

The Effect of Fluoride Administration on Rat Serum Osteocalcin Expression during Orthodontic Tooth Movement

(The Osteocalcin Project)



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A thesis submitted in partial fulfilment
of the requirements for the degree of

Master of Dental Science
(Orthodontics)

Discipline of Orthodontics
Faculty of Dentistry
University of Sydney

October, 2004

Dedication

To my wonderful parents Horst and Margaret, whose example I have always tried to follow and whose unending love, strength and selfless devotion to me has kept me going through life's ups and downs. A son could not have wished for better parents. Thank you for giving me every opportunity in life and for helping me believe that everything is possible.

To my adorable sister Monika whose love and friendship I have always held dear to my heart.

To darling Raya whose love and understanding I cherish.

Success is the result of many small victories that most people will never know about.

Anon

Acknowledgements

I would like to express my gratitude to the following for their generous help and support in the preparation of this thesis:

My final year colleagues Matthew Foo, Debora Harris, Angelina Zea and Wanjira Chutmaniskul

Professor M.A. Darendeliler

Mrs Margaret Wilkinson and the staff at The Royal North Shore Hospital Endocrinology Laboratory

Dr. Ross Matthews, Head Veterinarian and his staff at The Westmead Hospital Animal Research Facility

The staff at The Westmead Institute for Dental Research

Dr. Peter Petocz, Statistician

The University of Sydney Dental Research Foundation

The Australian Society of Orthodontists' Foundation for Research

Declaration

CANDIDATE CERTIFICATE

This is to certify that the aforementioned candidate has carried out the work described in this thesis under the auspices of the Discipline of Orthodontics at The University of Sydney. This work has not been submitted to any other university or institution for a higher degree.

A handwritten signature in black ink, appearing to read 'M. E. B. A. H.', is positioned above a horizontal dotted line.

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Abbreviations

ALP	Alkaline phosphatase
BP	Bone Particle
BMP	Bone Matrix Particle
BMU	Bone Multicellular Unit
$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	Hydroxyapatite
Ca^{2+}	Calcium ion
CEJ	Cementoenamel Junction
^{14}C	Carbon-14
cAMP	Cyclic Adenosine monophosphate
CTGF	Connective Tissue Growth Factor
DMP-1	Dentine Matrix Protein-1
DOPC	Determined Osteoprogenitor Cell
DPP	Dentine Phosphoprotein
ELISA	Enzyme-Linked Immunosorbent Assay
ER- β	Oestrogen Receptor Beta
FGF	Fibroblast Growth Factor
GCF	Gingival Crevicular Fluid
GLAST	Glutamate Aspartate Transporter
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
IGF	Insulin-like Growth Factor
IL	Interleukin
IOPC	Inducible Osteoprogenitor Cell
M-CSF	Murine Colony Stimulating Factor
MGP	Matrix Gla Protein
NaF	Sodium Fluoride
NCP	Non-collagenous Protein
NOS	Nitric Oxide Synthetase
OPG	Osteoprotegerin
OPN	Osteopontin
PDGF	Platelet-Derived Growth Factor
PDL	Periodontal Ligament
PG	Proteoglycan
PGE_2	Proteoglycan
PGHS-2	Prostaglandin G-H Synthetase E_2
RANK	Receptor Activator of Nuclear Factor

RANK-L	Receptor Activator of Nuclear Factor Ligand
RER	Rough Endoplasmic Reticulum
RIA	Radioimmunoassay
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TRAP	Tartrate Resistant Acid Phosphatase
1, 25-(OH) ₂ D	Calcitriol

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Abstract

Osteocalcin is a bone protein that has previously been used as a marker of bone turnover. The precise role of this protein in bone remodelling has as yet not been fully elucidated, however, it has been shown to be instrumental in the activation of osteoclastic bone resorption via its effect on osteoblasts. Fluoride influences bone growth by acting as a mitogenic agent for osteoblasts. This study utilises a rodent model to determine the effects of fluoride administration on systemic osteocalcin during orthodontic tooth movement. Thirty-two, eight week old, female Wistar rats were divided into four groups for this experiment. The first experimental group of rats (n=10) were fed a normal laboratory diet and distilled water (MilliQ). The second experimental group of rats (n=10) had the same diet with the addition of fluoridated water. The third (n=6) and fourth (n=6) groups were used as control groups. These did not undergo orthodontic tooth movement and were given fluoridated (100ppm NaF) and non-fluoridated water *ad libitum*. Both fluoridated and non-fluoridated experimental samples had 100g NiTi coil springs secured to the lower incisor and left first molar tooth to instigate orthodontic tooth movement for a period of 14 days.

Phlebotomy was performed via the lateral tail vein prior to application of the orthodontic appliance and final blood samples were obtained via cardiac puncture following sacrifice two weeks later. Serum was isolated from the samples following centrifuging at each stage of phlebotomy and an enzyme-linked immunosorbent assay (ELISA) was subsequently performed to determine osteocalcin levels in the various sample groups.

Orthodontic tooth movement using high, continuous forces was found to have a positive, statistically significant correlation with serum osteocalcin expression in the rodent model. The use of fluoride as a variable was found to increase the mean osteocalcin concentration, however, this was not statistically significant.

Keywords: Osteocalcin, fluoride, cementum, tooth movement, ELISA.

1. Introduction

1.1 Evolution of The Osteocalcin Project

The initial objective of this project was to identify a marker in the local periodontal environment that might be representative of hard tissue dissolution/metabolism in a non-invasive, quantitative manner.

Gingival crevicular fluid (GCF) represents a powerful fluid vehicle for the assessment of the biological state of the deeper-seated tissues of the periodontium diagnostics since it contains (depending upon the clinical situation) an array of biochemical and cellular factors which feature as biomarkers of the state of the periodontium.

References to analyses of GCF in the orthodontic literature are sparse. Interleukin 1- β , interleukin -6, tumour necrosis factor- α , β_2 -microglobulin (Uematsu *et al.*, 1996a), Prostaglandin E (Grieve *et al.*, 1994) and transforming growth factor- β_1 (Uematsu *et al.*, 1996b) have been measured in the GCF of teeth undergoing tooth movement. Other factors that have been studied within an orthodontic framework include proteoglycans (Waddington *et al.*, 1994; Waddington and Embury, 2001), acid and alkaline phosphatases (Insoft *et al.*, 1996), pH (Miyajama *et al.*, 1991), collagen cross-links pyridinoline and deoxypyridinoline (Griffiths *et al.*, 1998), cAMP-dependent protein kinase subunit (RII) (Burke *et al.*, 2002) and dentine phosphoproteins (Mah and Prasad, 2004).

The loss of root structure proceeds in a sequential manner to the loss of cementum, prior to the dissolution of dentine. Cementum breakdown products in the GCF (Dentine Matrix Protein 1, DMP1) have been studied; however, they were detected in both treatment and control groups, rendering them too insensitive to function as markers for orthodontic root resorption (Liu *et al.*, 2000).

Most of the aforementioned substances, however, are limited in their application as they are non-specific biomarkers of inflammation and/or soft tissue degradation and, therefore, are not specific for particular mineralised tissues. Furthermore, some of these have been shown to be undetectable in GCF (Griffiths *et al.*, 1998).

Dentine phosphoprotein (DPP) has recently shown more promise with respect to providing a quantifiable GCF marker of dental hard tissue dissolution; however, problems with sensitivity of the Enzyme-Linked Immunosorbent Assay (ELISA) (related to a lack of homology of rat with human DPP) hampers the use of this protein as a diagnostic aid at present (Mah and Prasad, 2004).

In the infancy of this project, osteocalcin was chosen as a marker to detect hard tissue dissolution due to the presence of this extracellular matrix component in cementum, dentine and bone (Bronckers *et al.*, 1998), a unique property amongst previously investigated diagnostic substances.

Griffiths *et al.* (1998) previously investigated GCF osteocalcin within the context of orthodontic movement, however, there was found to be high variability in levels of the protein between subjects, as well as a lack of a consistent pattern associated with the initial fitting of the appliance, active orthodontic movement and retention periods. Problems with sampling, including contamination of GCF samples from multiple sites hindered interpretation of the data from this investigation.

An initial pilot study was undertaken to investigate the *in vivo* concentration of osteocalcin in GCF associated with ten human premolar teeth (in addition to sham controls) undergoing intrusive experimental force over a four-week experimental period (prior to subsequent premolar extraction as part of planned orthodontic treatment). The relationship between GCF and serum levels of osteocalcin was to be investigated concomitantly in a separate cohort of periodontal patients who were enrolled in a concomitant, separate study directed by the Department of Periodontics within the Sydney Dental Hospital. Approval for the projects was obtained from the Central Sydney Area Health Ethics Committee.

Gingival crevicular fluid volumes were measured before and after the application of a sectional orthodontic cantilever appliance and these were quantified with a Periotron machine before subsequent centrifugation and analysis using ELISA.

Unfortunately, the sensitivity of the human osteocalcin kit used ($>0.5\text{ng/mL}$) was not sufficient to detect quantifiable levels of osteocalcin in the GCF of our sample of adolescent orthodontic patients.

This reflected the results of Wilson and associates (2003) who were unable to detect osteocalcin in human GCF but were able to isolate representative samples of the bone protein from oral trabecular and cortical bone biopsies.

Similarly, Kanimatsu *et al.* (1993) found no detectable levels of osteocalcin in GCF of gingivitis patients, while patients with untreated periodontitis had osteocalcin levels 200 to 500 times higher than in serum.

It was therefore decided to use a rodent model to investigate the effect of orthodontic tooth movement on serum osteocalcin levels using the variable of fluoride intake. It was thought that the use of this model might further help to elucidate the nature of this bone protein in hard tissue metabolism within the context of orthodontic tooth movement.

Fluoride was introduced as a variable in order to explore any possible interaction that may exist with the expression of osteocalcin during orthodontic tooth movement.

2. Review of the Literature

2.1 The Heterogeneity of Bone Tissue

Bone serves a dual role in the body due to its mechanical and metabolic properties. To provide these functions, the skeletal compartment is constantly remodelling in response to both gravitational and hormonal signals from the environment.

Morphologically, the skeleton consists of two macroscopically different envelopes: cortical bone and cancellous bone (80% and 20% of the skeleton, respectively). Cortical bone is composed of Haversian systems comprising lamellae arranged in a concentric manner around a central (Haversian) canal containing blood vessels. Cancellous bone, however, consists of trabeculae that are interconnected in a honeycomb formation to maximise the mechanical properties of the bone (Stepensky *et al.*, 2003). Due to differences in spatial organisation, the percentage of surface covered by cells is smaller for cortical bone as compared with cancellous bone. Hence, the two systems differ in metabolic activity, drug disposition and responses to metabolic change (Eriksen *et al.*, 1993).

The composition of bone changes with growth and the process of mineralization, however, mature bone in a given species is fairly constant in composition (Posner, 1996). Physiological activity of bone does not remain constant at varying tissue sites due to the bone remodelling process that is performed within the basic multicellular units (BMU).

Bone remodelling serves to maintain the mechanical integrity of the skeleton and also provides a mechanism by which calcium and phosphate ions may be released from or conserved within the skeleton. It consists of the osteoclastic removal of a quantum of bone, followed by the osteoblastic formation of osteoid within the cavity thus created. This osteoid is then subsequently mineralized.

In normal adult bone, the processes of resorption and formation are coupled in both space and time; therefore, bone resorption always precedes formation, and in the young adult skeleton, the amounts of bone formed and resorbed are quantitatively similar. The lifespan of each remodelling unit in humans is believed to be between two and eight months, with most of this period occupied by bone formation (Compston, 2002).

Sequential bone remodelling in the 3-month-old rat, however, has a resorption cycle of 1.4 days followed by a reversal phase of 4.2 days and a formation cycle of 33 days, as measured from the vertebrae (Baron *et al.*, 1984).

It is estimated that the adult human skeleton contains more than one million BMUs at any one time, with nearly five-fold more occurring in trabecular bone versus cortical bone. The net result is an exchange of 10% of the skeleton over a one-year period.

It follows that trabecular bone is more susceptible to conditions of turnover. This is an important concept when considering the impact of remodelling on the bone of the oral cavity, which has a higher cortical-to-trabecular bone ratio than vertebrae (McCauley and Nohutcu, 2002).

2.1.1 The Structure of Bone

Bone is a collection of several cell types embedded in an extracellular matrix composed of collagen and other proteins, with calcium phosphate crystals and hydroxyapatite providing structural integrity. The primary cells of the bone are the osteoblasts, the osteocytes and the osteoclasts, which respectively produce, reside in, and degrade the bone matrix, and have a complex system of differentiation and regulation. The proteins that comprise the extracellular matrix can be divided into two categories: the collagenous and the non-collagenous.

The composition of bone matrix is as follows: bone tissue wet weight is 70% mineralized matrix, 25% organic matrix and cells and 5% water. 40% of the dry weight of the bone is organic component and 90% of organic matrix is collagen. Collagen consists of a triple helix of tropocollagen in which pores exist within the collagen fibrils to enable mineral deposition.

The collagenous proteins consist almost entirely of Type I collagen in addition to 3% Type III collagen. The non-collagenous proteins are much more diverse, consisting of 15% osteonectin, 15% osteocalcin, 3% osteopontin, 12% bone sialoprotein II, 5% proteoglycan I, 2% matrix gla-protein, in addition to assorted bone morphogenetic factors, bone growth factors, serum proteins and other unidentified proteins (Horton, 1991).

The matrix proteins are osteonectin, osteocalcin and osteopontin. These have previously been thought to promote mineralization and bone formation. In contrast to the hard tissue specificity of osteocalcin, these matrix proteins exhibit much wider tissue distribution. Osteonectin is found in non-osseous locations including platelets and osteopontin is expressed in the renal medulla as well as in bone. Osteonectin is produced by osteoblasts and may mediate the deposition of hydroxyapatite, bind to growth factors, and influence the cell cycle. Most of these proteins have only recently been discovered and investigations are currently in progress to determine their specific structures and functions.

Roach (1994) investigated the non-collagenous bone proteins via immunohistochemistry during *de novo* mineralization and bone resorption. Both osteopontin and bone sialoprotein were localised ahead of the mineralization front, suggesting that both proteins are necessary for the initiation of bone mineralization. This supports previous suggestions that bone sialoprotein acts as a crystal nucleator. The role of osteopontin is less certain, but may be related to ensuring that the correct crystal structure is formed. Hence, osteopontin, although not associated specifically with bone, may also be implicated in regulating mineralization (Rodan, 1992). Roach (1994) also found that osteocalcin and osteonectin were not present in areas of first crystal formation, but were present in the fully mineralized matrix. Their role may, therefore, be to control the size and speed of crystal formation. Osteopontin, bone sialoproteins and osteocalcin (but not osteonectin) were also found to be present at reversal lines.

Interpreting this localisation together with information from the literature, the following functions are suggested during bone resorption: osteocalcin may act as a chemoattractant for osteoclasts, while both osteopontin and bone sialoprotein may facilitate the binding of osteoclasts.

Immunohistochemical studies have shown that bone sialoprotein, osteopontin and osteocalcin have unique distributions within the periodontium. They are all secreted at different stages of osteogenic cell differentiation (Ivanovski *et al.*, 2001). Both osteopontin and bone sialoprotein have been identified in cementum and may play regulatory roles during the process of cementogenesis (MacNeil *et al.*, 1995).

Osteopontin, a phosphorylated glycoprotein, is produced by osteoblasts and incorporated into matrix during bone formation. This noncollagenous protein (NCP), however, differs from osteocalcin in that it belongs to a family of unique phosphorylated glycoproteins that contain the tri-peptide sequence Arg-Gly-Asp common to cell attachment type proteins that bind integrins. Integrins are expressed in osteoblasts and osteoclasts and are requisite for attachment of these cells to bone (Horton and Davies, 1989).

Attachment, differentiation, and activation of osteoclasts were enhanced in the presence of osteopontin *in vitro*, and osteopontin has been localized to sites of osteoclast attachment in developing rat bone (Reinholt *et al.*, 1990). Ingram *et al.*, (1994) showed that the presence of an osteocalcin- and osteopontin-rich matrix increased the recruitment and binding of osteoclasts.

However, given the limited number of osteons isolated containing osteocalcin-rich surfaces compared with those rich in osteopontin (16% vs. 95%, respectively), osteocalcin was postulated to represent a site-specific regulator of bone remodelling.

Other bone proteins include: Bone acidic glycoprotein which is also thought to play a role in mineralization, Biglycan and Decorin which are proteoglycans that may bind to collagen (and various growth factors in the matrix) and finally, matrix Gla-protein (MGP) which may be involved in cartilage metabolism. The rest of the organic matrix is made up of proteoglycans, non-collagenous matrix protein (glycoproteins, phospholipids, phosphoproteins), growth factors and cytokines.

2.1.2.1 The Osteoblast

Osteoblasts [Gr. *osteo*, bone; *blastos*, germ] play a principal role in bone formation. These cells not only regulate new bone formation, but also indirectly mediate osteoclastic bone resorption. Previous investigations examining the effects of mechanical stress and electrical energy on bone remodelling (Rubin and McLeod, 1994) support the notion that osteoblasts transform stimuli into chemical signals that trigger either bone formation or resorption in accordance with Wolff's law. In health, osteoblasts maintain equilibrium between bone formation and bone resorption, which allows the skeleton to maintain its structural and anatomical functions.

2.1.2.1.1 Origin and Differentiation of Osteoblasts

Osteoblasts are of mesenchymal origin, arising primarily from stromal cells found in cancellous bone. The periosteum may serve as an important reservoir of osteoblasts, particularly during growth, following skeletal fracture, or with bone-forming tumours.

