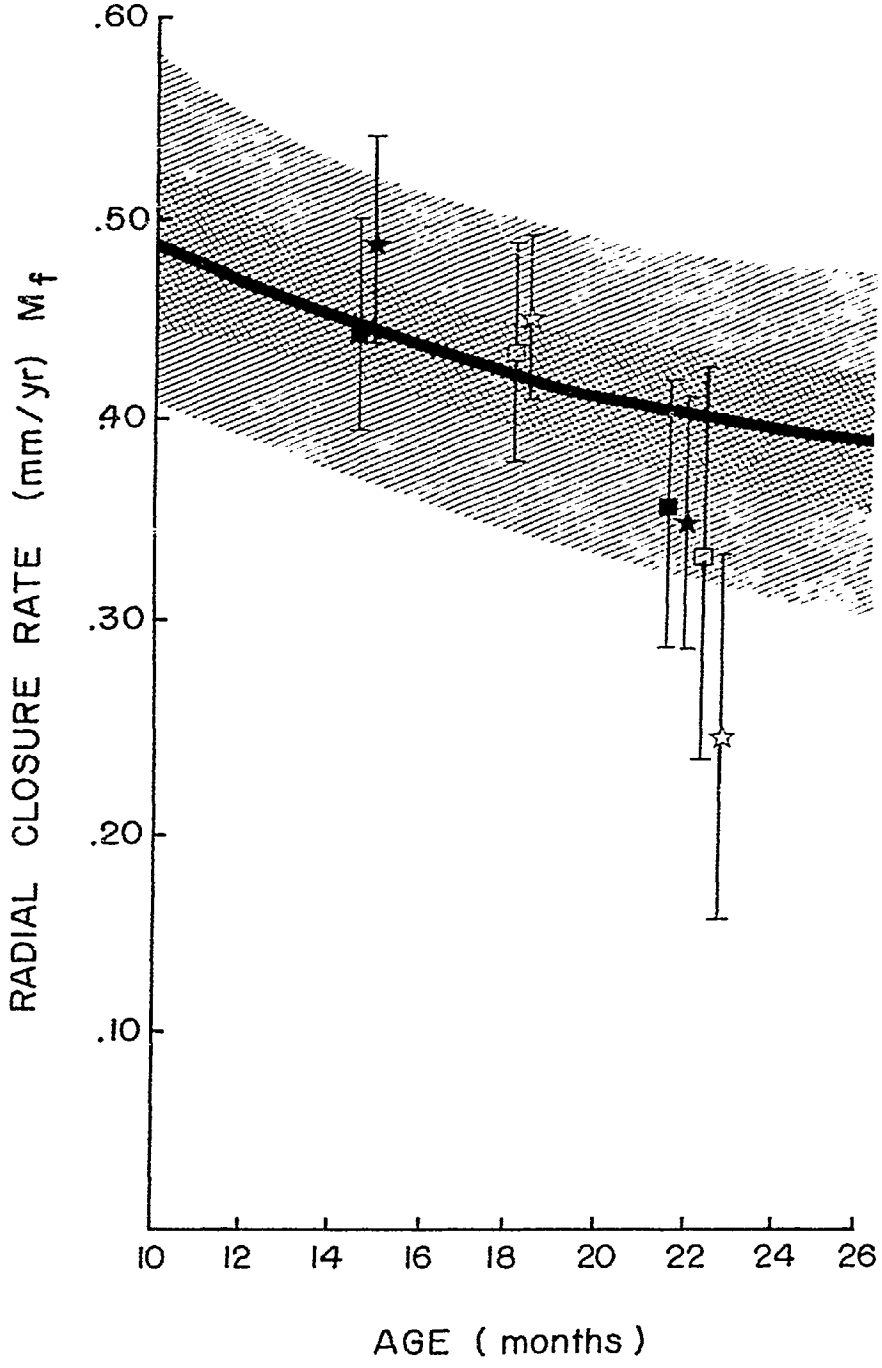
APPENDIX 7(vii)

LEAD EXPERIMENT "B"

RADIAL CLOSURE RATE, mm/yr (M_f)



There are two points in this graph for each dog. During the period between the points all dogs received 7 mg Pb/d orally.


.60
.50
.40
.30
.20
.10
RADIAL CLOSURE RATE (mm/yr) M_f
10
12
14
16
18
20
22
24
26
AGE (months)

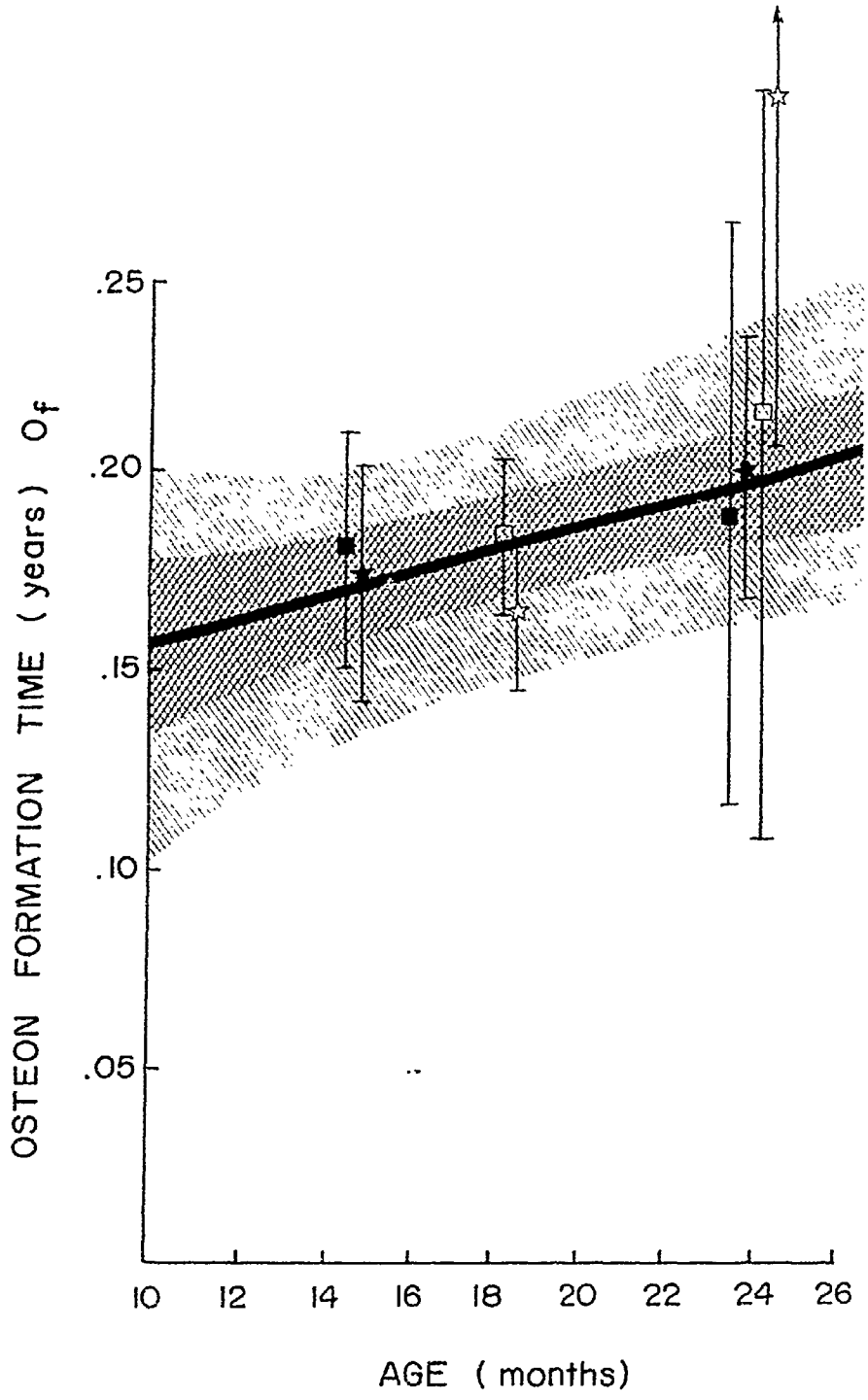
APPENDIX 7(viii)

LEAD EXPERIMENT "B"

OSTEON FORMATION TIME, yrs (O_f)

■ Dog JO16
★ Dog JR16
□ Dog CC16
☆ Dog CB16

There are two points in this graph for each dog. During the period between the points all dogs received 7 mg Pb/D orally.


.25
.20
.15
.10
.05
OSTEON FORMATION TIME (years) O_f
10
12
14
16
18
20
22
24
26
AGE (months)

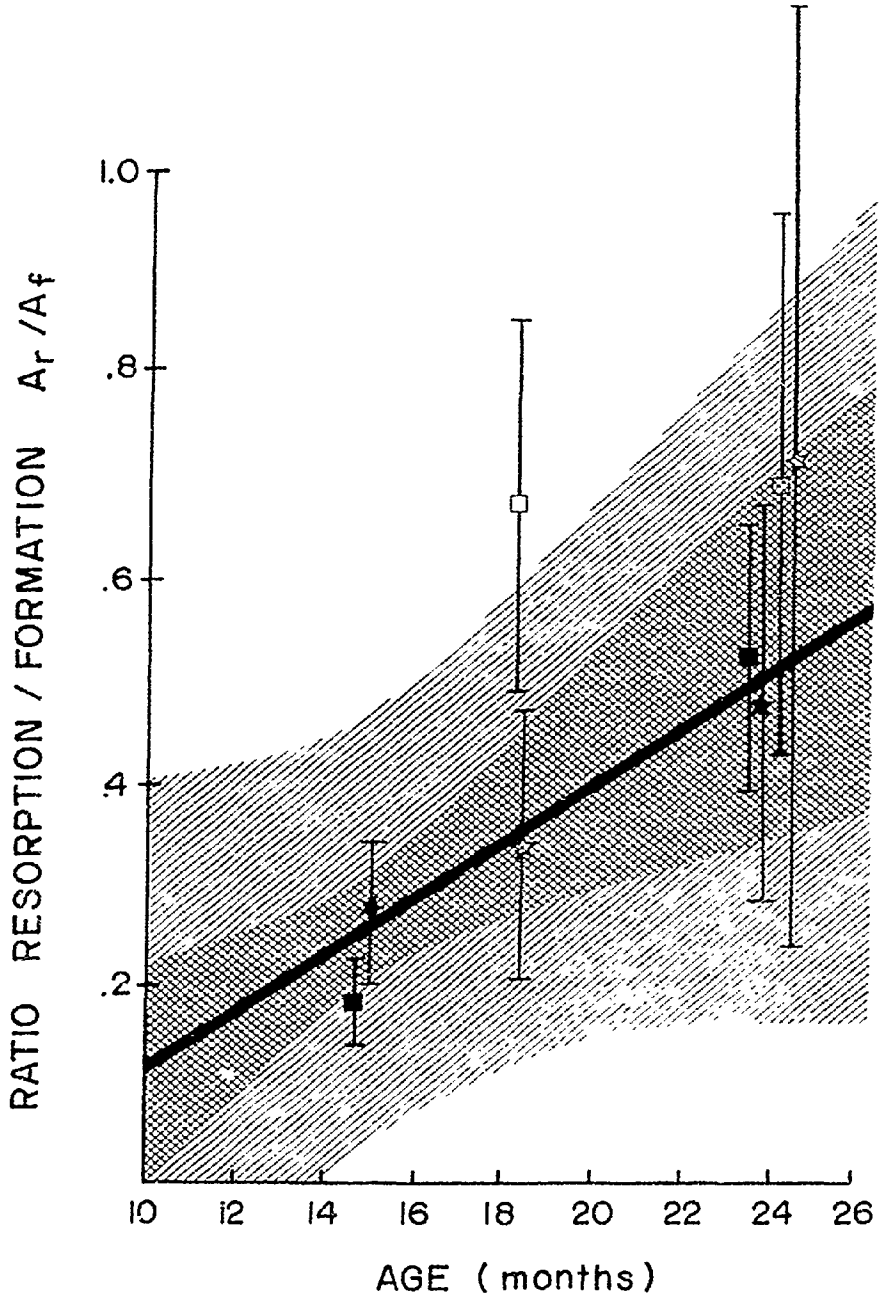
APPENDIX 7(ix)