Ontogeny occurs along a continuum, beginning with pluripotent stem cells and ending with terminally differentiated osteocytes. Pluripotent stromal stem cells have an endless capacity for renewal but are unable to synthesize many of the proteins necessary for bone formation. Some of these stromal precursors, under the influence of growth factors and other stimuli, evolve into the more differentiated osteoblasts (Tenenbaum, 1990).

With differentiation, new genes are activated while others are inhibited, and the cells gradually and systematically change their phenotypes. From the undifferentiated, pluripotent stromal stem cells arises a group of mesenchymal cells that have been termed "inducible osteoprogenitor cells" (IOPC's) (Urist *et al.*, 1983). The IOPC's respond to bone morphogenic protein (BMP) and other growth factors, eventually differentiating into "determined osteoprogenitor cells" (DOPC's).

The DOPC's, under the influence of a variety of systemic and bone-derived growth factors, mature into osteoblasts. Interestingly, both the IOPC and DOPC populations appear to have a limited number of cell divisions and, therefore, unlike the pluripotent stem cells, are not completely self-renewing (Stutzman and Petrovic, 1982).

Both the IOPC's and DOPC's are found within cancellous bone and give rise to orthotopic (intra-skeletal) bone formation.

Synthesis, deposition, and mineralization of the organic matrix of bone are complex processes that require the ordered expression of a number of osteoblast genes. The maturation sequence of osteoblasts has been divided into three consecutive phases: proliferation, extracellular matrix maturation, and mineralization.

2.1.2.1.2 The Synthetic Function of Osteoblasts

The principal role of osteoblasts is to form new bone via the synthesis of various proteins and polysaccharides. Other functions include the regulation of bone remodelling and mineral metabolism. Studies of osteoblastic cells isolated from trabecular bone, embryonic calvaria, and osteosarcoma have established a set of phenotypic markers of osteoblastic function. (Rodan and Rodan,1983). These include the synthesis of type I collagen, the expression of alkaline phosphatase (ALP), the secretion of osteocalcin, and the production of mineralized matrix (Manolagas and Jilka, 1995; Skjodt and Russell, 1992).

The phenotype is modulated by 1, 25-(OH)₂ D₃ (vitamin D₃), parathyroid hormone (PTH), glucocorticoids, prostanoids, and a number of cytokine growth factors (Beresford and Gallagher, 1984; McSheehy and Chambers, 1984).

These bone-regulating factors appear to exert their initial effect on the osteoblasts which are thereby stimulated to produce factors that activate osteoclasts (Reid *et al.*, 1986). Indeed, osteoclasts do not have receptors for 1, 25-(OH)₂ D₃ or PTH. Another consideration concerns the effects of calcitriol and PTH on bone metabolism and calcium homeostasis. Calcitriol can stimulate osteocalcin synthesis at concentrations that inhibit bone formation parameters, such as alkaline phosphatase and collagen synthesis.

Although physiological levels of vitamin D maintain normal mineralization of bone, elevated 1, 25-(OH)₂ D (calcitriol) levels lead to increased osteoclast activity and bone resorption for regulation of serum Ca²⁺. The specific induction of osteoblastic osteocalcin synthesis in the face of decreasing collagen synthesis implicates osteocalcin in mediating the resorptive effects of 1, 25-(OH)₂ D on bone metabolism. It is hence possible that osteocalcin may be involved in the effects of 1, 25-(OH)₂ D₃ on the differentiation of progenitor cells to form osteoclasts (Bar-Shavit *et al.*, 1983). This complex organization of bone suggests that regulation of remodelling involves several levels of control.

Although the maintenance of mineral homeostasis relies on circulating hormones such as calcitriol and PTH, regulation of resorption sites must ultimately rely on micro-environmental signals such as cytokines generated by bone cells and on site-specific, immobilized signals present within the extracellular matrix such as osteocalcin (Ingram *et al.*, 1994). Interestingly, a potential osteocalcin receptor has been described in immortalized human osteoblasts (Bodine and Komm, 1999).

The culmination of histomorphologic and gene expression studies by Pavlin *et al.* (2001) provided the first direct evidence, in an *in vivo* system, that osteogenic mechanical stress increases deposition of a new bone matrix primarily by stimulating differentiation of precursor cells into the mature osteoblasts and, to a lesser extent, by increasing the number of these cells on a bone surface.

2.1.2.2 The Osteocyte

Osteoblasts that have become encased in mineralized matrix are termed osteocytes. The osteocyte is the most abundant cell type in bone. Morphologically, osteocytes resemble osteoblasts in every respect, having prominent nucleoli, abundant rough endoplasmic reticulum (RER), and a peri-nuclear Golgi apparatus. Biochemically, they produce the same proteins as fully mature osteoblasts. However, as more mineral is laid down and osteocytes become encased deeper within the matrix, biochemical and morphological changes gradually occur that distinguish them from their precursors. As a result of the decreased synthetic capacity and cytoplasmic volume, the nuclei become the most prominent osteocytic cellular features (Holtrop, 1990).

The precise role of this cell in the physiology of bone is poorly understood and has been hampered by the fact that the cells are embedded in the mineralized matrix, making the tissue technically difficult to handle.

Experimental studies now indicate that osteocytes are sensitive to stress applied to intact bone and may indeed function as mechanosensors with intermittent loading at physiological strain magnitude producing rapid changes in metabolic activity (Meikle, 2002).

Osteocytes are also thought to contribute to bone strength by an unknown mechanism that may be related to bone quality. Loss of osteocytes may disrupt the osteocyte canalicular network resulting in a failure to detect signals that normally stimulate replacement of damaged bone (O'Brien *et al.*, 2004).

Disruption of this network could also be expected to disrupt fluid flow within the network that could adversely affect the material properties of the surrounding bone independently of changes in bone remodelling or micro-architecture.

Studies that have utilized an antibody against the propeptide of osteocalcin have indicated that in human bone, osteocytes are a primary source of osteocalcin. It has also been shown that osteocalcin may function by signalling osteoclasts and/or their precursors to initiate bone resorption (Robey, 1996).

2.1.2.3 The Osteoclast

Osteoclasts [Gr. *osteo*, bone; *klastos*, broken] are multinucleate, highly motile cells formed by the fusion of mononuclear precursor cells that migrate from the bone marrow to skeletal sites via the vasculature. After exiting the circulation at specific sites in the skeleton they may fuse with existing osteoclasts or with each other to form *de novo* osteoclasts, or remain as mononuclear cells to constitute a precursor pool for future recruitment.

The bone microenvironment plays an important role in osteoclast formation and function and is dependent upon local signals from other cells, chiefly but not exclusively those of the osteoblast lineage, and by growth factors sequestered in the bone matrix (Meikle, 2002). Osteoclasts are relatively sparsely distributed in bone and present difficulties for cell culture since they represent the end point of a differentiation process, and are themselves, incapable of differentiation (Arnett, 1990).

Typical tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts expressing calcitonin receptors and capable of resorbing bone are formed in murine bone marrow cultures in response to the osteotropic hormones 1, 25-(OH)₂ D₃, PTH and IL-1. Calcitonin is the only calcitropic agent that directly acts on mature osteoclasts to inhibit resorption. (Takahashi *et al.*, 1988).

IL-1 is a potent stimulator of bone resorption, which has its primary effect on the enhancement of division and differentiation of committed pre-osteoclasts (i.e. the later stages of osteoclastogenesis). In orthodontic tooth movement using the rat model, IL-1 elevations in alveolar bone are transient and precede the appearance of increases in the osteoclast population by several days. This strongly suggests that recruitment of pre-osteoclasts may be more important during tooth movement than the stimulation of large, multinuclear osteoclasts already resident at the site (Hughes and King, 1998).

This theory is supported by the work of Rody *et al.* (2001) who used a similar rodent model to elicit orthodontic tooth movement. They suggested that osteoclasts in the periodontal ligament (PDL) originate by the fusion of recently recruited pre-osteoclasts from the alveolar marrow. The development of osteoclasts from haemopoietic precursors in bone marrow cultures does not occur unless cells of the osteoblast lineage are present.

The symbiotic relationship between osteoblasts and osteoclasts is evidenced by the fact that osteoblast cultures can stimulate osteoclast function through a porous membrane, indicating that paracrine signals, capable of crossing the membrane separating the two cell compartments, are released by the bone-forming cells (Teti *et al.*, 1991). The identity of these paracrine signals has not yet been ascertained; however, osteocalcin has been investigated as a protein of particular interest (Ingram *et al.*, 1994).

2.1.3 The Structure of Cementum

Cementum comprises water, organic matrix and mineral. By volume, the inorganic material comprises approximately 45% inorganic material, 33% organic and 22% water (Berkovitz *et al.*, 1992).

Like bone, the mineral component in human cementum is hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, with small amounts of amorphous calcium phosphates (Bosshardt and Selvig, 1997). The hydroxyapatite in cementum is not chemically pure but contains other elements that have been taken up from the tissue fluid during initial crystallization. The amount of these ions initially incorporated into the mineral phase reflects their concentration in the fluid environment during the process of mineralization. With the passing of time, the concentration may change by additional uptake or substitution with other ions (Bosshardt and Selvig, 1997).

Biochemical analysis of human cementum has shown that the organic matrix consists of approximately 90% type I collagen and 5% type III collagen (Christner *et al.*, 1977).

The collagens in cementum have an important structural and morphogenic role and provide a scaffold for subsequent mineralization (Christoffersen and Landis, 1991).

The noncollagenous proteins of cementum are made up of various glycoproteins, enamel-related proteins, proteoglycans and osteocalcin. Osteocalcin has been isolated throughout root cementum (Ivanovski et al., 2001). The glycoproteins in cementum include bone sialoprotein, osteopontin, osteonectin and fibronectin.

These proteins are thought to have a function in the process of mineralization. The proteoglycans of cementum are small proteins and biochemical analyses of human cementum have identified dermatan sulphate, chondroitin sulfate, and hyaluronic acid as the glycosaminoglycan constituents of these proteoglycans (Bartold *et al.*, 1988).

Morphologically two types of cementum are recognized: acellular (primary) and cellular (secondary) cementum; both forms are deposited in layers throughout life in response to physiological demands placed on the tooth within the periodontal ligament. The first formed cement is of the acellular type, and this is usually laid down on the surface of dentine, being most commonly found towards the cemento-enamel junction (CEJ). Cellular cementum is usually found in the apical part of the root, gradually increasing in thickness as the apex is approached.

When cementum is formed slowly it is usually acellular. However, when cementum is formed rapidly, cementoblasts may become incorporated in cementum and are then known as cementocytes. Since cementum is being continually formed throughout life, a layer of cementoblasts lines the surface of the cementum (Scott and Symons, 1982).

Interposed between the layer of cementoblasts and cementum is the uncalcified matrix, the cementoid. It has been demonstrated that root cementum is more resistant to resorption than bone, and that this resistance is due to the presence of cementoid. (Lindskog and Hammarström, 1980; Jones and Boyde, 1988).

2.1.3.1 The Cementoblast

Relatively little is known about the nature of the cementoblast phenotype and the cellular mechanisms that may regulate cementogenesis. McCulloch *et al.* (1987) have reported that progenitor cells may migrate into the periodontal ligament from endosteal spaces in the adjacent alveolar bone, and then give rise to both osteoblasts and cementoblasts in mice.

The suggestion that cementoblasts and osteoblasts may share a common progenitor cell is supported by the finding that osteoprogenitor cells from foetal rat calvariae form cementum-like tissue when cultured on tooth root slices, as judged by histological and ultrastructural criteria (Melcher *et al.*, 1986).

Tenorio *et al.* (1993) demonstrated phenotypical similarities between cementoblasts producing cellular (but not acellular) cementum and adjacent osteoblasts reflecting the expression of shared characteristics between these two different cell types.

Matsuda *et al.* (1998) showed that cultured cells of human periodontal ligament undergo osteoblastic-type differentiation upon receiving a cyclic stretch. The interaction between Epidermal Growth Factor (EGF) and Epidermal Growth Factor Receptor (EGF-R) is thought to be central to the differentiation of periodontal ligament cells, thereby regulating the source of cementoblasts and osteoblasts.

2.1.4 Cementum and Bone – a Comparison

There are many similarities between cementum and bone in their structural and biochemical composition. Many of the phenotypic markers of osteoblasts including osteocalcin, bone sialoprotein and osteonectin are also expressed in cementoblasts (D'Errico *et al.*, 1997; Ivanovski *et al.*, 2001), while other markers, such as cementum-derived attachment protein and cementum-derived growth factor, are specifically associated with cementoblasts (Metzger *et al.*, 1998).

There are, however, several differences between bone and cementum, the most obvious being the lack of vascularity and innervation in cementum. The most distinct difference between the two tissues is in their functions and their abilities to respond to environmental changes such as mechanical stress and injury.

While the alveolar bone readily responds to mechanical environmental changes with active remodelling and/or repair, cementum does not show an anabolic response to the same challenge and has a very limited repair capability (Bosshardt and Schroeder, 1996).

This is manifest in the response of the two tissues to orthodontic tooth movement where the cementum normally undergoes minimal or degenerative changes, while the extensive remodelling of the alveolar bone allows for spatial redistribution of teeth within the jaws (King *et al.*, 1997).

2.1.5 Regulation of Bone Remodelling

Bone remodelling or turnover is a cyclic process, which is occurring dyssynchronously at thousands of sites in the human skeleton at all times. This process serves to maintain the mechanical integrity of the skeleton and also provides a mechanism by which calcium and phosphate ions may be released from or conserved within the skeleton. Each cycle is initiated by the recruitment of a team of osteoclasts to a targeted site of the bone surface. A subsequently appearing team of osteoblasts refills a cavity excavated by these osteoclasts. This process is designated 'coupling'. In normal adult bone, the process of resorption and formation are couples in both space and time; therefore, bone resorption always precedes formation, and in the young adult, the amounts of bone resorbed and formed are quantitatively similar (Compston, 2002).

Systemic hormones, local factors such as cytokines and growth factors, regulate the finely tuned process of bone remodelling. This process is governed by a continuous supply of osteoblasts and osteoclasts.

Systemic hormones influence the formation of osteoblasts and osteoclasts via their ability to control the production and/or action of local mediators (Manolagas, 2000). These exert effects on cells of both the osteoclast and osteoblast lineages by:

- (a) Stimulating the proliferation of undifferentiated precursor cells,
- (b) Recruiting cells to sites of remodelling activity and
- (c) Regulating their differentiation and function.

Osteoclasts have a limited lifespan of about 12.5 days as opposed to the lifespan of active osteoblasts, which is approximately three months. Hence, the progression of bone remodelling requires the continual addition of osteoclast precursor cells. Evidence suggests a central role for osteoblastic cells in the regulation of osteoclastic resorption. To this end, osteoblasts release paracrine factors, in particular IL-1, IL-6 and annexin-II, which are concerned with osteoclast recruitment (Roodman, 1996).

Osteoclasts ultimately undergo apoptosis characterized by nuclear and cytoplasmic condensation in addition to fragmentation of nuclear DNA. Oestrogen blocks bone resorption and has been shown to promote the apoptosis of cultured osteoclasts. In contrast, hormones that stimulate bone resorption such as PTH and $1,25(\text{OH})_2\text{D}_3$, inhibit osteoclast apoptosis *in vitro* (Roodman, 1996), while cytokines that enhance osteoclast activity, such as IL-1 and M-CSF, do so in part by increasing osteoclast survival.

When osteoclasts complete the resorptive cycle they secrete proteins such as osteocalcin and osteopontin (BSP-1), which later serve as substrates for osteoblast attachment (McKee *et al*, 1993).

In vitro experiments suggest that the conversion of the initial resorption phase of the bone remodelling cycle to one of deposition is mediated by growth factors (Meikle *et al.*, 1991; Mundy, 1994).

TGF- β , FGFs, IGFs, PDGFs and BMPs released from the matrix as it resorbs, or by bone cells activated as a consequence of the resorption process, down-regulate osteoclast formation and exert anabolic effects by stimulating new bone formation by osteoblasts; these growth factors also promote osteoblast survival by preventing osteoblast apoptosis (Hill *et al.*, 1997).

TGF- β and IGF-1 are reported to have stimulatory effects on both osteoprogenitor cells and mature osteoblasts (Gowen, 1991). Fibroblast growth factors (FGF's), both acidic (a-FGF) and basic (b-FGF) stimulate the proliferation of osteoblasts in culture, while platelet derived growth factor (PDGF) acts as a potent mitogen for all cells of mesenchymal origin (Hauschka *et al.*, 1986). Moreover, both BMP and TGF- β have been shown to promote osteoblastic bone formation in animals and recent experimental work has shown that TGF- β can actually hasten fracture healing in a rat model (Mohan, 1990).

Osteogenesis is dependent on a number of factors capable of stimulating angiogenesis, proliferation, chemotaxis and differentiation. Since no single growth factor has been shown to be capable of inducing all these biological activities, it seems likely that the bone-inducing activity derived from bone matrix represents the combined action of multiple factors acting at specific stages during osteogenesis (Meikle, 2002).

2.1.5.1 Cellular and Molecular Mechanisms of Bone Resorption

Bone resorption is a complex multistage process that involves the removal of both the mineral and organic constituents of bone matrix, constituting an essential step in the sequential process of bone remodelling. The protagonist in this process is the osteoclast with the osteoblast playing an accessory role. The osteoblasts are involved, first, by retracting to expose the non-mineralized surface layer and, second, by releasing matrix metalloproteinases that degrade the surface layer of osteoid. This facilitates access of osteoclasts to the mineralized matrix, the latter of which is chemotactic for osteoclasts.