LEAD EXPERIMENT "B"

RATIO RESORPTION TO FORMATION (A_r/A_f)

■ Dog JO16
★ Dog JR16
□ Dog CC16
☆ Dog CB16

There are two points in this graph for each dog. During the period between the points all dogs received 7 mg Pb/d orally.


RATIO RESORPTION / FORMATION A_r / A_f
1.0
.8
.6
.4
.2
10
12
14
16
18
20
22
24
26
AGE (months)

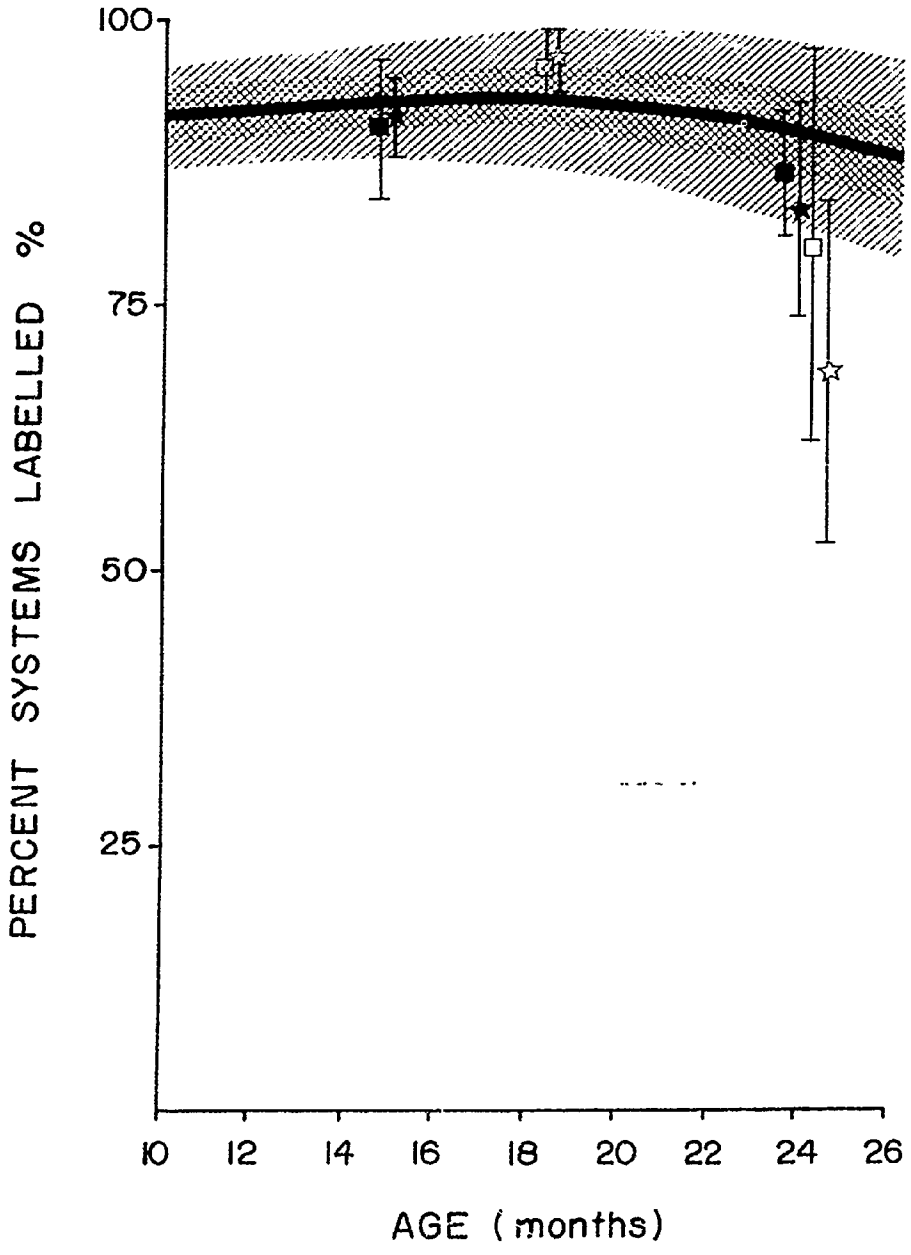
APPENDIX 7(x)

LEAD EXPERIMENT "B"

PERCENT LABELLED SYSTEMS (%)

■ Dog JO16
★ Dog JR16
□ Dog CC16
☆ Dog CB16

There are two points in this graph for each dog. During the period between the points all dogs received 7 mg Pb/d orally.


100
75
50
25
PERCENT SYSTEMS LABELLED %
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 8(i)

HAEMATOLOGICAL DATA FOR NTA DOGS

Dog	TFY5	TEY5	Normal Values*
White Blood Cell x 10^9/l	8.7	10.2	11.6±3.4
Red Blood Cell x 10^{12}/l	6.9	6.3	7.0±0.5
Haemoglobin g/dl	16.1	15.0	14.8±2.4
Hematocrit %	45.2	41.3	49.9±4.1
Mean Corpuscular Volume Fl	66.0	65.0	67.0±5.5
Mean Corpuscular Haemoglobin pg	22.9	23.3	23.0±1.5
Mean Corpuscular Haemoglobin Concentration g/dl	35.1	36.1	35.0±2.5
Differential			
Neutrophil	78.0	71.5	
Lymphocyte	14.0	23.5	
Monocyte	4.0	2.0	
Eosinophil	3.0	1.0	

This table shows the averages of the haematological results obtained on peripheral blood examination of each dog during the control and experimental period.

*The normal haematological values for the Beagle dog were obtained from Andersen (1970).

APPENDIX 8(ii)

BIOCHEMICAL DATA FOR NTA DOGS

	TFY5					TEY5				
	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range
Sodium	146	1.29	145.0	148.0	3.0	146	1.29	145.0	148.0	3.0
Potassium	4.0	0.13	3.8	4.1	0.3	4.1	0.22	3.8	4.3	0.5
Chloride	109	1.50	108.0	111.0	3.0	109	1.15	108.0	110.0	2.0
Carbon dioxide	22.0	2.45	19.0	25.0	6.0	22.0	1.83	20.0	24.0	4.0
Blood urea nitrogen	13	1.15	12.0	14.0	2.0	10	0.05	10.0	11.0	1.0
Creatinine	1.0	0.00	1.0	1.0	0.0	0.8	0.05	0.8	0.9	0.1
Total protein	6.1	0.81	5.5	7.3	1.8	6.1	0.24	5.8	6.4	0.6
Albumin	2.9	0.15	2.7	3.0	0.3	3.0	0.17	2.7	3.1	0.4
Calcium	10.2	0.33	9.7	10.4	0.7	10.3	0.43	9.8	10.8	1.0
Phosphate	3.5	0.46	3.1	4.1	1.0	3.9	0.27	3.5	4.1	0.6
Glucose	103	6.66	96.0	109.0	13.0	99	4.24	93.0	103.0	10.0
Uric acid	0.5	0.10	0.4	0.6	0.2	0.4	0.06	0.3	0.4	0.1
Total bilirubin	0.1	0.00	0.1	0.1	0.0	0.1	0.06	0.1	0.2	0.1
Alkaline phosphatase	45	9.90	38.0	59.0	21.0	29	5.19	25.0	36.0	11.0

APPENDIX 8(iii)

CIRCULATING LEVELS OF CANINE ANTI-BOVINE IMMUNOREACTIVE PARATHYROID HORMONE IN Na_3NTA TREATED DOGS

(micro liter equivalents/ml)

DOG	1st BIOPSY	2nd BIOPSY
TFY5	less than 20	32
TEYs	less than 20	less than 20

Circulating levels of canine anti-bovine iPTH were determined using the method described by Hruska et al (1975).

Normal levels of canine iPTH in the Beagle range from undetectable levels to 80 micro liter equivalents/ml, (Slatopolsky, personal communication, 1978).

APPENDIX 8(iv)

REMODELLING PARAMETERS IN HAVERSIAN BONE

FROM Na_3NTA TREATED DOGS

	Control		Experimental		S.E.(x_1-x_2)[a]	"t"	P
A_c (Cortical area/mm^2)	11.77	1.18	10.91	0.52	0.47	1.84	N.S.[b]
C/T (Ratio cortical-total area)	0.65	0.01	0.64	0.01	0.02	1.00	N.S.
A_f (Number of osteoid seams/mm^2)	4.35	0.66	2.69	0.26	0.66	2.54	N.S.
A_r (Number of resorption spaces/mm^2)	0.90	0.00	0.97	0.11	0.08	0.87	N.S.
S_f (Circumference osteoid seams, mm)	0.18	0.02	0.19	0.00	0.02	1.00	N.S.
M (Appositional rate, microns/day)	1.24	0.05	1.15	0.02	0.02	4.50	<0.05
M_f (Radial closure rate, mm/yr)	0.43	0.02	0.33	0.00	0.02	6.33	<0.025
U_f (Activation frequency, foci/yr)	24.51	9.02	13.87	1.08	7.14	1.49	N.S.
O_f (Osteon formation time, yrs)	0.18	0.02	0.20	0.01	0.01	2.00	N.S.
A_r/A_f (Ratio resorption to formation)	0.22	0.03	0.36	0.00	0.02	7.00	<0.025
V_f (Bone formation rate, $mm^2/mm^2/yr$)	0.33	0.01	0.17	0.01	0.01	16.00	<0.01
W.O.S. (Width osteoid seams, microns)	8.67	1.38	8.09	0.49	0.63	0.92	N.S.
% (Percent labelled systems)	94.15	0.07	81.20	0.05	0.06	259.00	<0.001
W.T. (Wall thickness, μm)	73.99	11.78	66.01	2.89	6.29	1.27	N.S.