The osteoclast is actively engaged in the synthesis, vectorial transport, and polarized secretion of lysosomal enzymes toward its apical ruffled-border membrane. These vesicles dissociate upon reaching the low pH established in the bone-resorbing compartment by the osteoclast. The apical bone-resorbing compartment is sealed off by the attachment of the osteoclast to the calcified matrix and is actively acidified by the osteoclast.

Once the osteoclast forms a sealed-off bone-resorbing compartment and secretes lysosomal enzymes and protons (producing a low pH environment), the mineral phase dissolves, exposing the organic matrix to the proteolytic action of the matrix metalloproteinases and cysteine proteinases (Baron, 1989).

Three resorption steps can be distinguished:

1. Recruitment of osteoclastic precursors
2. New osteoclast activation
3. Activation of mature osteoclasts present near the bone formation site.

Bone formation is then initiated. Finally, osteoclastic cells receive signals for termination of bone resorption (the nature of this end-signal is poorly defined and may depend on calcium levels or cytokines) and release many soluble factors that activate osteoblasts and ensure bone formation until the cessation of osteogenic stimuli (Heymann *et al.*, 1998).

A great deal of information about the systemic, and more recently the local regulation of bone has been accumulated until the present day. However, despite the exciting progress of recent years, we are still far from being able to integrate this knowledge into a coherent vision of just how bone turnover is regulated *in vivo*. Hence, we can only speculate with hypothetical models about the cellular interactions and molecular mechanisms involved in this complex sequence of events.

2.1.5.2 Modulation of Orthodontic Movement

Bone modelling is a mechanically- mediated adaptive process for changing a bone's size, shape, or position. Site specific, anabolic and catabolic modelling events are manifestations of overload hypertrophy and disuse atrophy, respectively. Catabolic bone modelling at the periodontal ligament (PDL) surface is the rate-limiting step in tooth movement.

In initiating tooth movement, connective tissue growth factor (CTGF) is expressed in osteoblasts and osteocytes, and osteopontin (OPN) is expressed by osteoclasts and osteocytes. Undermining resorption occurs by recruiting osteoclasts to the site of maximal PDL compression and a paravascular osteogenic response is initiated in widened areas of the PDL:

- (1) Pre-osteoblast formation at ~10 hours,
- (2) Peak DNA synthesis at ~20 hours,
- (3) Maximum rate of mitosis at ~30 hours, and
- (4) Initiation of bone formation at ~48 hours.

At other skeletal sites, additional genes have been linked to mechanical activation of bone: glutamate/aspartate transporter (GLAST), nitric oxide synthetase (NOS), prostaglandin G/H synthetase (PGHS-2), Msx1, and c-fos. An intricate series of endocrine, paracrine, and autocrine factors has been described.

Parathyroid hormone (PTH) enhances expression of both insulin-like growth factor I (IGF-I), and the oestrogen receptor beta (ER- β), which are both modulators of mechanical loading. Transforming growth factor beta (TGF- β) and the related bone morphogenic proteins (BMPs) help control anabolic modelling. PGE2, IL-1, and other inflammatory cytokines mediate catabolic modelling. Osteoclast differentiation and activation is controlled by genes related to tumour necrosis factor (TNF) and its receptor (TNF-R), colony stimulating factor 1 (CSF-1), osteoprotegerin (OPG), receptor activator of nuclear factor kappa (RANK), and RANK ligand (RANKL) (Roberts *et al.*, 2004).

2.1.5.3 Orthodontic Tooth Movement in the Rodent Model

Rat alveolar bone has frequently been used for the study of bone remodelling because the alveolar socket of the rat molar has a perfect balance between bone resorption and formation, a constant spatial orientation of these activities, and a very high rate of bone turnover. Rat molars drift distally under physiological conditions and this results in balanced bone turnover (Vignery and Baron, 1980). However, the normal pattern of bone resorption on the distal alveolar surface and the failure to demonstrate any peak in its activity is rapidly reversed with the application of a mesially directed orthodontic force on the rat molar (King *et al.*, 1991). In this model, an early wave of resorption is followed by a wave of formation with the relative amounts differing markedly (compared with controls).

Bone accrues at tension sites due to an inhibition of resorption and a stimulation of formation, with the most marked changes occurring late in the tooth movement.

At pressure sites, bone is lost due to the stimulation of resorption and the inhibition of formation occurs early in tooth movement. This suggests that the normal degree of coupling between bone formation and resorption is disturbed in orthodontic tooth movement (Melsen *et al.*, 1999). This occurs at an early stage at sites of pressure and later in sites of tension. These imbalances facilitate tooth relocation by removing bone ahead of the tooth and depositing it behind.

In this respect, this quantitative uncoupling of bone remodelling is similar to the process of osteoporosis. In short, the uncoupling of resorption and formation is manifested in a 14-day period as:

- a) A period of little change (1-3 days)
- b) A period that is predominantly resorptive (5-7 days)
- c) A period that is primarily formative (beginning between days 5 and 7 and lasting until the end of the experiment on day 14) (King *et al.*, 1991).

It is further suggested that enhancement of bone formation in orthodontic tooth movement occurs by increases in the numbers of active osteoblasts. It is equivocal as to whether a significant increase in their functioning also occurs. Significant late changes in osteoid perimeter may only reflect increased numbers of osteoblasts, since no changes in bone formation rate are detectable (King *et al.*, 1991).

Lu *et al.* (1999) applied orthodontic forces (50g) to mandibular rat molars, demonstrating higher distribution of osteoclastic cells on the alveolar bone than on the root surface, indicating that alveolar bone is more readily removed than the root surface. They also showed that cellular cementum may be resorbed more easily than acellular cementum due to its enhanced organic components and low mineralized structure.

King and Fischlschweiger (1982) demonstrated that maximum tooth movement was achieved with forces averaging 40g during day 7 (in the rat model), whereas substantially larger forces produced significantly less tooth movement; and that when appliances were loaded with increasing amounts of force, the specific activity of bone resorption stimulators increased progressively. This was highlighted by the study of Ashizawa and Sahar (1998) who moved rat molars mesially with a fixed coil-spring appliance using three different magnitudes of initial tensile force (27, 60 and 136g). They found little or no relationship between the magnitude of the initial tensile force and the amount of new bone formation in the alveolar wall on the tension side during the initial stage of tooth movement. However, a heavy initial force (136g) produced a wide necrotic area of periodontal ligament, and delayed the bone remodelling that occurred on the pressure side.

2.1.5.3.1 Influence of Rodent Age on Tooth Movement

Bridges *et al.* (1988) evaluated the effects of age on tooth movement and concomitant mineral density in the treated tissues of the rat. The absolute amount of tooth movement occurring in a cycle and the rate of tooth movement in the late part of the cycle were significantly greater in the younger animals (21-28 days old), while the total time required for a cycle was shorter in the younger animals.

The "instantaneous" tooth movement was greater in the young animals and the control tissue mineral density was lower, suggesting that bone density may play a key role in this phase of the cycle. The delay period was longer in the adult rat (90-100 days old), but bone densities were not significantly different between the young and adult groups at the end of this period, suggesting that there is a minimal tissue mineral density, unrelated to the remodelling, that occurs as a result of tooth movement. Mineral densities returned to control levels at approximately the same rate in the tissues of both groups during the late part of the cycle, but the rate of tooth movement in the adult was less than in the younger animal. This suggests that the proportions of bone formation to resorption are equivalent at comparable times in both groups, but that absolute amounts of each may be higher in the younger animals.

2.1.5.3.2 The Influence of Hormones on Rodent Tooth Movement

Sex hormones play important physiological roles in bone metabolism. Haruyama *et al.* (2002) showed that orthodontic tooth movement varies depending on the phase of the oestrus cycle in rats. It was shown that the serum oestradiol level varied according to the phase of the oestrus cycle, with a peak during pro-oestrus and a nadir during oestrus, and was inversely related to tooth movement. Oestrogen is known to inhibit osteoclasts both directly and indirectly (Harris *et al.*, 1996) and negative correlations were observed between oestradiol and markers of bone resorption (TRAP and pyridinoline) in the orthodontic rat study by Haruyama *et al.* (2002).

Since the rate of orthodontic tooth movement strongly depends on the activity of osteoclasts that resorb bone (Rygh, 1986), cyclic changes in serum oestrogen may account for the observed variation in tooth movement.

Haruyama *et al.* (2002) found that progesterone also exhibited oestrus-cycle-dependent fluctuation. This fluctuation did not seem to be related to tooth movement or to be significantly correlated with TRAP and pyridinoline. However, there was a significant positive correlation between this hormone and serum osteocalcin.

2.1.5.3.3 The Process of Orthodontic Root Resorption

Like bone metabolism, the precise mechanism of orthodontically-induced root resorption remains somewhat elusive, however, the fundamental component underlying the pathologic process is a local inflammatory reaction (Bosshardt *et al.*, 1998; Brezniak and Wasserstein, 2002).

The alveolar bone adjacent to the hyaline zone is removed by indirect resorption, *i.e.* by osteoclasts working from adjacent marrow spaces or on the surface of the alveolar bone (Reitan, 1960). A corresponding resorption of the tooth root occurs in the area bordering the hyaline zone (King and Fischlschweiger, 1982).

Resorption of the calcified dental tissues is believed to occur when the root-resorbing cell or 'odontoclast' is able to access the mineralized tissue through a breach in the formative cell layer covering the tissue (Tronstad, 1988; Jones and Boyde, 1988), if the mineral and matrix surfaces coincide (Jones and Boyde, 1988), or if the precementum is mechanically damaged or denuded. These denuded root areas attract hard tissue resorbing cells to colonize the damaged areas of the root (Tronstad, 1988).

Orthodontic-related root resorption is associated with local over-compression of the periodontal membrane and concomitant development of a sterile coagulation necrosis which has been termed the hyaline zone (Nakane and Kameyama, 1987; Tanaka *et al.*, 1990).

The displacement of the periodontal ligament is a two-step process: an initial morphometric change (compression) followed by biochemical changes. The histochemical changes seen in the periodontal ligament involve fibre coalescence and hyalinization (Reitan, 1985) as well as degradation of connective tissue matrices by both an extracellular pathway (matrix metalloproteinases), and an intracellular phagocytic pathway (lysosomal enzymes) (Sodek and Overall, 1988). Concomitantly, there is also demineralization of Sharpey's fibres (Jones and Boyde, 1988).

In the rodent model, the first cells to be involved in the initial stage of root resorption at the periphery of the necrotic zone have been shown to be mono-nucleated, TRAP-negative (non-clast) cells resembling macrophages and/or fibroblasts (Brudvik and Rygh, 1993a,b). TRAP staining is used as a marker of osteoclastic cells and their precursors (Cole and Waters, 1987).

Underneath the main hyaline zone in the latter stage of root resorption, both multi-nucleated, TRAP-positive, giant cells without a ruffled border and mono-nucleated, TRAP-positive, macrophage-like cells appear to be responsible for removal of the necrotic tissue and resorption of the surface part of the root cementum (Brudvik and Rygh, 1994a,b). It is believed that the multi-nucleated giant cells presenting without a ruffled border in this destructive process are derived from the mono-nucleated phagocytic system (Brudvik and Rygh, 1994b).

In a study involving rat molar intrusion, Lu *et al.* (1999) demonstrated that distribution of TRAP-positive cells was higher on the alveolar bone than on the root surface, which is in agreement with a previous study concluding that the former is more readily resorbed than the latter (Lindskog and Hammarström, 1980). This may be an explanation for the observation that clast cells (including osteoclasts from haematopoietic progenitors of bone marrow) may be induced quickly (by mechanical stimuli) on the alveolar bone surface facing the periodontal space.

Rodent studies have confirmed that orthodontic root resorption is a consequence of the hyaline zone elimination process (Brudvik and Rygh, 1993a,b; 1994a,b; 1995a,b).

It has been reported that the uncalcified mineral tissues, osteoid, precementum, and predentine are resistant to resorption and may initially prevent loss of root tissue (Reitan 1985). However, continuous pressure will eventually lead to resorption of these areas (Reitan 1985, Tronstad 1988).

Facilitating the movement of molar teeth in the rodent model, Hellsing and Hammarström (1996) showed that limited areas of hyalinized tissue were seen in the periodontal ligament (PDL) as early as one day after application of the orthodontic appliance and root resorption could be seen as early as seven days after active treatment. This supported the results of Engström *et al.* (1988), who further concluded that the observed root resorption in their rat model was clearly related to the degradation process occurring in the vicinity of the hyaline zone.

The cementum underlying the dense hyaline zone showed evidence of outer surface degradation, possibly due to enzymes released from the mechanically damaged PDL. This suggested that the unmineralized cementoid had been degraded and the mineralized cementum exposed. In the periphery of the hyaline zone multinucleated cells were found, having been possibly attracted to the region by the exposed mineralized cementum. Degradation of the osteoid layer has previously been found to precede osteoclastic bone resorption (Chambers and Fuller, 1985).

Hellsing and Hammarström (1996) also showed that there is a complex pattern on the root surface when a tooth is exposed to an orthodontic force. Smooth areas and resorption cavities were found to alternate with normal cementum. A postulated explanation was that a varying thickness and density of the hyaline zone resulted in differences in elimination time. The subsequent access of resorption cells to the exposed surface would then occur at varying times.

2.1.6 Markers of Bone Metabolism

The rate of formation and resorption of bone can be assessed by measuring enzymatic activity related to bone-forming (alkaline phosphatase) or resorbing (acid phosphatase) cells. An alternative is to measure bone matrix components released into the circulation, either after their secretion by osteoblasts (osteocalcin, procollagen Type I) or after their release (as breakdown products) during osteoclastic bone resorption (urinary hydroxyproline, pyridinoline cross-links).

It must be emphasized that the level of biochemical markers of bone formation and resorption represents primarily the whole body rate of bone remodelling. They allow little to be gleaned regarding the function of individual teams of osteoclasts and osteoblasts, and give no information about the activity of individual cells (van Daele *et al.*, 1994).

i.e. In disease states in which resorption and formation are coupled and in balance, either of the markers will reflect the overall rate of bone turnover.

Osteoblastic activity is closely associated with serum osteocalcin, one of the proteins found in relatively high concentration in bone. Bone specific alkaline phosphatase (measured by radioimmunoassay, not by iso-enzyme analysis of alkaline phosphatase) is more sensitive and specific than alkaline phosphatase as this enzyme is made only by active osteoblasts. Another relatively more recent test is the propeptide of type I collagen (PICP). Like many other proteins, collagen is secreted as a propeptide, and the ends are removed to synthesize collagen. These tests have been shown to correlate with bone formation as measured by bone histomorphometry or calcium kinetic studies in normal individuals.

A recent serum-based marker of bone resorption is the collagen cross-linked N-telopeptide which, in a randomized, double-blind, parallel group, placebo-controlled trial of antiresorptive therapy in osteopenic women, was significantly correlated with serum osteocalcin (Gertz *et al.*, 1998).

2.2. Osteocalcin

Osteocalcin is one of three known vitamin K-dependent proteins produced by osteoblasts, the other proteins being matrix gla-protein and Protein S. Vitamin K is a co-factor of γ -glutamyl carboxylase for the gamma-carboxylation of glutamic acid residues in a large number of proteins. There are essentially two classes of vitamin-K dependent proteins. One class is involved in protein coagulation. The γ -carboxyglutamic acid residues (Gla) in the peptide chain of the blood coagulation proteins bind to calcium ions, and this binding is thought to give these vitamin K –dependent proteins various functions. The other class of vitamin K-dependent proteins includes the calcium-binding bone protein osteocalcin.

Osteocalcin is also known as bone Gla protein (BGP) or “the vitamin K-dependent protein of bone” and accounts for 15-20% of the non-collagen proteins in the bone matrix in humans and almost 10% in rats (Bronckers *et al.*, 1998).

The name osteocalcin (Gr.*osteo*, bone; Lat.*calc*, lime salts; *in*, protein) derives from:

- 1) The Ca^{2+} affinity of this protein and
- 2) The abundance of this protein in bone tissue.

Osteocalcin is a calcium-binding peptide synthesized almost exclusively by osteoblasts and odontoblasts undergoing mineralization.

It is a small protein about 49-50 residues in size (5,900 daltons), and is synthesized mainly by osteoblasts as they synthesize the rest of the bone matrix, and then gamma-carboxylated post-translationally at three positions, residues 17, 21, and 24, by the vitamin K-dependent carboxylase system in the rough endoplasmic reticulum (Figure B). This modification leads to a conformational change, stabilizing the α -helical portion of the protein and conferring a greater affinity for calcium and hydroxyapatite (Figure A).