[a]Significant difference between the means of the two control and the two experimental biopsies as determined by a two-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with two degrees of freedom at 0.05 level is 4.3027; the "t" value with two degrees of freedom at 0.025 is 6.2053; the "t" value with two degrees of freedom at 0.01 is 9.9248; the "t" value with two degrees of freedom at 0.001 is 31.598.

[b]NS, Not significant.

APPENDIX 8(v)

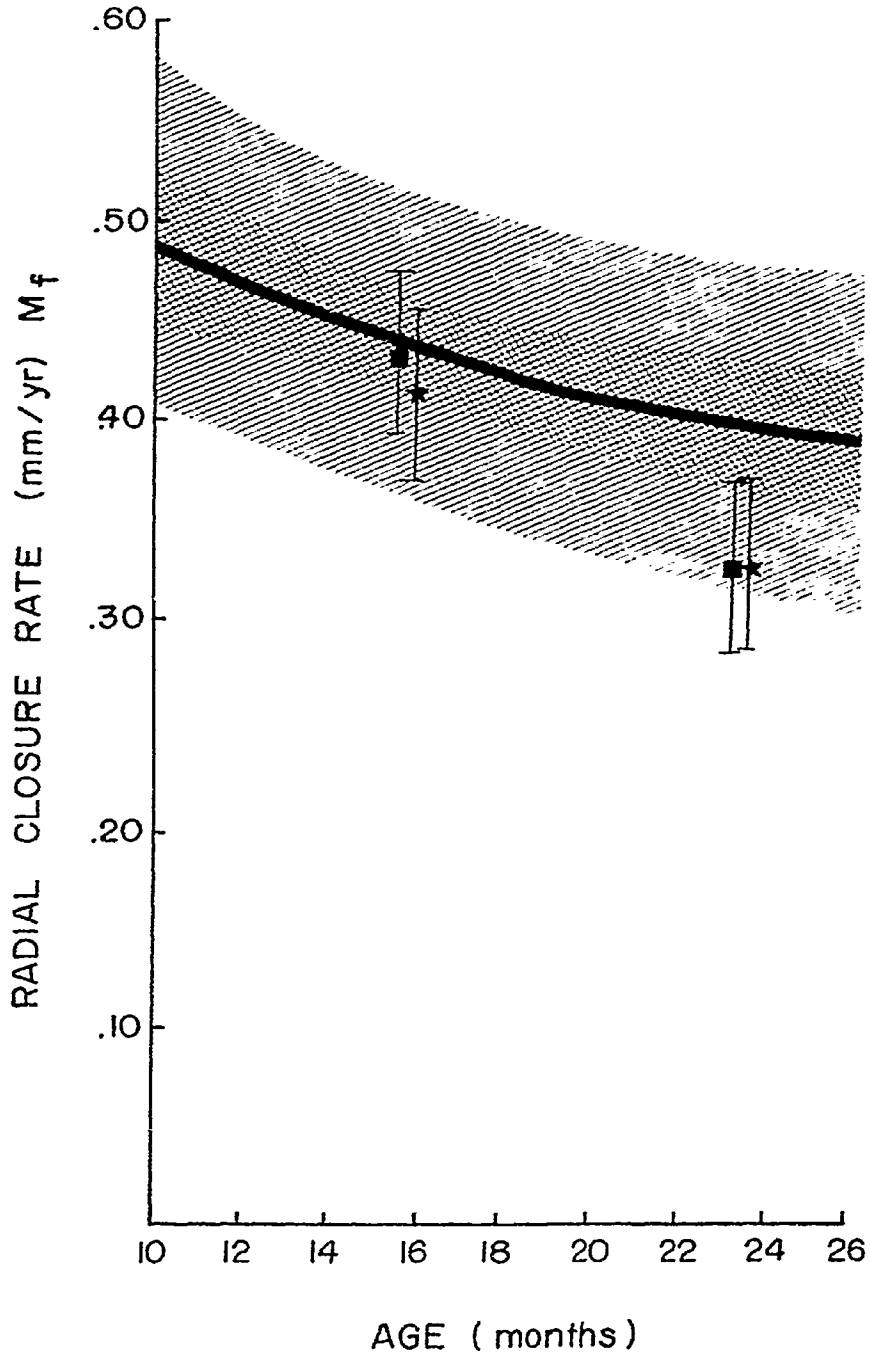
Na_3NTA EXPERIMENT

RADIAL CLOSURE RATE, mm/yr (M_f)

■ DOG TFY5

★ DOG TEY5

There are two points on this graph for each dog. During the period between these points each dog consumed approximately 35 mg Na_3NTA per day in its drinking water.