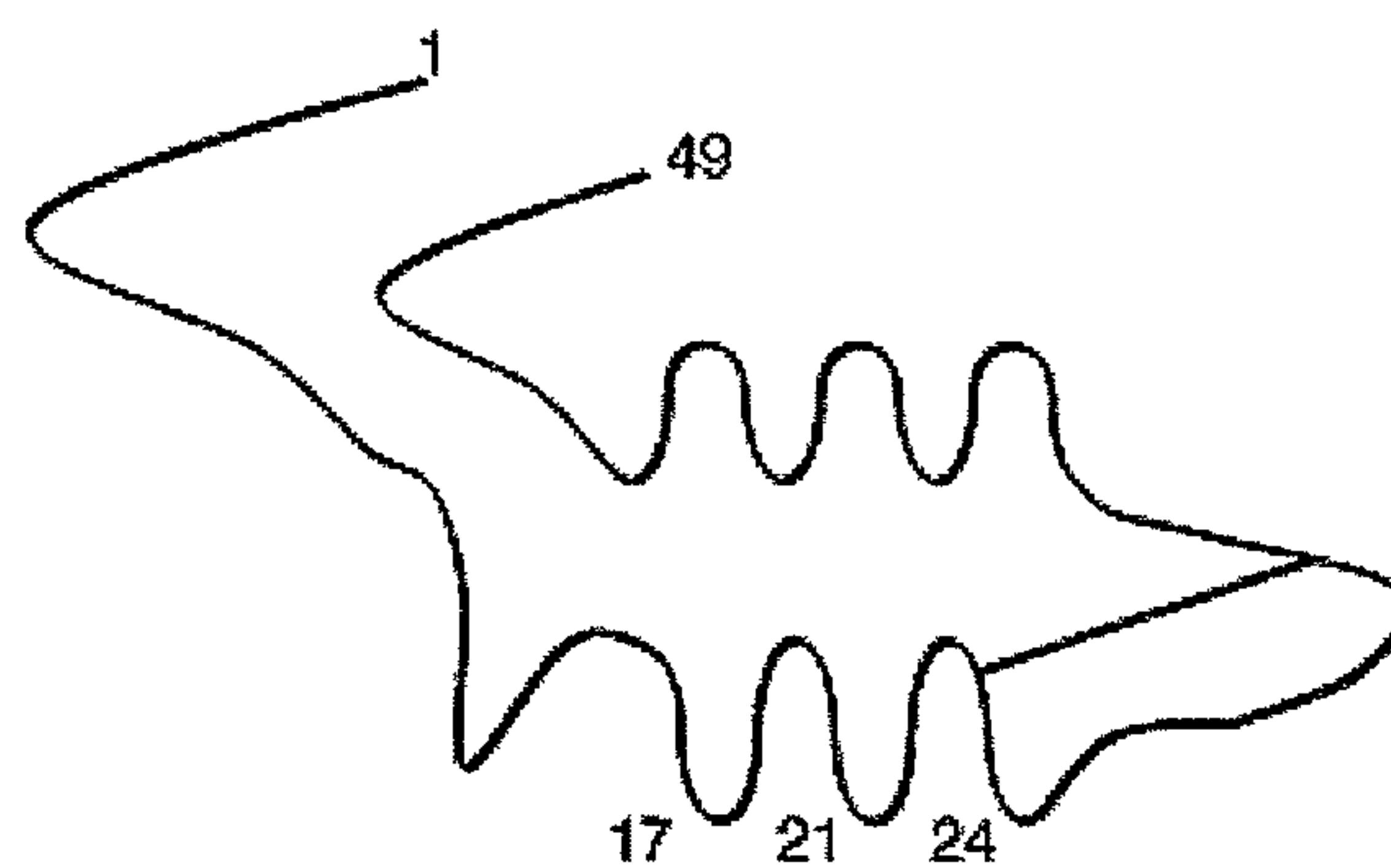


Figure A- Diagram of the secondary structure of osteocalcin. Gla residues are present at positions 17, 21 and 24 and a disulphide bridge is present between residues 23 and 29 (Source: Lee *et al.*, 2000).

This amino acid sequence is conserved among various diverse vertebrate species including the human, calf, rat and swordfish (Hauschka, 1986).

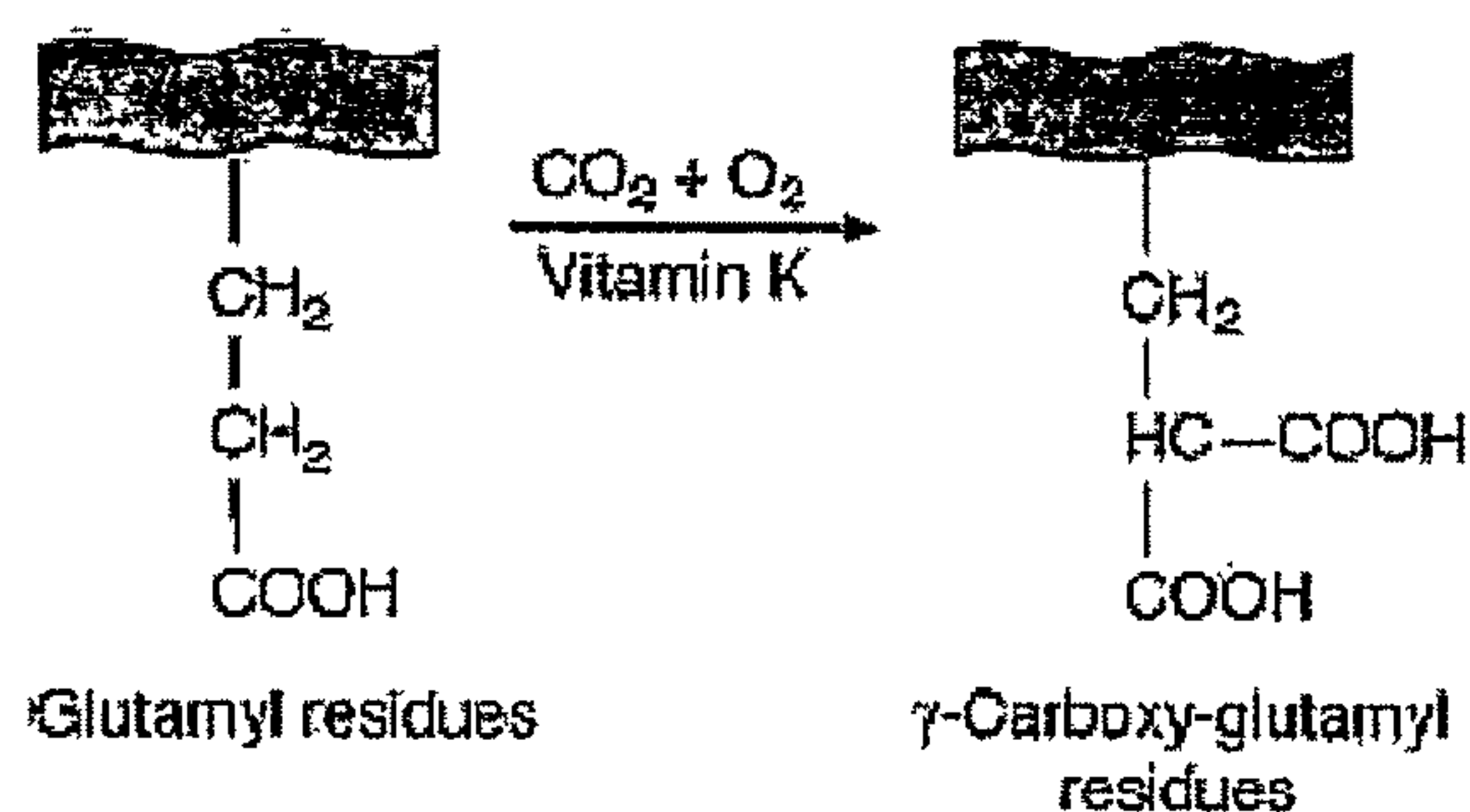


Figure B- The gamma-carboxylation of glutamyl residues. This step is catalysed by the vitamin K-dependent carboxylase with vitamin K as a co-factor (Source: Lee *et al.*, 2000).

2.2.1 Synthesis and Catabolism of Osteocalcin

Osteocalcin has long been accepted as an osteoblast, cementoblast and odontoblast-specific product (Calvo *et al.*, 1996), however, there have been reports of megakaryocyte and adipocyte expression of osteocalcin mRNA and protein (Benyahu *et al.*, 1997). It should be noted that this expression has been reported *in vitro*, in immortalized cell lines, and not *in vivo*. At this point in time, osteocalcin is thought to be exclusively restricted to mineralized tissue *in vivo*.

Osteocalcin appears to be more closely related to formation of osteoid than to formation of mineralized bone. Thus, in osteomalacia or disease where there is excess osteoid, such as Paget's disease, the osteocalcin value may be inaccurate. Osteocalcin is excreted by the kidneys, and serum values are increased in renal failure.

This may occur in elderly patients with normal creatinine values who have low glomerular filtration rates. Assays which measure the intact protein are less influenced by the glomerular filtration rate. Bone specific alkaline phosphatase is not influenced by renal failure (it is frequently elevated, but that is because the bone formation rate is actually high) (Weaver, 1997).

Most of the osteocalcin produced is incorporated into the bone matrix where, due to the high affinity of the Gla-residue for calcium ions and hydroxyapatite, it is bound (Hauschka, 1986).

Serum osteocalcin reflects the 10-40% of osteocalcin produced that is not incorporated into the bone matrix. The proportion of osteocalcin incorporated into the matrix varies from >90% in the young to 70% in adults (Swaminatham, 2001). It is postulated that newly synthesized osteocalcin is released into the circulation as the intact (1-49) molecule. Osteocalcin fragments may be derived from bone resorption and catabolism of the molecule in vivo before clearance by metalloproteases in the kidneys and the liver.

The osteocalcin knockout mouse reported by Ducy *et al.* (1996) showed no abnormal phenotype until 6 months of age, at which time there was a marked increase in bone formation. This suggests that osteocalcin may have a role in the regulation of osteoblast function. The majority of osteocalcin secreted by the osteoblast is deposited in extracellular bone matrix; serum osteocalcin represents the fraction of total osteocalcin that has not adsorbed to hydroxyapatite.

Sensitive radioimmunoassays for osteocalcin have shown that this protein circulates in blood at concentrations ranging from 2 to 15 ng/ml in normal adult humans (Tanaka *et al.*, 1986) to as much as 900ng/ml in young rats (Price and Williamson, 1981).

The enormous reservoir of osteocalcin in the skeleton can be appreciated from the fact that only $10^{-5} - 10^{-4}\%$ of the total body osteocalcin is circulating. Comparable levels of osteocalcin are found in both serum and plasma samples of humans and animals (Hauschka *et al.*, 1989).

The half-life of osteocalcin in the circulation is a few minutes and is rapidly cleared by glomerular filtration in serum. It has a half-life of five minutes in the rat and, therefore, if kidney function is normal, variations in serum levels reflect variations in production rate (Price *et al.*, 1981).

Animal studies have shown that the chief route of circulating osteocalcin is renal glomerular filtration and degradation. Administration of radiolabelled osteocalcin to rats shows primarily renal uptake with a moderate level of radioactivity being concentrated in bone and lesser amounts in liver and other soft tissues (Farrugia and Melick, 1986). The plasma half-life is five minutes in the rat, and the protein is degraded to its constituent amino acids by the proximal renal tubule. Less than 0.2% of the total daily urinary output of osteocalcin derives from circulating osteocalcin; hence the residual may be presumed to come primarily from osteocalcin released by osteoclasts in situ during bone resorption (Hauschka *et al.*, 1989).

2.2.2 Factors Affecting Osteocalcin Expression

It has been demonstrated that several factors affect expression of osteocalcin. Vitamin K and 1,25 (OH)₂ vitamin D₃ are directly involved in the regulation of osteocalcin synthesis (Price and Baukol, 1980). Dexamethasone promotes the expression of the osteoblastic phenotype including osteocalcin in rat marrow stromal cell culture (Leboy *et al.* 1991). Furthermore, osteocalcin secretion has been shown to increase with cyclic strain in culture of human osteoblast-like osteosarcoma cells (Harter *et al.* 1995).

2.2.3 Biological Properties of Osteocalcin

The precise function of osteocalcin has yet to be elucidated; however, the key undoubtedly lies in its unique structure. The molecule displays a number of biological properties that suggest a physiologically relevant role in bone growth (Hauschka *et al.*, 1989; Delmas, 1990):

1. Inhibitor of hydroxyapatite precipitation
2. Effector molecule for 1,25(OH)₂ D₃ action
3. Chemoattractant for monocytes and other cells
4. Competitive inhibition of leukocyte elastase
5. Biosynthesis by osteoblasts

6. Appearance in bone more or less coincident with the onset of mineralization
7. Low circulating levels in blood, which are modified by physiological and pathological conditions
8. Induction of membrane potential changes in mononuclear leukocytes

The amino-terminal portion of the molecule shows considerable interspecies variation, but the structural motif for the central portion of the molecule is conserved.

This conserved nature among species and its detection early in the process of mineralization suggest a fundamental role for osteocalcin in attainment and maintenance of the bone mineral matrix, as well as in bone remodelling (Power and Fottrell, 1991).

The γ -carboxylation of the three glutamic acid residues on osteocalcin (OC) gives the protein its mineral binding properties, so that it is able to adsorb onto the hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) crystals at the mineralization front of the bone. Osteocalcin binds weakly to free calcium ions and more tightly to calcium in hydroxyapatite crystals. Spectroscopic studies suggest that the molecule undergoes a conformational shift when associated with hydroxyapatite, partly as a result of electrostatic attraction between specific anionic residues in the protein and the calcium ions embedded in the face of the crystal (Cole and Hanley, 1991).

These unique structural features of osteocalcin that dictate the association with calcium ions and Ca^{2+} mineral surfaces could certainly confer on this protein the ability to modulate the mineral dynamics of bone.

Osteocalcin could inhibit or stimulate nucleation, precipitation, or metamorphosis of Ca^{2+} -phosphate mineral phases or it could mobilize Ca^{2+} from crystal surfaces. (Hauschka *et al.*, 1989).

Studies that have utilized an antibody against the propeptide of osteocalcin have indicated that in human bone, osteocytes are the primary source of osteocalcin, and that it may function by signalling osteoclasts and/or their precursors to initiate bone resorption (Robey, 1996). However, it should be noted that the localization of osteocalcin in rodent bone is quite different and it is found in the osteoblastic layer on the endosteal surfaces (Robey and Boskey, 1996).

2.2.4 Investigation of Osteocalcin Function

Bone resorption and subsequent formation involve complex events that are mediated in part by the unique proteins of bone matrix (Malone *et al.*, 1982). The specific contributions of bone matrix to resorption and formation of bone has remained nebulous due to the complex orchestrated activity of osteoblasts, osteoclasts and calcium-regulated hormones.

In 1981, Rodan and Martin first hypothesized a role for osteoblastic involvement in the hormonal control of bone resorption. The first part of the hypothesis suggested that resorbing agents, such as PTH and prostaglandins, induce a shape change in osteoblasts (via cAMP and Ca^{2+}), which uncovers matrix for osteoclastic digestion, further enhancing subsequent resorption by releasing osteocalcin.

The second aspect of the hypothesis is the potential direct action of osteoclasts by products of hormone action on the osteoblast, contributing to chemotaxis as osteocalcin has been shown to do (Rodan and Martin, 1981).

Devitalized, mineral-containing bone particles (BPs) have contributed greatly to understanding these aspects of bone biology. The implantation of BPs elicits the recruitment and differentiation of osteoclasts that resorb the BPs. Bone particle resorption reflects the state of bone metabolism in a more concentrated, homogenous form that is more suitable for quantitative analysis than is the intact skeleton (Glowacki, 1982).

Knowledge that osteoclasts are derived from a monocytic progenitor and that monocytes can be recruited by matrix degradation products of resorbing bone (Malone *et al.*, 1982), suggested investigation of the role of bone matrix components in bone resorption in vivo. Osteocalcin has been shown to be a chemoattractant for human peripheral monocytes and macrophages (Malone *et al.*, 1982).

Subcutaneous implantation of bone particles that were 99% deficient in osteocalcin have been shown to be poorly resorbed (to 60% of the levels of resorption of normal BP after 12 days of implantation) when quantitatively evaluated by histomorphometric techniques (Lian *et al.*, 1984).

The proposition that osteocalcin may function as a negative regulator for mineral apposition is supported by the observation that purified osteocalcin inhibits the spontaneous conversion of brushite to hydroxyapatite and inhibits the formation of hydroxyapatite crystals in supersaturated calcium/phosphate solutions in vitro (Hauschka *et al.*, 1989; Price 1989).

The post-translational carboxylation of the glutamate residues of osteocalcin is vitamin K-dependent and thus can be inhibited by warfarin. Warfarin treatment results in formation of an uncarboxylated osteocalcin molecule that cannot accumulate in mineral (Bronckers *et al.*, 1998).

A rat study by Glowacki and Lian (1987) clearly showed decreased recruitment of osteoclasts by bone particles that had been prepared from warfarin-treated rats compared with normal bone particles. Their data also demonstrated that the activity of the multinucleated cells that eventually appeared around the osteocalcin-depleted BPs was greatly diminished compared with the cells resorbing normal BPs.

The slower and diminished migration of cells to the bone particles and diminished activity of the osteoclasts was believed to be due to the reduced content of osteocalcin (which was the only difference observed between the two groups).

Glowacki *et al.*, (1991) used subcutaneous apatite with/without osteocalcin treatment in the rat model to further substantiate this hypothesis that osteocalcin may function as a matrix signal in the recruitment and differentiation of bone-resorbing cells.

Liggett *et al.*, (1994) provided further support for this hypothesis with the observation that osteocalcin promotes differentiation of osteoclast progenitor cells from murine long-term bone marrow cultures in the presence of colony-stimulating factor (M-CSF) and granulocyte-macrophage stimulation factor (GM-CSF), but not in the absence of these factors. M-CSF has been shown to be a crucial factor in osteoclastogenesis.