.60
.50
.40
.30
.20
.10
RADIAL CLOSURE RATE (mm/yr) M_f
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 8(vi)

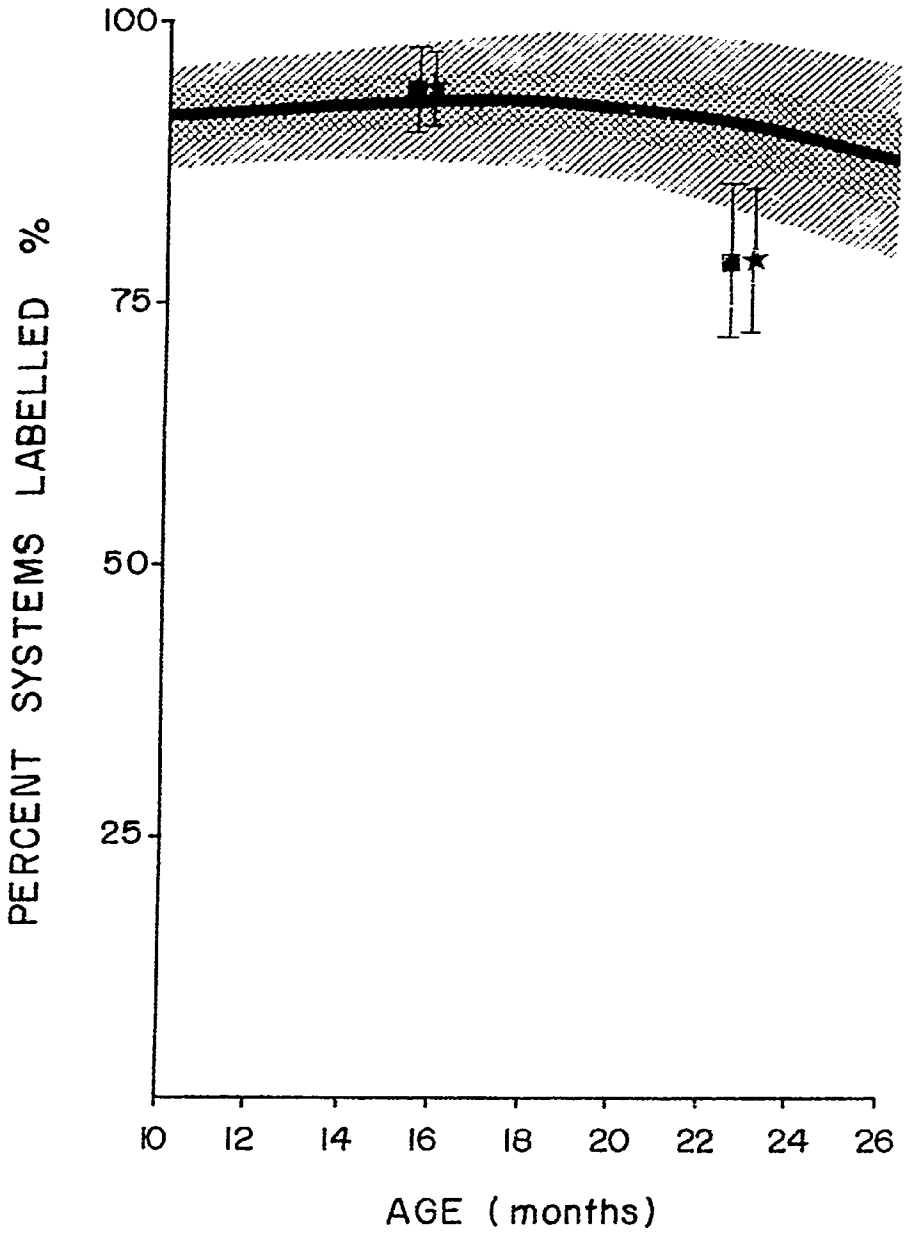
Na_3NTA EXPERIMENT

PERCENT LABELLED SYSTEMS (%)

■ DOG TFY5

★ DOG TEY5

There are two points on this graph for each dog. During the period between these points each dog consumed approximately 35 mg Na_3NTA per day in its drinking water.


100
75
50
25
PERCENT SYSTEMS LABELLED %
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 9(i)

HAEMATOLOGICAL DATA FOR ZINC DOGS

Dog	RRX5	RQX5	FK16	FJ16	Normal Values*
White Blood Cell x 10^9/l	12.7	15.4	9.8	10.9	11.6±3.4
Red Blood Cell x 10^{12}/l	7.3	6.3	6.8	6.9	7.0±0.5
Haemoglobin g/dl	16.8	14.2	15.3	16.2	14.8±2.4
Hematocrit %	45.9	39.6	41.5	44.7	49.9±4.1
Mean Corpuscular Volume Fl	64.0	63.5	62.0	66.0	67.0±5.5
Mean Corpuscular Haemoglobin pg	22.8	22.8	22.6	23.3	23.0±1.5
Mean Corpuscular Haemoglobin Concentration g/dl	36.9	35.7	37.4	36.4	35.0±2.5
Differential					
Neutrophil	71.0	62.0	82.0	70.0	
Lymphocyte	21.0	29.5	11.0	17.0	
Monocyte	4.0	3.0	5.0	1.0	
Eosinophil	4.0	5.5	2.0	3.0	

This table shows the averages of the haematological results obtained on peripheral blood examination of each dog during the control and experimental period.

*The normal haematological values for the Beagle dog were obtained from Andersen (1970).