These CSFs are produced by fibroblasts and osteoblasts, and their production is greatly enhanced by IL-1 α and IL-1 β . During orthodontic tooth movement IL-1 α and IL-1 β are produced on the pressure side in response to applied force and CSFs may also be produced on the pressure side as a result of increased IL-1. Therefore, it is likely that osteocalcin promotes the osteoclastogenesis in concert with the CSFs (Kobayashi *et al.*, 1998).

There are other suggestions that osteocalcin may mediate bone resorption. Rats chronically treated with warfarin show premature closure of the epiphyseal growth plate and a denser cortical shaft (Hauschka, 1989). Warfarin treatment results in formation of an uncarboxylated osteocalcin molecule that cannot accumulate in mineral (Bronckers *et al.*, 1998). This could imply a direct inhibitory activity of normal osteocalcin on mineralization or an over-mineralization resulting from decreased resorption in the osteocalcin-deficient bone. Furthermore, in studies with rat osteopetrotic mutants that have deficient numbers of osteoclasts and decreased osteoclastic activity, bone osteocalcin levels were decreased by 30-50% (Hauschka, 1989).

Chenu *et al.* (1994) used human osteoclastic cells derived from giant cell tumours of bone to study the biological effects of osteocalcin on osteoclasts. In this study, osteocalcin promoted adhesion and spreading of these cells, triggering the release of bone sialoprotein (BSP), osteopontin (OPN) and fibronectin (FN). Furthermore, osteocalcin induced chemotaxis of osteoclasts in a dose-dependent manner. This action of osteocalcin was mediated by an intracellular calcium increase. These results provide evidence that osteocalcin exerts a role in the resorption process, inducing intracellular signalling, migration and adhesion, followed by synthesis and secretion of the aforementioned endogenous proteins.

A landmark study investigating the role of osteocalcin was performed by Ducy *et al.*, (1996). These investigators generated osteocalcin-deficient mice using embryonic stem cell technology. These mice were normal at birth and had no skeletal-patterning defects and no ectopic bone formation.

At six months, however, the mutant mice developed bone-remodelling abnormalities. They displayed long bones with increased cortical thickness and density, consistent with an increased amount of mineralized bone matrix. They also displayed an increase in width of cancellous bone of 150% compared with their wild-type littermates. Histomorphometry indicated that the lack of osteocalcin led to an increase in bone formation. Remarkably, the number of osteoblasts was not increased in the mutant animals.

Another important finding of this histomorphometric analysis was the increase in osteoclast number in the bones of the osteocalcin-deficient mice. This raised the possibility that the osteoclasts were poorly functioning in mutant mice. To test the function of osteoclasts *in vivo*, 5-month old wild-type and mutant mice were ovariectomized (Ducy *et al.*, 1996). Ovariectomy, and the oestrogen depletion it causes, creates a rapid increase in bone resorption that ultimately leads to osteoporosis.

Consistent with the increase in osteoclast surface in osteocalcin deficient mice before ovariectomy, these animals developed a more severe osteoporosis than their wild-type littermates following oestrogen depletion.

Vital dye staining performed in these two groups of animals indicated that the absence of osteocalcin did not affect bone mineralization.

Thus, osteocalcin is postulated to limit bone formation without the impairment of bone resorption or mineralization. The molecular mechanism by which osteocalcin controls bone matrix deposition is unknown. The increase in bone formation rates occurs without a comparable increase in osteoblast surface, indicating that each osteoblast is laying down more matrix. This suggests that osteocalcin, like other members of the family of proteins to which it belongs, may bind to a specific (yet to be determined) receptor to fulfil its function. Thus, osteocalcin has been identified as a negative regulator of bone formation and suggests that an antagonist of osteocalcin synthesis or function may be of some therapeutic use in the treatment of osteoporosis.

To the same end, Wolf (1996) used mice that lacked the genes for osteocalcin to study the function of the bone protein. These osteocalcin-deficient mutants had normal bones at birth but had increased bone density and thickness at six months. Their osteoblasts deposited more bone matrix than those of the wild-type mice, though mineralization was not affected. This information was used to infer that osteocalcin is a negative regulator of bone formation.

2.2.5 Applications of Osteocalcin

2.2.5.1 Non-Clinical Applications using Osteocalcin

Scientists have long considered it unlikely that proteins in fossils more than a million years old would be found. The discovery of osteocalcin in dinosaur bones by a team of scientists in the Netherlands and the USA, however, makes this the oldest protein ever detected (Muyzer *et al.*, 1992).

Osteocalcin is tightly bound to the hydroxyapatite matrix of bone, and as a consequence is relatively more stable than the dominant protein, collagen. Its distribution in nature is limited to vertebrates. Osteocalcin and collagen have previously been isolated from modern and fossil bone samples of different organisms in different depositional environments for analysis of their carbon-14 (^{14}C) content. Whereas collagen is susceptible to aqueous weathering, hydrolysis, solubilization and removal, as well as contamination by soil amino acids and peptides, osteocalcin is more strongly bound to the apatite matrix of the bone and hence less prone to loss or replacement by contaminants.

Ajie *et al.*, (1991) presented evidence that osteocalcin is, therefore, a more suitable protein fraction for obtaining accurate ^{14}C (carbon -14) estimates for paleodietary reconstruction from bone samples.

In forensic odontology, osteocalcin has also been applied successfully to determine age at death. Bone osteocalcin can hence be used as a permanent 'aging' peptide of the organic bone matrix using its D-aspartic acid content. (Ritz *et al.*, 1996).

2.2.5.2 Clinical Applications using Osteocalcin

Assays for intact osteocalcin have been shown to be related to growth velocity in children; however, there are a number of factors that may complicate interpretation of the results. These include variations in the immunochemical reactivity, the possible presence of degradation fragments in serum and the dependence of vitamin K status for adequate enzymatic carboxylation (Robins, 1994). Evidence of a correlation with growth rates comes from the observation that serum osteocalcin levels parallel the height velocity curve, with higher values in childhood and during adolescence, that later fall to adult values. There is an age and sex –dependent change in serum osteocalcin levels in children and adolescents with a pattern resembling height velocity curves for children as well as serum osteocalcin elevation coinciding with the pubertal growth spurt (Kanbur, 2002).

Serum osteocalcin is decreased in children with growth hormone deficiency but is normal in those treated with human growth hormone. Taken together, these data suggest that serum osteocalcin may be a relevant marker for skeletal growth and maturation (Delmas *et al.*, 1986).

Osteocalcin has also been investigated as a possible method of predicting periodontal disease activity in the gingival crevicular fluid microenvironment (Kunimatsu *et al.*, 1993; Eley and Cox, 1998; Mc Cauley and Nohutcu, 2002). This will be discussed in the section- *Tissue Distribution of Osteocalcin*.

Plasma osteocalcin concentrations have been found to decrease significantly in situations of acute physiological stress, such as following myocardial infarction, major abdominal/orthopaedic surgery and even marathon running. The exact cause of this decrease is not known but glucocorticoid concentrations, which are increased in situations of acute physiological stress, inhibit osteoblastic activity (Nicholson *et al.*, 2002). This highlights the importance of osteocalcin sample collection in the rested state.

2.2.6 Osteocalcin and Metabolic Bone Disease

During production, a certain amount of osteocalcin leaks into the circulation, where it can be measured by radio-immunoassay (RIA) or enzyme-linked immunosorbant-assay (ELISA).

Theoretically, serum osteocalcin could originate from bone matrix degradation, but it has been suggested that most osteocalcin originates from new cellular synthesis (Price *et al.*, 1981). This implies that the osteocalcin level may be a valid marker of bone turnover if resorption and formation are coupled, and act as specific marker of bone formation if these are uncoupled. This is illustrated in patients with multiple myeloma (uncoupling of formation and resorption), where an inverse relationship between osteocalcin levels and disease severity has been observed.

The lowest values are exhibited by patients with extensive lytic bony lesions, suggesting the presence of marked osteoblastic inhibition. During remission, osteocalcin levels return to normal values (Van Daele *et al.*, 1994).

In women with postmenopausal osteoporosis, serum osteocalcin is increased, reflecting the histological heterogeneity of osteoporotic patients where osteocalcin correlates with histological variables of bone formation (Brown *et al.*, 1984). An elevation of 10% has been reported in the mean osteocalcin concentration in postmenopausal osteoporosis, in contrast to the greater elevation reported in bone resorption markers, such as deoxypyridinoline, which is increased by 50% (Eastell *et al.*, 1988). Serum osteocalcin concentrations correlate with the rate of bone loss from the distal forearm during the course of osteoporosis (Christiansen *et al.*, 1997).

However, the overlap of results is too great for the diagnosis of osteoporosis in individuals and the scatter around the regression line is too great to identify individuals with accelerated bone loss.

It has also been shown that in untreated post-menopausal women serum osteocalcin is the best single biochemical marker reflecting the spontaneous rate of bone loss as assessed by repeated bone mass measurements over a 2-4 year period (Delmas, 1992).

In osteomalacia, serum osteocalcin correlates with parameters of osteoid formation and is significantly elevated. This is due to secondary hyperparathyroidism and to the defect in mineralization in osteomalacia, which prevents the incorporation of *de novo* osteocalcin into the bone and results in elevated serum concentration. (Hannon *et al.*, 1995).

Circulating osteocalcin is increased in hyperparathyroidism and decreased in hypoparathyroidism. Osteocalcin concentration correlates with parathyroid hormone concentration better than does serum alkaline phosphatase activity (marker of bone formation) and urinary hydroxyproline (marker of bone resorption) (Lee *et al.*, 2000).

Duda *et al.*, (1988) studied concurrent assays of serum osteocalcin and bone alkaline phosphatase, finding that these measurements gave concordant results in osteoporosis and primary hyperthyroidism, but discordant results in patients with glucocorticoid excess, chronic renal failure, osteolytic metastases and Paget's disease of bone. They suggested that these discrepancies occurred because these two biochemical markers reflected different aspects of osteoblast function.

In Paget's disease, serum osteocalcin concentrations are significantly raised, but the magnitude is smaller than for other markers of bone formation such as bone alkaline phosphatase. The reason for this discrepancy is not known, but it could relate to the greater affinity of osteocalcin for the woven bone of Paget's disease *i.e.* due to increased binding of the protein by the woven bone matrix. This in turn, would reduce the fraction of synthesized osteocalcin released into the circulation (Merle & Delmas; 1990).

2.2.7 Tissue Distribution of Osteocalcin

Bronckers *et al.*, (1987) examined the distribution of osteocalcin in tooth germs, calvaria and alveolar bone of the rat. Osteocalcin was observed in young odontoblasts and in early predentine at a stage prior to initial mineralization. Similar results were obtained for bone: young osteoblasts as well as osteoid were immunopositive for osteocalcin prior to formation of the first mineralized bone.

Histochemical staining revealed that differentiating osteoblasts and odontoblasts exhibited alkaline phosphatase activity at stages prior to the appearance of osteocalcin and that cells adjacent to odontoblasts and osteoblasts (not directly involved in the formation of predentine/ dentine and osteoid/bone) stained for alkaline phosphatase, but not for osteocalcin. They concluded that osteocalcin is a more specific marker for bone and dentine formation than alkaline phosphatase, since only the cells directly involved in these processes were immunostained for osteocalcin.

De Vries *et al.* (1988) investigated the immunocytochemical localization of osteocalcin in human and bovine teeth. They found that osteocalcin immunoreactivity was localized at the dentino-enamel junctions, whereas the major part of the dentine was unstained. This suggested that there is possibly some involvement of amaeloblasts in the deposition of osteocalcin.

They surmised that the low concentration of the protein suggested that it has no direct function in dentine formation. The almost complete absence of osteocalcin from bovine and human dentine is in contrast with its abundance in rat dentine.

More recently, however, Papagerakis *et al.* (2002) have also shown that osteocalcin is present in human odontoblasts, detected throughout the length of the odontoblast processes, and located within the enamel matrix, specifically at the dentinoenamel junction. This suggests that osteocalcin moves inside the canaliculi via odontoblasts cell processes becoming localized to specific extracellular locations during dentine and enamel formation.

An indication for the role of osteocalcin might be found in its presence at the dentino-enamel junction, which seems to be common to all species encountered (De Vries *et al.*, 1988).

It has also been found that osteocalcin is not found in acellular cementum of developing rat incisor and molar teeth. In contrast, cellular cementum in molar teeth was found to contain large amounts of osteocalcin. Consistent with these observations, the cells engaged in the formation of cellular cementum (cementoblasts and cementocytes) were also found to be associated with large amounts of osteocalcin. Hence, the dissolution of tooth structure as occurring during the process of physiologic tooth movement may result in the release of osteocalcin (De Vries *et al.*, 1988).

Using immunohistochemical methods, rat crown and root odontoblasts, as well as dentine were found to stain strongly for osteocalcin in advanced stages of dentinogenesis (Bronckers *et al.*, 1994).

Odontoblasts first synthesize and deposit an unmineralized collagenous matrix, the predentine, which resembles osteoid in many ways. At a discrete distance from the odontoblast cell body, this predentine transforms into a mineralizing dentine that (unlike bone) does not undergo remodelling. The composition of dentine matrix is quite similar but not identical to that of bone (Butler and Ritchie, 1995), and odontoblasts have been shown to synthesize and deposit osteocalcin into mineralizing dentine.

Bronckers *et al.* (1994) concluded that cementoblasts and cementocytes of cellular cementum produce osteocalcin and express an osteoblast-like, not an odontoblast-like phenotype. The cells responsible for the production of acellular cementum were not found to express osteocalcin. In contrast, cells and matrices of surrounding alveolar bone were found to strongly express the bone protein.

This finding was refuted by Kagayama *et al.* (1997) who investigated the expression of osteocalcin in Wistar rats. They found that all cells lining cellular cementum were positive for osteocalcin. They suggested that the osteocalcin expression of cementoblasts forming acellular cementum is similar to that of cells forming cellular cementum as well as osteoblasts and odontoblasts. Furthermore, they speculated that osteocalcin has a role in the calcification of acellular cementum.

Human periodontal ligament fibroblasts have been found to exhibit in vitro phenotypic characteristics consistent with those of osteoblast-like cells (i.e. immunofluorescence against osteocalcin), thus suggesting that such cells have the potential to differentiate into osteoblasts and/or cementoblasts (Basdra and Komposch, 1997). Support for this hypothesis was strengthened by the demonstration of the same findings in the murine model by MacNeil *et al.* (1998) and later within the human periodontal ligament (MacNeil *et al.*, 2001).

2.2.7.1 Osteocalcin in Gingival Crevicular Fluid

Osteocalcin has also been investigated in gingival crevicular fluid (GCF) as a potential marker of bone resorption. Kunimatsu *et al.*, (1993) investigated the potential relationship of GCF osteocalcin levels to periodontal disease severity by performing a cross-sectional study on 14 chronic periodontitis and five chronic gingivitis patients. No significant amounts of osteocalcin were found in gingivitis patients, whereas in periodontitis patients varying levels of osteocalcin were found and these significantly correlated with the clinical parameters. Patients with untreated periodontitis had osteocalcin levels 200 to 500 times higher than in serum. This suggested that GCF osteocalcin levels reflected the degree of inflammation at the sampled sites.

Another cross-sectional study investigated 17 patients and compared the GCF osteocalcin levels at healthy, gingivitis and periodontitis sites (Nakashima *et al.*, 1994).

They found that osteocalcin was present at both healthy and diseased sites and the mean concentrations in GCF were more than ten times greater than normal serum levels. They also found that total amounts of GCF osteocalcin significantly correlated with clinical parameters. In the aforementioned investigations GCF osteocalcin levels were correlated with gingival inflammation but correlations with actual bone turnover were not tested.

Griffiths *et al.* (1998) investigated GCF osteocalcin within the context of orthodontic movement of human canine teeth, however, there was found to be high variability in levels of the protein between subjects, as well as a lack of a consistent pattern associated with the initial fitting of the appliance, active orthodontic movement and retention periods. Problems with sampling, including contamination of GCF samples from multiple sites hindered interpretation of the data from this investigation.

Wilson *et al.* (2003) collected concomitant trabecular and cortical bone as well as GCF samples from 14 subjects with untreated periodontal disease to compare these with serum markers of bone metabolism including osteocalcin. They were unable to detect osteocalcin in GCF (RIA assay sensitivity reported as 0.05ng/mL) but were able to demonstrate that there was considerable local production of osteocalcin. The serum osteocalcin levels were higher than in cortical bone but significantly lower than in trabecular bone.

There have been no longitudinal studies in humans relating GCF osteocalcin levels to periodontal disease activity, however, there has been one animal study relating these levels to the progression of ligature-induced experimental periodontitis in beagle dogs (Giannobile *et al.*, 1995). The GCF osteocalcin levels increased significantly after two weeks following initiation of the disease. These levels peaked at eight and ten weeks following ligature placement at levels nearly tenfold greater than those at contralateral sites. Following removal of the ligatures the GCF osteocalcin levels dropped precipitously and approached control levels.

2.2.8 The Heterogeneity of Circulating Osteocalcin

The heterogeneity of circulating osteocalcin was first reported in 1985 (Gundberg *et al.*, 1985). When serum osteocalcin is hydrolysed in the kidney and liver the C-terminal fragment is easily cleaved and the N-terminal mid-fragment shows greater apparent stability. Residues 19-20 and 43-44 are susceptible to tryptic hydrolysis. It has been proposed that the arginyl-arginyl residues at positions 19-20 are protected from proteolysis by their incorporation in the Gla helix or that this protection is derived from the highly negatively charged g-carboxyglutamyl residues at positions 17, 21 and 24 (Rosenquist *et al.*, 1995).