APPENDIX 9(ii)

BIOCHEMICAL DATA FOR ZINC DOGS

	FJ16					FK16					RRX5					RQX5				
	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range	Mean	S.D.	Minimum	Maximum	Range
Sodium	145	0.71	144.0	146.0	2.0	140	10.32	120.0	146.0	26.0	143	2.49	139.0	145.0	6.0	145	3.20	142.0	151.0	9.0
Potassium	4.3	0.3	3.9	4.6	0.7	4.4	0.15	4.3	4.6	0.3	4.4	0.37	4.0	5.0	1.0	4.2	0.27	3.7	4.5	0.8
Chloride	107	1.82	105.0	110.0	5.0	108	1.41	107.0	110.0	3.0	107	1.95	105.0	110.0	5.0	110	1.22	109.0	112.0	3.0
Carbon dioxide	21.9	2.33	18.7	25.0	6.3	20.0	2.88	16.1	24.0	7.9	20.8	1.13	19.8	22.0	13.0	20.3	3.57	17.0	26.4	9.4
Blood urea nitrogen	17	5.43	13.0	26.0	13.0	19	6.89	10.0	29.0	19.0	14	5.17	9.0	22.0	13.0	13	5 0	9.0	21.0	12.0
Creatinine	0.7	0.13	0.5	0.8	0.3	0.7	0.13	0.6	0.9	0.3	0.8	0.16	0.70	1.1	0.4	0.9	0.08	0.5	0.7	0.2
Total protein	6.9	0.32	6.4	7.2	0.8	6.4	0.18	6.2	6.6	0.4	6.2	0.61	5.7	7.2	1.5	6.0	0.43	5.6	6.7	1.1
Albumin	2.7	0.21	2.5	3.0	0.5	2.6	0.15	2.4	2.8	0.4	3.1	0.94	2.5	4.8	2.3	2.7	0.23	2.	2.9	0.6
Calcium	10.4	0.37	9.9	10.7	0.8	10.0	0.44	9.3	10.4	1.1	9.6	0.40	9.1	10.1	1.0	10.2	0.60	9.5	11.0	1.5
Phosphate	3.7	0.34	3.2	3.9	0.7	3.8	0.36	3.3	4.2	0.9	3.9	0.19	3.7	4.2	0.5	3.7	8.76	2.4	4.6	2.2
Glucose	94	12.99	81.0	111.0	30.0	88	7.80	81.0	100.0	19.0	89	13.66	67.0	104.0	37.0	96	8.60	81.0	102.0	21.0
Uric acid	0.5	0.08	0.4	0.6	0.2	0.5	0.80	0.4	0.6	0.2	0.5	0.05	0.4	0.5	0.1	0.6	0.42	0.3	1.3	1.0
Total bilirubin	0.1	0.05	0.1	0.2	0.1	0.1	0.05	0.0	0.1	0.1	0.1	0.00	0.1	0.1	0.0	0.1	0.06	0.1	0.2	0.1
Alkaline phosphatase	64	11.90	54.0	81.0	27.0	48	13.40	32.0	64.0	32.0	29	5.41	22.0	37.0	15.0	39	6.34	31.0	47.0	16.0

APPENDIX 9(iii)

CIRCULATING LEVELS OF CANINE ANTI-BOVINE IMMUNOREACTIVE PARATHYROID HORMONE IN ZINC TREATED DOGS

(micro liter equivalents/ml)

DOG	1st BIOPSY	2nd BIOPSY
RRX5	33	27
RQX5	26	less than 20
FJ16	less than 20	less than 20
FK16	25	30

Circulating levels of canine anti-bovine iPTH were determined using the method described by Hruska et al (1975).

The normal levels of canine iPTH in the Beagle range from undetectable levels to 80 micro liter equivalents/ml, (Slatopolsky, personal communication 1978).

Appendix 9(iv)

ZINC EXPERIMENT

BONE ZINC CONCENTRATION IN THE ZINC TREATED DOGS

DOG	1st BIOPSY	2nd BIOPSY
RQX5	130.67	141.60
RRX5	132.40	218.79
FJ16	178.55	*
FK16	146.19	164.60
	146.95 ± 22.18	175.00 ± 39.63

The bone zinc concentration obtained from the rib of 18 dogs not treated with zinc was found to be 119.59 ± 34.32. (Standard Deviation)

* Sample lost during tissue processing.

APPENDIX 9(v)

REMODELLING PARAMETERS IN HAVERSIAN BONE FROM ZINC TREATED DOGS

	Control		Experimental		S.E.(x_1-x_2)[a]	"t"	p
A_c (Cortical area/mm^2)	9.63	1.24	9.74	1.07	0.42	0.27	N.S.[b]
C/T (Ratio cortical-total area)	0.62	0.01	0.62	0.05	0.02	0.10	N.S.
A_f (Number of osteoid seams/mm^2)	4.67	1.55	1.95	0.74	0.66	4.09	<0.05
A_r (Number of resorption spaces/mm^2)	0.95	0.38	0.86	0.24	0.15	0.58	N.S.
S_f (Circumference osteoid seams, mm)	0.19	0.01	0.22	0.02	0.01	2.32	N.S.
M (Appositional rate, microns/day)	1.31	0.07	1.25	0.10	0.07	0.91	N.S.
M_f (Radial closure rate, mm/yr)	0.45	0.03	0.38	0.06	0.03	2.34	N.S.
U_f (Activation frequency, foci/yr)	29.08	11.08	10.82	4.22	5.23	3.49	<0.05
O_f (Osteon formation time, yrs)	0.17	0.01	0.19	0.02	0.01	1.36	N.S.
A_r/A_f (Ratio resorption to formation)	0.22	0.05	0.38	0.10	0.04	6.10	<0.01
V_f (Bone formation rate, mm^2/mm^2/yr)	0.41	0.16	0.17	0.08	0.08	2.85	N.S.
W.O.S. (Width osteoid seams, microns)	7.26	0.57	8.48	0.71	0.48	2.52	N.S.
% (Percent labelled systems)	94.98	0.70	83.58	7.79	3.58	3.16	N.S.
W.T. (Wall thickness, µm)	73.80	0.85	67.69	5.07	2.64	2.31	N.S.