Garnero *et al.* (1994) used mono- clonal antibodies to identify circulating fragments in healthy subjects and patients with metabolic bone disease.

The intact molecule and the N-terminal mid-fragment were the most abundant immunoreactive forms in normal and osteoporotic subjects. Additionally, they found that there was an increase in the N- terminal mid-fragment in patients with Paget's disease, and concentrations of both intact and fragmented osteocalcin were increased in patients with chronic renal failure. Hence, different metabolic bone diseases may have different characteristic patterns of immunoreactive forms of osteocalcin highlighting the possibility of measuring different forms in the clinical investigation of bone pathologies (Lee *et al.*, 2000).

The half-life of intact osteocalcin is short and it is mainly removed from circulation by glomerular filtration into urine. There is evidence that the production of some of the fragments found in urine occurs before renal clearance and is not a result of it. Due to the rapid clearance from the circulation, serum osteocalcin could reflect the acute changes in hard tissue metabolism, while some of the urine osteocalcin might serve as an index of long-term changes (Ivaska *et al.*, 2003).

2.2.9 Measurement of Osteocalcin

Urinary measurement of osteocalcin is inconvenient in the rodent model and requires expensive metabolic cages. Hence, serum-based measurement is a much more acceptable and widely used method.

Bovine osteocalcin is more abundant and more stable than human osteocalcin. As osteocalcin is highly conserved between species, many assays are directed against bovine osteocalcin. In 1980 Price *et al.* described the first assay for osteocalcin (Price *et al.*, 1980). This assay employed a rabbit antibody directed against calf osteocalcin to detect human osteocalcin in plasma, with a sensitivity of 0.1 ng.

By 1985 there were several methods for the determination of osteocalcin. Gundberg *et al.*, (1985) compared two commercial kits with an in-house assay and showed a good correlation between methods when the assay standard and normal ranges were used. This was the first report to draw attention to differences between methods and the first to suggest that one assay cannot be substituted for another. In 1986 Tanaka *et al.* reported the first enzyme immunoassay (ELISA) developed for osteocalcin.

This assay correlated well with the method of radioimmunoassay (RIA). There are now several commercial immunoassays available in RIA and enzyme-linked immunoassay (ELISA) formats for the detection of total (fully and under-carboxylated) osteocalcin.

Srivastava *et al.* (2000) compared rat osteocalcin concentrations measured with a radioimmunoassay (RIA) to a sandwich enzyme-linked immunoassay (ELISA).

They proposed a number of potential explanations for the observed differences in serum osteocalcin concentrations between the various assays that include:

1. differences in osteocalcin calibration preparation;
2. differences in the *in vitro* degradation of osteocalcin during sample handling and incubation with assay reagents; and
3. differences in the reactivity of the different antibody (employed in various assays) toward the smaller fragments of osteocalcin, which are generated by *in vivo* or *ex vivo* degradation of intact osteocalcin.

These investigators deemed that further studies are needed to evaluate whether the observed differences in serum osteocalcin among the various assays could be explained on the basis of types and quantities of various osteocalcin fragments present in rat serum.

2.2.9.1 Osteocalcin Sample Stability and Handling

Osteocalcin is markedly unstable *in vitro* (Garnero *et al.*, 1994; Swaminatham, 2001), rapidly degrading in samples at room temperature and at 4° C. Garnero *et al.*, (1994) reported an *in vitro* degradation of 17% of intact osteocalcin over two hours incubation at room temperature. This group also described a 4% *in vitro* degradation of intact and N-terminal mid-fragment after two hours incubation at room temperature. This instability may occur in samples, standards or tracers and it has been suggested that there may even be degradation of these during the assay procedure itself (Blumsohn *et al.*, 1995).

Protease inhibitors may reduce deterioration of osteocalcin in samples (Noonan *et al.*, 1996) but it is recommended that samples are kept at 4° C immediately upon venepuncture with serum or plasma separated as soon as possible, and samples frozen until assay. For short-term storage e.g. one month, freezing at -20° C will suffice, but for long-term storage it is advisable to store samples at -70° C.

Osteocalcin is sensitive to freeze-thaw cycles and haemolysis. Repeated freeze-thaw cycles have been shown to reduce immunoreactivity by up to 40% (Gundberg *et al.*, 1985). Plasma osteocalcin concentrations measured by enzyme immunoassay or radioimmunoassay are also influenced by the nature of the anticoagulants used.

Power *et al.* (1991) investigated the effects on plasma osteocalcin concentrations of different anticoagulants used to collect the blood samples. They found that osteocalcin concentrations were relatively stable in plasma and serum at -20° C for two freeze/thaw cycles. Haemolysis decreases apparent osteocalcin concentrations, possibly due to proteolysis by enzymes released from lysis of red blood cells (Tracy *et al.*, 1990).

Most commercial assays measure osteocalcin in either serum or plasma, but the anticoagulant used in plasma samples may affect the results obtained. In particular, potassium oxalate and sodium fluoride should be avoided (Power *et al.*, 1991). Furthermore, some commercial assays may also be sensitive to calcium ions. Unfortunately, most manufacturers do not make available information on the calcium content of their buffers. This has clear implications for samples collected using EDTA or other calcium chelators (Lee *et al.*, 2000).

The diversity of the osteocalcin molecule itself has an evident contribution to its immunoreactivity. Multiple proteolytic forms of osteocalcin have been found in circulation and urine. The osteocalcin fragments might be produced by osteoblasts or released from bone resorption or they might result from degradation in the circulation or peripheral organs, such as kidneys, liver and lungs (Farrugia and Melick, 1986).

Several proteases such as plasma, trypsin and cathepsins, have been reported to cleave osteocalcin into smaller fragments *in vitro* (Baumgrass *et al.*, 1997). In addition, the carboxylation of glutamic acid residues contributes to the heterogeneity of osteocalcin.

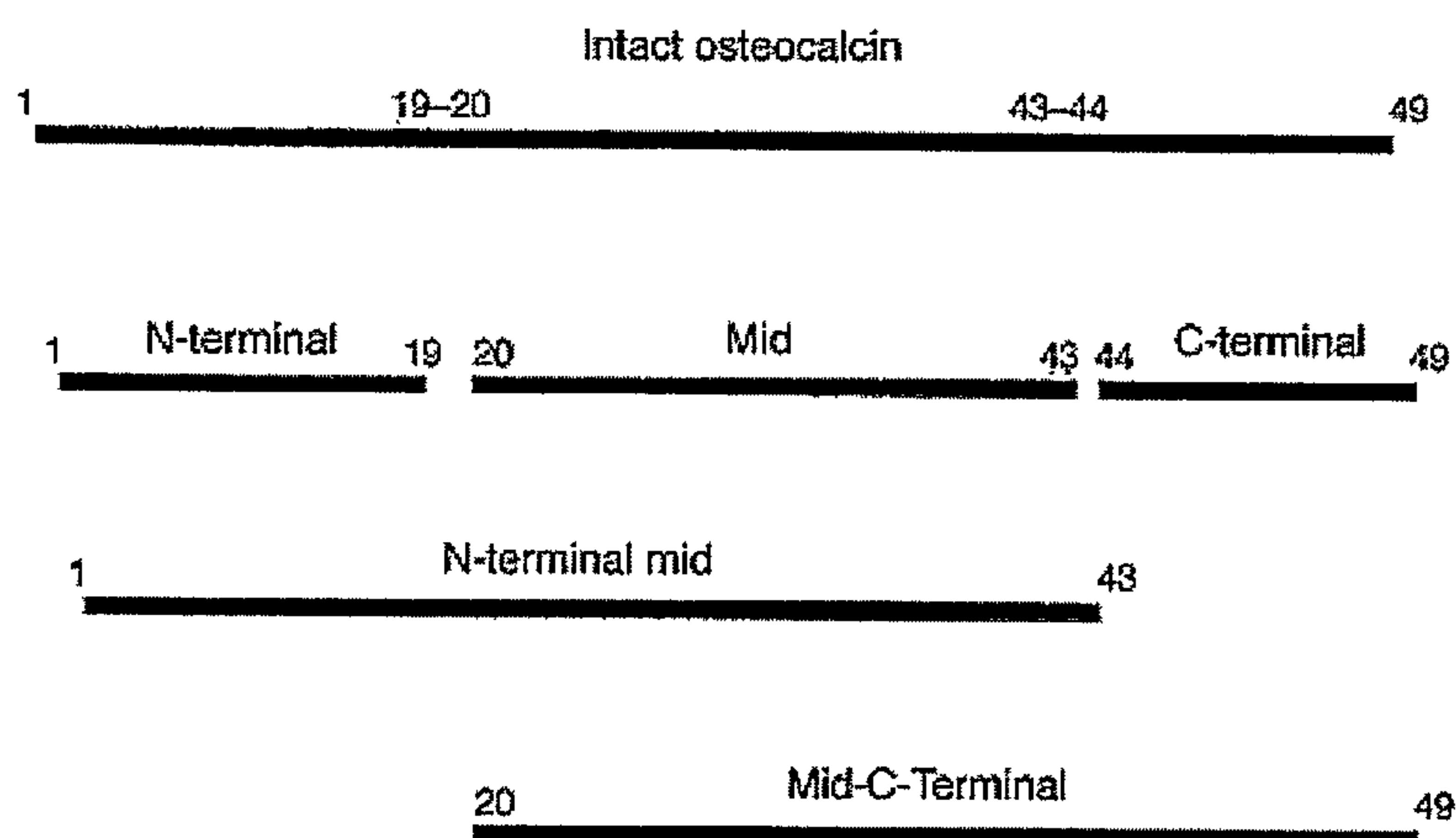


Figure C- The various fragments of osteocalcin (Source: Garnero *et al.*, 1994)

When Ca^{2+} binds to Gla residues an α -helical structure is known to form, while the conformation of non-carboxylated osteocalcin lies somewhere between the random coil and helical form.

Furthermore, impaired γ -carboxylation of glutamic acid residues has been described (Ivaska *et al.*, 2003). Thus, a single conformation cannot be defined for the osteocalcin peptide in solution.

2.2.9.2 Biological Variability of Osteocalcin

Human serum osteocalcin has been shown to have a large diurnal variation (peaking in the early morning at approximately 4a.m. with lowest levels occurring at night) (Markowitz *et al.*, 1987; Tracy *et al.*, 1990). Samples for osteocalcin determination should be collected at the same time of day if comparisons between serial samples are required.

According to a Danish study there is also a seasonal variation of 23%, with concentrations increasing to a peak in winter from a nadir in summer (Thomsen *et al.*, 1989; Nielson *et al.*, 1990).

During the menstrual cycle it reaches its highest levels during the luteal phase (Nielson *et al.*, 1990). Serum osteocalcin concentrations are higher in children than in adults, rising during puberty and then falling to adult levels (Gundberg *et al.*, 1983). Most studies show a higher concentration of osteocalcin in adult males than in adult females and show an age-related increase after the fifth decade of life in women. The postmenopausal rise in serum osteocalcin can be reversed by hormone replacement therapy (Hannon *et al.*, 1998).

2.2.9.3 Standardization for Commercial Osteocalcin Assays

Various assays exist for assessment of osteocalcin and concentrations in the same serum or plasma sample may vary enormously (Fassbender *et al.*, 2002).

Several studies have compared commercial assays; all have found a marked difference between methods irrespective of whether they were commercial kits or in-house assays.

In 1985, Gundberg *et al.*, demonstrated that three different assays gave widely differing results for identical samples; nevertheless, the results were consistent when the reference ranges for the different assays were taken into account.

In 1990, Delmas and co-workers highlighted the poor comparability of osteocalcin assays in a series of blinded serum samples and a reference standard analyzed by eight in-house assays and two well-characterized commercial assays. Different assays were found to have different absolute concentrations of osteocalcin, sometimes differing by up to 50%. However, all of the assays gave acceptable results when compared by assay-to-assay correlations and when the results were expressed as a percentage of the mean of two controls.

A similar analysis of commercially available assays, which used Z scores ($Z \text{ score} = \frac{\text{sample osteocalcin} - \text{assay mean osteocalcin}}{\text{SD}}$) to compare the different kits, found that this procedure reduced the differences between results (Masters *et al.*, 1994).

The divergent results from different assays arise from several factors, including cross-reactivity of the antibodies to other molecules, especially at lower concentrations of the analyte, differences in immunorecognition of fragments and differences in standards.

(Blumsohn *et al.*, 1995). Enzyme-linked immunosorbent assays utilizing monoclonal or polyclonal antibodies have been described and both provide clinically useful results (Morris *et al.*, 1993). Some of the variation in absolute serum osteocalcin levels arises from variable cross-reactivity with osteocalcin fragments, but monoclonal assays do not necessarily provide increased specificity compared with polyclonal antibody-based assays (Tracy *et al.*, 1990).

The detection of intact osteocalcin or fragments is an important issue in the choice of a commercial assay. Assays detecting only intact osteocalcin will be particularly sensitive to *in vitro* degradation, whilst assays detecting fragments may, depending on the fragments recognized, overestimate the concentration of intact osteocalcin. Another difficulty arises from recent studies which suggest that the specificity of commercial assays may differ from that claimed by the manufacturers (Colford *et al.*, 1997).

The clinical interpretation of osteocalcin measurements relies, to a certain extent, upon methodological differences, but there is no consensus regarding the most clinically informative fragment(s) of osteocalcin to measure. This may depend on the sample group for which the assay is requested.

Hence, it can be seen that the application of osteocalcin assays to routine clinical practice has been hampered by poor comparability between different methods and the instability and heterogeneity of circulating osteocalcin. Osteocalcin assays are available in many formats using both polyclonal and monoclonal antibodies and different standards. Given this, it is not surprising that osteocalcin values determined by different assays give discordant results.

Given the inherent problems of sample stability and biological variability, there is an absolute need for continuity in the methodology for sample collection. This includes the avoidance of haemolysis and freeze-thaw cycles. The poor comparability of methods and problems with sample handling are compounded by a lack of consensus regarding the most clinically informative fragment(s) of osteocalcin.

2.3. Fluoride

2.3.1 Fluoride Distribution

Fluoride is the thirteenth most abundant element in the earth's crust and a natural component of the biosphere. Fluoride is the ionic form of fluorine, a halogen and the most electronegative of the elements of the periodic table (Whitford,1996). In air, the dominant species is hydrogen fluoride, which is rapidly absorbed from the respiratory tract. Fluoride is a bone-seeking anion.

Up to 99% of the body burden of fluoride is found in bone and other calcified tissues such as enamel and dentine (Whitford, 1990; Rao *et al.*, 1994). It has been found that fluoride distribution in the skeleton is not homogenous, increases with age and is sex-dependent. Fluoride concentration in trabecular bone is significantly higher than in cortical bone, both in humans and in rats (Eble *et al.*, 1992).

Most fluoride in bone is not readily exchangeable, but can be mobilised through the process of bone modelling and remodelling. Thus, net flow of fluoride from or into the bone can be positive or negative, depending on the level of fluoride intake, hormonal status and other factors (Whitford, 1994; Li *et al.*, 1995).

2.3.1.2 Fluoride Distribution in the Dental Hard Tissues

Kato *et al.*, (1997) found that fluoride concentration was higher in the cementum and dentine (in an age-dependent, cumulative way) when comparing the tooth roots of anterior human teeth from fluoridated and non-fluoridated areas in the United Kingdom.

Ishiguro *et al.* (1994) investigated the distribution of fluoride in the dental hard tissues and the supporting mandibular bone within the same individuals. They determined that the average fluoride concentration is greater in cementum than in alveolar bone and then decreases in the following order: cancellous bone (mandible), dentine and enamel.

Nakagaki *et al.* (1988) found that the distribution of fluoride in human cementum reflected that found in mineralized tissues, with fluoride concentrations tending to be highest toward the external surface. It was also found that fluoride concentrations were high in acellular and low in cellular cementum.

Cementum has been reported to have the highest fluoride content of all the calcified tissues, up to five times as much as enamel, and three times as much as alveolar bone. Concentrations up to 0.9% (9000ppm) of the ash weight have been reported, while some authors have found considerably lower values (Yardeni *et al.*, 1963; Gedalia *et al.*, 1965; Zipkin, 1970).

There could be several reasons why cementum has higher fluoride content than all other calcified tissues. The crystallinity of the mineral component in cementum is lower than in other hard tissues, which means that cementum is more permeable and has a greater capacity for adsorption of fluoride over time. While bones have a remodelling capability, cementum seems to have little resorption capacity (aside from that induced through orthodontic tooth movement). Cementum, therefore, increases in thickness with age and thus continues to absorb fluoride. It is also possible that fluoride from alveolar bone could be taken up by the cementum surface (Nakagaki *et al.*, 1985).

Ito *et al.* (1997) investigated the fluoride profiles in rat cementum for a period of up to 25 weeks and found that the average fluoride concentrations in cementum increased significantly with increasing dose and duration of fluoride administration. There was also found to be a relative reduction in the average fluoride concentrations after fluoride administration was ceased. Contributing factors to the latter observation were speculated to involve new cementum formation and subsequent fluoride release from cementum surfaces following cessation of the fluoride administration. They concluded that withdrawal of fluoride administration reduced the fluoride concentration on the outermost cementum layers as opposed to bone, where reduction occurred across the entire thickness.

Cementum is also exposed to the circulating tissue fluids in the periodontal ligament from which fluoride can be absorbed (Sugihara *et al.*, 1991). Human tooth samples, however, are frequently taken from individuals with periodontal disease and hence subsequent exposure to oral fluids may obfuscate cementum fluoride analyses.

Kondo *et al.*(1995) measured the molar cementum fluoride concentration in rats administered water containing 100ppm fluoride. They found a marked increase with age, both in outer and inner regions. It was concluded that the fluoride distribution in rat molar cementum may increase throughout life with respect to the consumption of drinking fluorided water.

Kato *et al.* (1990) investigated the fluoride distribution in the cementum and neighbouring hard tissues of rats receiving drinking water containing different concentrations of fluoride. Fluoride concentrations in rat cementum were found to be higher than those of dentine and alveolar bone and these values increased throughout the tissues with increasing fluoride intake. Similar distributions of fluoride were also observed in the cementum of contralateral teeth from the same animal.

2.3.2 Sources of Fluoride

Fluorides may be found occurring naturally in drinking water or added at concentrations of 0.7 to 1.2 mg/litre in municipal water. Other sources of fluoride exposure in humans include the use of fluoride-containing dental products, tea and fish. Fluoride is an important element for mineralization of body tissues. It can be applied systemically or locally for prevention and treatment of dental caries (W.H.O. 1984), to promote healing of bone disorders or in the treatment of osteoporosis, although its therapeutic effect in the latter condition is controversial (Ringe and Rovati, 2001).

Primary osteoporosis, in both males and females, results from an imbalance between new bone formation and resorption of trabecular bone, leading to bone loss and eventual mechanical failure (Kassem *et al.*, 1994). This disease process has been the catalyst for much of the fluoride-related bone research that has been carried out thus far.

The cause for the relative unresponsiveness of some osteoporotic patients to NaF is unknown, partly because of the precise mechanism of action of fluoride at the cellular level remains elusive. Fluoride appears to have distinct effects on skeletal metabolism. There is an increase in osteoid surface and thickness and an increase in matrix apposition rate (Lundy *et al.*, 1986).

2.3.3 Distribution and Metabolism of Fluoride

Absorption of fluoride entering the gastrointestinal tract is affected by a number of factors such as the chemical and physical nature of the ingested fluoride as well as other concomitantly ingested foods. Solutions of fluoride salts such as sodium fluoride (NaF) are rapidly and almost completely absorbed from the gastrointestinal tract, probably by simple diffusion (Carlson *et al.*, 1960). After ingestion of sodium fluoride, human plasma fluoride levels have a half-life of about three hours (Ekstrand *et al.*, 1977). Approximately 99% of the fluoride in the body is localized in the skeleton. The rest is distributed between the blood and soft tissues (W.H.O., 1984).

Whitford (1996) showed that absorption of ingested fluoride in rats was in the order of three times higher than that of the group given fluoride by injection, which was actually negative in value. This observation indicated that there was net fluoride secretion into the gastrointestinal tract following daily intraperitoneal injection and that systemic absorption was enhanced via oral administration.

It is known that the pharmacokinetics and toxicity of fluoride depend on the stage of growth and development. When weaning occurs, the clearance rate of fluoride from plasma is about ten times higher in rats and six times higher compared with animals that have reached the stage of maturity.

The explanation for this occurrence results from the enhanced ability of the developing skeleton, with its numerous, relatively small and loosely organized crystallites, to remove fluoride from the extracellular fluids (Kato *et al.*, 1990).

2.3.4 Fluoride - Mechanisms of Action

2.3.4.1 Effect on Bone Formation.

The predominant effect of fluoride on bone is to stimulate bone formation. Both direct and indirect cellular mechanisms have been proposed as explanations for the observed increase in bone formation during chronic fluoride administration. Bone cells are responsive to changes in electrical currents and hence bone crystals may act as transducers for mechanical stress and may generate piezoelectric currents in bone that may affect osteoblastic activity.

The increased rigidity of crystalline fluorohydroxyapatite may enhance these piezoelectric currents. This could explain the more pronounced effect of fluoride on the weight-bearing axial skeleton (vertebral column) than on nonweight-bearing bone such as the radius (Riggs *et al.*, 1989). It has also been demonstrated that fluoride is generally more readily incorporated into cancellous bone than cortical bone (Zerwekh *et al.*, 1992). Compared to other anabolic factors, fluoride remains the most potent agent inducing uncoupling between bone resorption and formation in favour of formation (stimulation of the osteoblast proliferation and bone matrix synthesis), thus resulting in an increased bone volume (Caverzasio *et al.*, 1998).

2.3.4.2 Effects of Fluoride on Osteoblastic Cells

In 1983, Farley *et al.* first made the observation that fluoride can directly influence the activity of osteoblastic cells in culture (Farley *et al.*, 1983). This research showed that micromolar concentrations of fluoride increased the proliferation and alkaline phosphatase activity of bone cells derived from chick embryonic calvaria as well as enhanced collagen synthesis.

These initial results were supported by the work of Marie *et al.* in 1992, which was able to demonstrate a positive histologic response of osteoblastic cells isolated from osteoporotic subjects following 12 to 24 months of NaF therapy. These investigators concluded that the nett increase in bone formation in the responders resulted from the enhancement of osteoblastic proliferation rather than increased osteoblastic differentiation.

Conflicting results for human cells were obtained by Kopp and Robey (1990) as well as Baylink *et al.* (1992). From these negative results and because osteoblasts derived from bone of either fluoride-treated osteoporotic patients or fluoride-treated experimental animals exhibited a higher capacity to proliferate *in vitro*, it has been concluded that, *in vivo*, fluoride may act indirectly through the local synthesis of some growth factors or may exert a preferential action on a subpopulation of osteoblastic cells (Bellows, 1991).

The latter hypothesis is supported by the observance that osteoblast precursors are more sensitive to fluoride action than the mature osteoblast. This was demonstrated by Kassem *et al.* in 1994, who suggested that the *in vivo* effects of NaF on bone formation may be mediated by stimulating proliferation and differentiation of committed osteoblast precursors in bone marrow. These investigators described a stimulatory effect on osteocalcin production in human marrow-derived stromal cells. Hence, variability in fluoride response may be explained on the basis that different stages of osteogenic cell differentiation have particular degrees of variability in responsiveness to fluoride.

However, although the osteoblasts in fluoride-affected bone are active, there are fewer plump, cuboidal, highly secretory osteoblasts. This suggests that, whereas fluoride is mitogenic to osteoblast precursors, it is toxic to individual osteoblasts at the same concentrations (Bonjour *et al.*, 1993).

Chavassieux *et al.* (1991) investigated the dose effects of short-term NaF administration on ewe bone remodelling and concluded that fluoride had a stimulatory effect on osteoblasts. Fluoride was also found to prolong the lifespan of osteoblasts that had reduced activity.

In a different experiment involving the administration of sodium fluoride (3.5mg/kg) to lambs for 120 days with subsequent histomorphometric analysis, Chavassieux *et al.* (1991) confirmed that fluoride induced an increase in the birthrate of osteoblasts and bone formation compared with controls.

This increase in bone formation at the tissue level (accompanied by serum osteocalcin levels twice as high in treated animals compared with controls) was associated with a toxic effect at the individual cell level.

Following long-term exposure to fluoride in human subjects, osteoblasts assume a flat, inactive appearance, and the amount of osteoid surface covered by osteoblasts decreases, indicative of reduced osteoblastic activity (Briancon and Meunier, 1981). At physiological serum levels, however, fluoride was not found to increase expression of mRNA (i.e. cause greater protein expression) by osteoblasts, although it did cause an increase in the amount of unmineralized osteoid (Li and Den Besten, 1993). The relative increase in bone osteoid may, therefore, be related to other mechanisms such as altered matrix mineralization.

Fluoride has been demonstrated to inhibit phosphotyrosyl acid phosphatase in osteoblasts (Lau *et al.*, 1989). The consequent enhancement of tyrosyl phosphorylation of cellular signalling proteins may perhaps account for osteoblastic proliferation or synthesis of essential growth factors. In addition, fluoride elicits a rapid increase in cytosolic ionic calcium in human osteoblasts, thereby increasing the concentration of inositol polyphosphates (Zerwekh *et al.* 1990). The rise in intracellular calcium and activation of the phosphatidyl-inositol pathway may thence lead to osteoblastic proliferation by stimulating protein phosphorylation or synthesis of essential growth factors.

A further calcium-related postulate as to the mechanism of fluoride-induced up-regulation of osteoblastic activity is provided by Khokher & Dandona (1990). They postulated that fluoride's metabolic effects might be dependent on extracellular calcium, and moreover, be associated with a change in the transmembrane potential of the osteoblast via cation-conducting channels in the cell's membrane.

This mechanism would be consistent with that of parathyroid hormone (PTH), which is a potent stimulator of bone resorption. PTH affects cell morphology, collagen synthesis and calcium metabolism in osteoblasts (Yamaguchi *et al.*, 1987), and alters the membrane potential of these cells (Fritsch *et al.*, 1988). Indeed, a number of osteotropic agents such as cyclic adenosine monophosphate (cAMP), prostaglandin E₂ (PGE₂), hydrocortisone, and calcitonin have all been shown to similarly affect membrane potential in osteoblastic cells (Ferrier *et al.*, 1985).

Using electrophysiological experiments on human osteoblasts, Gofa and Davidson (1996) tested this hypothesis. They demonstrated that NaF potentiates a K⁺-selective ion channel in osteoblastic cells. This effect was absent in calcium-free medium and could be blocked by a combination of organic and inorganic calcium channel blocking agents. Although the underlying cellular mechanisms remain unclear, their findings suggested that activity of calcium and/or potassium selective channels via second messenger cascades may mediate many of the early events involved in the fluoride-induced activation of osteoblastic cells.

The most recent postulate regarding the effect of fluoride on osteoblasts relates to the role of Type 5 Tartrate-resistant acid phosphatase (TRAP), which is a different isoenzyme from the eponymous biochemical marker of osteoclasts. Two properties of osteoblastic type 5 TRAP may be relevant to its physiological functions:

1. It acts as a protein-tyrosine phosphatase under physiologic conditions, and
2. It is sensitive to inhibition by clinically relevant concentrations of fluoride.

Due to the fact that fluoride stimulates osteoblastic proliferation/differentiation and because protein tyrosine phosphorylation plays an important regulatory role in cell proliferation/differentiation, these unique properties have led Lau and Baylink (2003) to propose that the osteogenic action of fluoride is mediated, at least in part, by the fluoride-mediated inhibition of osteoblastic type 5 TRAP/ protein tyrosine phosphorylation. This leads to a stimulation of osteoblast proliferation and differentiation and subsequently, an increase in bone formation.

2.3.4.3 The Effect of Fluoride on Osteoclasts

The effect of fluoride on osteoclasts is not as well understood as its effect on osteoblasts, and is complicated by a possible effect of fluoride-induced hyperparathyroidism (Bonjour *et al.*, 1993). There is some evidence that sodium fluoride decreases the number of resorption lacunae as well as the amount of bone resorbed per osteoclast (Okuda *et al.*, 1990).

No effect of fluoride on osteoclasts is seen at concentrations of less than 500 μ M and a decreased number of osteoclasts is seen at higher concentrations, where cytotoxicity is also seen in osteoblast experiments (Bellows *et al.*, 1991). Previous to the work of Oguro *et al.* in 2003, histomorphometric studies in general did not support a major effect of NaF on parameters of bone resorption. The stimulatory effects of NaF on bone formation had previously been explained solely by its activation of osteoblasts. This study involved the use of NaF with primary cultures of progenitor cells derived from murine bone marrow. It was subsequently found that NaF induces early differentiation of bone marrow haemopoietic progenitor cells along the granulocytic pathway but not the monocytic pathway that is linked to osteoclast formation.

Therefore, in addition to its potent stimulatory effects on osteoblastic bone formation, NaF could be expected to indirectly reduce osteoclastic bone resorption. This property, to promote osteoblast recruitment without prior stimulation of osteoclastic activity, is seen only with fluoride and aluminium (Kleerekoper, 1996).

Indeed, observations from the laboratory of Caverzasio *et al.* (1997) suggest that the molecule responsible for the effect of fluoride on the replication of bone-forming cells is a combination of these two elements i.e. a fluoroalumino complex (AlF_x). It is thought that the fluoro-alumino complex enhances protein tyrosine phosphorylation in osteoblast-like cells enhancing tyrosine kinase activity.

Burgener *et al.* (1995) showed that AlF_x might specifically activate a G protein dependent tyrosine phosphorylation of several cellular signalling proteins in osteoblasts.

This gives further support to the hypothesis that this signal transduction mechanism is involved in the osteogenic effects of fluoride. The type of tyrosine kinase(s) involved in the mitogenic response induced by AlF_x in osteoblast-like cells has not yet been elucidated.

There is also some *in vitro* evidence that NaF decreases the number of resorption lacunae as well as the amount of bone resorbed per osteoclast (Okuda *et al.* 1990). Changes in apatite crystallinity could explain the inhibition of bone resorption seen after fluoride treatment.

Increased resistance to dissolution by osteoclastic enzymes as well as high levels of fluoride liberated from fluoride-containing bone during the resorptive process, however, might inhibit osteoclastic activity and explain this effect of fluoride (Melsen *et al.* 1996).

2.3.4.4 The Effect of Fluoride on Extracellular Bone Matrix

In addition to its cellular components, a functional extracellular matrix is an important requisite for the formation and mineralization of hard tissues. The major components of the extracellular matrix which may be affected by fluoride include Type I collagen and non-collagen proteins such as bone sialoproteins and phosphoproteins, osteonectin, osteocalcin, and proteoglycans (PGs) (Klaushofer and Peterlik, 1996). The effect of NaF on bone matrix formation has previously been investigated (Kassem *et al.*, 1994).

No significant effects of NaF were observed on the synthesis of Type I collagen; however, in the presence of 1,25- dihydroxyvitamin D₃, NaF was found to enhance the formation of Type I collagen by osteoblasts in culture.

Fluoride has previously been shown to have effects on the PGs of mineralized tissues (Waddington and Langley, 1998). The exact role that PGs play in bone has not been elucidated. It has been proposed that PGs may be involved in mineralization and bone remodelling. This proposal is assessed by studies demonstrating that PG aggregates inhibited the mineralization process and that they must be removed to allow the occurrence of mineralization (Dean *et al.*, 1994). Thus, it has been described that, under conditions favourable for mineralization *in vitro*, matrix vesicles produced by osteoblasts become enriched in metalloproteinases capable of digesting PGs, which are potential inhibitors of matrix calcification.

Rodriguez and Rosselot (2001) evaluated the effect of NaF on the synthesis and/or structural characteristics of PGs produced by avian osteoblasts. They postulated that the stimulatory effect of fluoride on bone mineralization may be mediated in part by changes in the rate of synthesis or of the structural characteristics of bone PGs. They found that the effects observed in the rate of synthesis of PGs and the proliferation rate of avian osteoblasts, were evidenced at a narrow and low range of NaF concentrations. This is in concordance with observations that elevated concentrations of NaF cause negative effects in mineralized tissues such as bones and teeth (Meunier, 1992).

Turner *et al.* (2001) postulated an age-dependent response to fluoride in the rodent model, with fluoride intake tending to have the greatest effect on the amount of unmineralized osteoid present (suggesting an effect on mineralization) in younger animals. The effects of fluoride on mineralization were deemed significant only in older rats.

Hence, in addition to its effects on bone cells involved in bone remodelling, fluoride may induce a modification in bone matrix components which may partially explain the mineralization aberrations observed in the aforementioned studies.

2.3.4.5 The Effect of Fluoride as a Mitogen and its Effect on Growth Factors

As previously discussed, sodium fluoride has been shown to be mitogenic for osteoblastic and pre-osteoblastic cells. A unique property of the *in vitro* mitogenic action of fluoride is that the mitogenic dose is very low (micromolar) and at least two orders of magnitude lower than the doses of fluoride required for the effects on other biological systems (millimolar levels) (Lau and Baylink, 1998). A concentration of 10^{-5} M NaF has been shown to initiate osteogenesis from mandibular mesenchyme in the presence of serum (Hall, 1987). A more extensive range of concentrations of NaF has since been utilized and the effective concentration is now shown to be precisely placed between 10^{-5} and 10^{-6} M. Lower or higher concentrations fail to initiate osteogenesis (Hall, 1994).

A minimum of six days exposure to 10^{-5} M NaF is required for osteogenesis to occur and no increase in osteogenesis is seen with exposure beyond six days. The implication is of a threshold for exposure to NaF, and with NaF acting as a switch, directing mesenchymal cells into an osteogenic pathway. Additionally, the mitogenic action of fluoride is highly sensitive to changes in phosphate concentration (Farley *et al.*, 1988).

Cytosolic ionic calcium in osteoblasts increases linearly from 25ng of fluoride per millilitre to 200ng/mL (approximating the toxic cellular threshold), reaching a peak at 500ng of fluoride per millilitre (Zerwekh *et al.* 1990).

When fluoride concentrations are raised within the range of 500-1000ng/mL, the intracellular calcium concentration declines to a level below that observed at 100ng of fluoride per millilitre. Nodule formation in cultured foetal rat calvarial cells (a manifestation of osteoblastic proliferation and matrix synthesis) is stimulated at modest fluoride concentrations, but suppressed at high (toxic and non-physiologic) concentrations (Bellows *et al.* 1991).

Osteogenesis is *not* initiated when mesenchyme is cultured in the presence of NaF in *serum-free* medium, implying that NaF initiates osteogenesis in concert with one or more other factors present in serum. One possibility, as NaF is a mitogen, is that NaF provides a mitogenic signal and that a second factor(s) is (are) required to provide the differentiation signal(s).