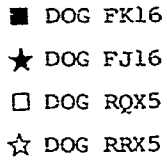
[a]Significant difference between the means of the four control and the four experimental biopsies as determined by a two-tailed "t" test, using the pooled square deviations of each group to compute the standard error of the difference. The "t" value with four degrees of freedom at 0.05 level is 2.7764; the "t" value with four degrees of freedom at 0.01 level is 4.6041.

[b]NS, Not significant.

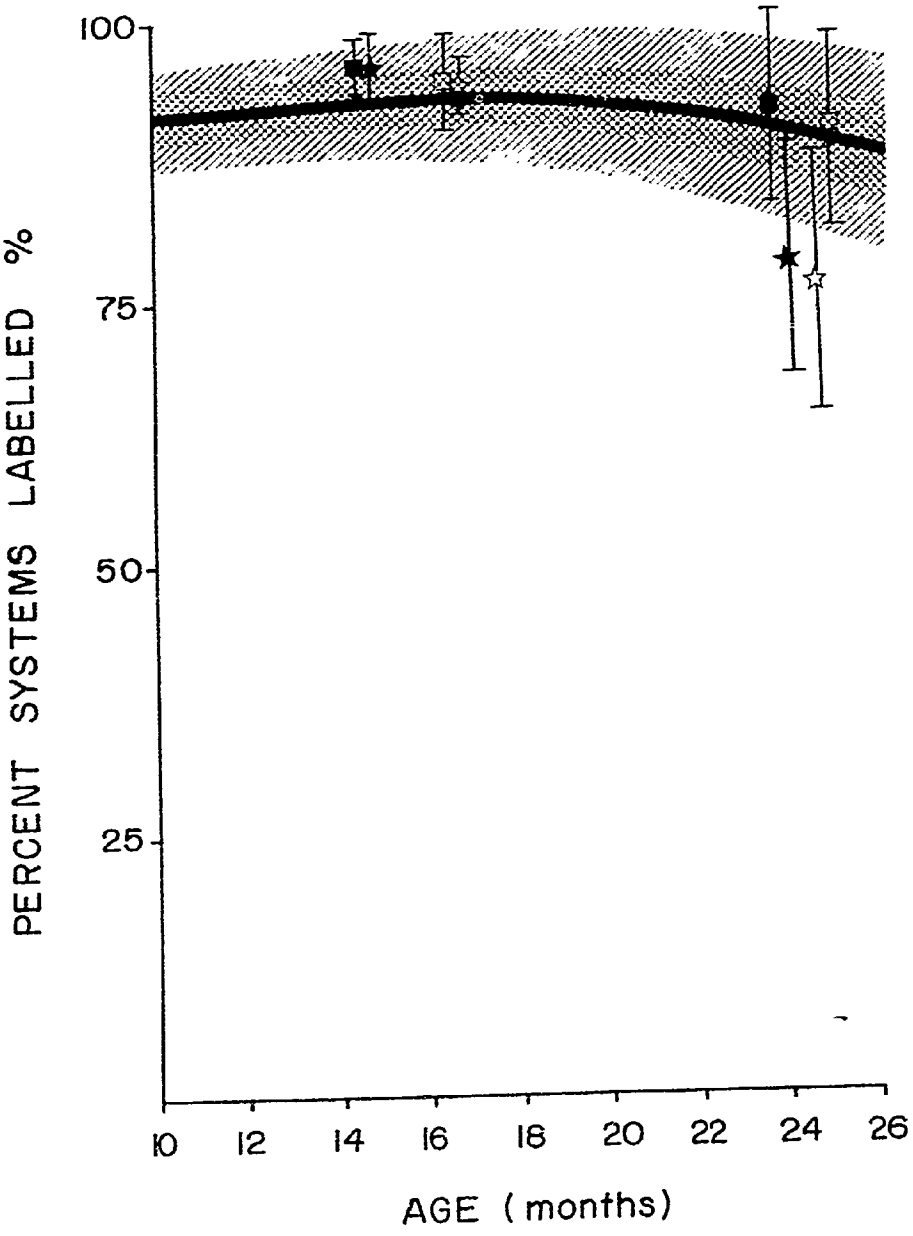
APPENDIX 9(vi)

ZINC EXPERIMENT

PERCENT LABELLED SYSTEMS (%)



There are two points on this graph for each dog. During the period between points all dogs had their normal drinking water substituted by a solution containing 100 ppm Zn.


100
75
50
25
PERCENT SYSTEMS LABELLED %
10
12
14
16
18
20
22
24
26
AGE (months)

APPENDIX 10(i)

NORMAL BLOOD SERUM BIOCHEMISTRY IN THE BEAGLE

Sodium (meq/liter)	147.30 ± 3.10
Potassium (meq/liter)	4.70 ± 0.50
Chloride (meq/liter)	107.00 ± 9.00
CO_2 (bicarbonate) (meq/liter)	12.20 ± 4.00
Blood Urea Nitrogen (mg/100 ml)	15.60 ± 7.10
Creatinine (mg/100 ml)	.81 ± .39
Total protein (g/100 ml)	6.70 ± 1.10
Albumin (g/100 ml)	3.40 ± 0.40
Calcium (mg/100 ml)	9.90 ± 1.60
Phosphorus (mg/100 ml)	5.40 ± 1.80
Uric acid (mg/100 ml)	.67 ± .42
Total bilirubin (mg/100 ml)	.19 ± .20
Glucose (mg/100 ml)	99.00 ±22.00
Alkaline phosphatase (Bodansky U/100 ml)	3.25 ± 2.17

Normal blood serum biochemistry in the Beagle as cited by Andersen (1970).

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