Amman and co-workers (1998) were able to give some credence to this hypothesis with their findings that the administration of fluoride could enhance insulin-like growth factor-I (IGF-I) osteogenic effects of bone mass *in vivo*. Hall (1994) was able to demonstrate that NaF and Epidermal Growth Factor (EGF) can act synergistically and after minimal exposure to elicit osteogenesis from mandibular mesenchyme, proving that they can do this without mandibular epithelium and without serum factors.

At present, Bone Morphogenic Protein (BMP) is being investigated with respect to its ability to provide the proliferative and the differentiation signals provided by NaF and growth factors *in vivo*.

In addition to having its mitogenic action accentuated in the presence of IGF-I, fluoride modulates the activity of calcitonin and parathyroid hormone (PTH) on osteoblasts (Farley *et al.*, 1988). Fluoride-induced stimulation of osteoblastic proliferation has also been found to be abolished by an antibody to transforming growth factor- β (TGF- β) (Reed *et al.*, 1993).

A detailed discussion of the hypothesized molecular mechanisms of fluoride on bone cells is beyond the scope of this thesis, suffice to say that the proposed cellular mechanisms of fluoride action are myriad and have yet to be fully understood (Lau and Baylink, 1998).

Although each of these models merits further investigation, none is able to account for all the aforementioned observations, including the low (micromolar) effective dose of fluoride, the cell and tissue specificity of fluoride's mitogenic action, and/or the absolute requirements of an appropriate growth factor.

2.3.4.6 The Physicochemical Action of Fluoride

As well as influencing bone growth on a biological level by acting as a mitogenic agent, fluoride exerts an effect on a physicochemical level. At all doses, fluoride has a physicochemical effect on bone: as it is incorporated in the bone mineral, it substitutes for hydroxyl groups in the carbonate-apatite structure to produce hydroxyfluoroapatite.

Indeed, nearly all dietary fluoride is built into the crystals, where it substitutes for the hydroxyl lattice sites (Eanes and Reddi, 1979).

The crystal lattice of fluoroapatite is quite different from that of hydroxyapatite and the changes render the crystal more stable. Fluoride ions, unlike hydroxyl ions, reside in the plane of the calcium ions, resulting in a structure that is electrostatically more stable and structurally more compact. This enhanced stability makes the skeletal structures more resistant to osteoclastic resorption, further altering the normal remodelling cycle. A mixture of fluoroapatite and hydroxyapatite has been shown to be less soluble than either component individually. (Chachra, 1999).

Examination of bone mineral from both fluoride-treated human subjects and experimental animals has revealed the presence of fluoroapatite, with a larger crystal size and lower solubility compared with hydroxyapatite-rich tissue of untreated animals or subjects (Posner *et al.*, 1963). There is no evidence that the formation of fluoroapatite *per se* alters bone mineralization and, therefore, the impairment in bone mineralization produced by fluoride is most likely due to the aforementioned cellular action of fluoride, rather than to its physicochemical effects (Pak *et al.*, 1995).

2.3.5 Effect of Fluoride on Bone Structure and Functional Qualities

2.3.5.1 Human Bone

Previous studies have indicated that *in vivo* fluoride exposure can increase bone mass without increasing whole bone failure load, leading to the conclusion that the material strength of fluoridated bone is less than normal (Turner, 1995; Sogaard *et al.*, 1995).

The structural integrity of bone is a function of fluoride dose, with bone shown to be microradiographically abnormal at a NaF dosage of 60mg/day, but normal at a dosage of 45mg/day (Jowsey *et al.*, 1972).

Histomorphometric studies in growing rats demonstrated that fluoride increased cortical and trabecular bone mass at therapeutic doses, but reduced trabecular bone mass at toxic doses (Turner *et al.*, 1989). In addition, these researchers showed that incorporation of fluoride into rat bone mineral has a dose-specific effect on bone strength so that bone-strength followed an inversely proportional relationship with bone fluoride content: positive effects for lower fluoride intakes and negative effects on bone strength for high intakes.

The increase in bone volume at low fluoride doses resulted from stimulated periosteal bone formation and apposition rates.

In a study of human osteoporotic subjects, NaF therapy increased cancellous bone by 19% and trabecular thickness over a period of 41 months, however, new bone was added only to existing trabecular surfaces with no evidence, quantitative or qualitative, for the outgrowth of new trabeculae into the marrow space (Balena *et al.*, 1998).

These investigators concluded that new bone formation induced by NaF is exclusively appositional. NaF could thicken, and possibly strengthen, horizontal trabeculae, but cannot replace them once they have been lost. This would explain the inconsistent effect of fluoride in reducing vertebral fracture frequency, despite its consistent effect in increasing vertebral bone mass (Häuselmann and Rizzoli 2003).

Two randomized, placebo-controlled, double-masked clinical trials (Riggs *et al.*, 1990; Kleerekoper *et al.*, 1991) demonstrated that NaF given continuously at a daily dose of 75mg for four years did not reduce the vertebral fracture rate in patients with spinal osteoporosis, despite increasing spinal bone density by 35%. Several explanations have been proposed for these seemingly paradoxical results.

The most popular explanation is that (at least in the dose used in these trials) the new bone formed under the influence of NaF is biomechanically unsound. Other postulates include altered collagen fibre orientation, defective mineralization or some other (as yet unknown) subtle abnormality (Balena *et al.*, 1998).

2.3.5.2 Effect of Fluoride on Bone Structure and Functional Qualities in the Animal Model

Fluoride also exerts a biphasic effect on the mechanical property of bone. In rats maintained on a normal diet, administration of 200mg or more of fluoride per litre of drinking water caused a significant reduction in the mechanical strength of bone (Wolinsky *et al.*, 1972). The exposure of up to 75mg of fluoride per litre of drinking water in rats did not affect the mechanical properties of the tibia (Einhorn *et al.*, 1992).

Chachra *et al.*, (1999) found that fluoride administration at 100ppm in the drinking water of young rabbits between the ages of 3 ½ and 6 months caused profound changes to bone mineral and mineralization. In addition to altering the mineral directly (increasing the width of crystals), bone was found to be hypermineralized, as evidenced by a shift towards denser bone in the mineralization profiles and increased hardness. The structure of the bone was minimally affected, however, the mechanical properties of the bone were severely affected.

Thus, high fluoride exposure (100mg of fluoride per litre) reduces mechanical strength of bone, whereas lower fluoride exposure (< 75mg of fluoride per litre) improves or does not affect mechanical strength. This concurs with the work of Lees and Hanson (1992) who demonstrated an improvement of rabbit femur bone quality at low fluoride dosages.

According to the published literature, the response of the rat to fluoride may be slightly different to that of other animal models; fluorosis in the rat may be characterized by reduced bone resorption with secondary Haversian remodelling (Ream, 1981).

Cheng and Bader (1990) concluded that fluoride affects rat cortical and cancellous bone differently, however, even with reduced bone formation, NaF increases rat cancellous bone volume through trabecular thickening in a dose-dependent manner, probably due to a more severely reduced bone resorption. In addition, NaF increases the bone mineral packing density and perhaps the size of individual bone mineral crystallite. Both modes of action are capable of producing a more brittle bone. This mirrors the findings in human subjects (Compston, 2002).

The lack of intracortical remodelling in the rat may explain any difference in bone mass when compared with humans (Giavaresi *et al.*, 1999). Fluoride incorporation into the skeleton is largely dependent upon remodelling (formative) surfaces and, therefore, very little response should be expected in cortical bone in rats.

Furthermore, because fluoride therapy has been shown to increase peripheral bone fragility in humans (Schnitzler *et al.*, 1990), it may be that the action of fluoride must be expected to be slightly different in the rat due to the fact that this animal experiences very little Haversian remodelling. An increase in cancellous mass will not necessarily increase whole bone strength if the cortical bone compartment is diminished, and especially not if the new bone formed is of poor quality.

At high dosages or exposure, there may be a mineralization defect, which may lead to the formation of non-lamellar, mosaic bone. The strength of bone may not change or increase at low fluoride dosages, but it may deteriorate following high dosages.

The effect of the incorporation of ingested fluoride into bone apatite in young rats exposed to 50mg/mL sodium fluoride was found to have no effect on trabecular volume but increased the amount of unmineralized osteoid (Turner *et al.*, 2001). This impairment of mineralization by fluoride appeared to be the primary cause of diminished vertebral bone strength. This was not evident at lower fluoride doses.

Giavaresi *et al.*, (1999) showed that fluoride-containing bone presented an important decrease in mechanical properties characterized by a shorter deformation to load and a greater structural rigidity with increased stiffness. The mechanical properties of fluoride-treated bone can be different depending on the type of bone tissue and the biomechanical tests used. Some investigators have reported raw failure load data, whereas others have reported failure data corrected for bone size (Sogaard *et al.*, 1995).

This is an important aspect due to the fact that fluoride may affect bone size and because failure load and failure stress display different trends in fluoride studies. Site-specific differences may be due to previously discussed differences in the effect of fluoride on cortical and trabecular bone.

Fluoride may, therefore, be seen to have little effect on, or even deteriorate, bone quality depending on bone site, dosage and duration of exposure.

2.3.5.3 Investigation of Fluoride Effects in Animal Models

The effects of fluoride on bone have been studied in different animal models (see Table 1.). There are some variations in the reported results, which may be due either to a different response to fluoride according to the species or to the dose of fluoride.

The bone of the rat differs from the human bone by the aforementioned near absence of the ability to remodel intra-cortically. A review of the bone effects of fluoride in animal models revealed a great disparity in dosage varying from a therapeutic dose (1mg/kg/day) to a toxic dose. The lethal dose of fluoride in the rat has been calculated at 85.5mg/kg (Whitford, 1987). The duration of treatment ranged from 15 days to 33 months. Radiological examinations of treated animals showed bony exostoses and an increase in cortical thickness of fluoride-treated bone (Chavassieux, 1990). Refer to Table A.

All histomorphometric analyses are in agreement in that they conclude that fluoride induces an increase in bone formation parameters. After fluoride treatment, osteoblast numbers increase, however, the activity of each osteoblast decreases as manifested by the decrease in bone formation rate at the cellular level. This effect is noted whatever the animal model and the amount of fluoride used, however, the toxic effect of fluoride on osteoblasts increases with the dosage level.

An augmentation of bone resorption is reported by some authors at high fluoride levels. To account for this stimulation of bone resorption, several authors suggested that it was due to a hyperactivity of parathyroid glands induced by fluoride (Melsen *et al.*, 1996).

Using a subcutaneous endochondral bone formation model in rats, Eanes and Reddi (1979) found delayed uptake of radio-labelled calcium in fluoride-treated animals. On the basis of histological and biochemical data, they suggested that the mineralization defect was caused by retarded matrix development, leading to a decreased number of matrix sites for crystal nucleation.

Table A. Effects of Fluoride on Bone Remodelling in experimental studies

Authors	Animal Model	Bone Formation	Bone Resorption	Bone mineralization
Baylink et al. (1970)	Rat	▲ (Periosteum)	▲ (Endosteum)	▼
Ream (1991)	Rat	▲	▲	▼
Faccini (1967)	Rabbit		▼ (On F ⁻ containing bone	
Snow & Anderson (1986)	Dog	▼ (Cancellous)	▲ (Cancellous)	► (Cancellous)
Kragstrup et al. (1984)	Pig	▲ (Cancellous)	▲ (Cancellous)	► (Cancellous)
Chavassieux et al. (1988)	Ewe	▲ (Cancellous)	▲ (Cancellous)	▼ (Cancellous)

Hence, due to the wide range of fluoride dosages used across a range of different animal models, with varying treatment duration, and considering the dosage-dependent bivalent response of fluoride itself, it is not possible to provide general conclusions with respect to this previous body of research.

2.3.6 The Effect of Fluoride on Tooth Movement in Rats

As well as stimulating bone formation at the cellular level, fluoride has been shown to inhibit osteoclastic activity in rats (Lindskog *et al.*, 1989). These researchers found that, after administration of a single high dose of sodium fluoride, there were reduced numbers of active osteoclasts and a locally increased number of inactive osteoclasts not attached to bone surface. This was supported by the work of Hellsing and Hammarstrom (1991) who used a rat model to investigate the role of pregnancy and fluoride in the orthodontic movement of teeth. This latter study showed that a NaF concentration of 15mg/kg body weight over 21 days inhibited tooth movement in the rat compared with controls.

Taylor *et al.* (1989) showed that osteoclasts, isolated mechanically from chick bones, had decreased depth of resorption lacunae and inhibition of osteoclastic activity in culture containing 15mg/L NaF. Okuda *et al.* (1990) found that 0.5-1.0mM NaF (equivalent to 1mg of NaF/kg body weight per day) decreased the number of resorption lacunae made by individual osteoclasts and decreased the resorbed area per osteoclast. These studies all highlight the negative effect of NaF on orthodontic tooth movement.

Singer *et al.* (1967) found that indirect resorption occurred in the absence of periodontal ligament hyalinisation during tooth movement induced in fluoride-treated rats. There was found to be considerably less bone loss and osteoclastic activity in the fluoridated rats than in the non-fluoridated group, thus interfering with experimental tooth movement in the former.

2.4. Diurnal Variation in Biological Systems

Various studies concerning diurnal rhythmic phenomena have been performed in the field of bone physiology. Diurnal rhythms have been found in various parameters related to bone formation and bone resorption, including, calcium-regulating hormones, plasma calcium and phosphate, serum markers of type I collagen synthesis and degradation, urinary excretion of bone collagen cross-links and osteocalcin.

Delmas (1992) found that human serum osteocalcin undergoes a circadian rhythm within a narrow range, with a nightly peak at 4 a.m., a nadir at 5 p.m., and a 15% difference between peak and trough values. This circadian rhythm is likely to be due to an increase in human bone turnover during the night, as has been shown in the case of other markers of human bone turnover. The mean circadian osteocalcin pattern is conserved over time when studies are performed under similar conditions in a test-retest clinical research design (Markowitz *et al.*, 1987).

It has been shown that the events related to matrix production by osteoblasts occur around midday in rats (Simmons, 1992). In growing rats, the proliferation of chondrocytes and collagen-synthetic activities of osteoblasts in the rat mandibular condyle fluctuate with a peak in the middle of the light period and a nadir in the middle of the dark period (Oudet & Petrovic, 1978). Under physiologic conditions, maximal cell proliferation in the periodontal ligament occurs during the daytime (Roberts *et al.*, 1984).

Like these other events that contribute to bone formation, bone resorption has been considered to be active during daytime when the animal is resting (Simmons, 1992). Shinoda and Stern (1992) have shown that the release of calcium from bone is most active during the middle to late light period in rats. A morphologic study revealed that osteoclast-bone surface contact area is more extensive during the light period than in the dark period (Simmons *et al.*, 1988). Thus, in rats, both bone formation and bone resorption are most active in the environmental light period and are minimal in the environmental dark period.

2.4.1 Diurnal Variation in Orthodontic Tooth Movement

Diurnal variations have also been found in various local events that influence alveolar bone turnover, such as proliferation and matrix-synthetic activities of osteogenic cells, osteoclast-bone surface contact, and enzyme activities related to bone formation and bone resorption (Igarashi *et al.*, 1998).

Considering such diurnal fluctuations in bone physiology, biological responses to the external force exerted by orthodontic appliances (which inevitably include mechanically induced bone modelling in the alveolar bone) may vary depending on the time of day such forces are applied.

The results of the rat study performed by Igarashi *et al.*, (1998) indicate that there is a diurnal variation in tooth movement in response to orthodontic external force and that the application of force during the animal's rest period may be more effective than that while it is active.

In this study, the amount of new bone formed on the tension side in the light-period group of orthodontically treated rats was greater than that in the dark-period group. This indicates that a greater bone-formative response can be obtained when orthodontic force is applied during the light period, the active period of bone formation, rather than during the dark period. Therefore, the magnitude of the response may depend on the underlying physiologic activity of bone formation, which varies with the time of day. However, bone resorptive lacunae, rather than new bone formation, were observed on the tension side in the dark-period group.

The authors explained this as follows: in the dark-period group, teeth received orthodontic force during the period of minimal bone metabolism. When the spring was removed, relapse of the teeth may have occurred, i.e., the teeth would have moved to their original positions during the period of maximum activity. This may have resulted in minimal tooth movement with minimal bone formation and traces of bone resorption on the tension side. Another possible explanation for this variation is that the susceptibilities of mechanosensors in the target tissues could vary with the time of day. At present, however, the precise mechanisms are not known.

The causes of skeletal diurnal rhythms have not yet been clarified. Several endogenous and exogenous factors have been suggested to be involved. In rats, rhythms of cell proliferation in cartilage and bone parallel the serum corticosterone level, while the rhythm of matrix synthesis in bone is parathyroid-dependent (Simmons, 1992).

It has been shown that diurnal rhythms in calcium metabolism in both rats and human beings are regulated by undefined serum factor(s) (Lakatos *et al.*, 1995). Therefore, it is reasonable to assume that the observed variation in tooth movement is also caused by these hormonal rhythms.

Another possible cause may be a biomechanical rhythm, i.e., the diurnal variation in masticatory function. In the study by Igarashi *et al.* (1998), the animals consumed ten times more food during the dark period than during the light period, indicating that masticatory activities increased at night. A 24-hour electromyogram study has revealed that masseter muscle activity in rats increased during the dark period (65% of the whole-day activity) (Ishizuka & Tanne, 1995). Therefore, it is conceivable that increased masticatory activity during the dark period might interfere with orthodontic force, and thus may have inhibited tooth movement in the dark-period group.

These results indicate that diurnal variation should be considered an important factor that may affect tooth movement and hence osteocalcin expression.