THE DIRECT EFFECTS OF
PORPHYROMONAS GINGIVALIS 2561 ON BONE FORMATION AND MINERAL RESORPTION
IN VITRO.

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Dentistry
University of Toronto

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Dedicated in loving memory of my father, Harry Loomer, and my brother, Dr. Mark Loomer.

Always in my thoughts and in my heart.
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The Direct Effects Of \textit{Porphyromonas Gingivalis} 2561 On Bone Formation And Mineral Resorption \textit{in Vitro}.

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\textbf{ABSTRACT}

\textit{Porphyromonas gingivalis} has been implicated in the pathogenesis of periodontal disease. I undertook this study to examine the effects of \textit{P. gingivalis} 2561 on \textit{in vitro} bone formation in the chick periosteal osteogenic (CPO) model. Compared with controls devoid of bacterial products, osteogenesis was inhibited significantly in cultures treated with either conditioned medium or extracts obtained from \textit{P. gingivalis}. Varying amounts of inhibitory activity were observed, depending on the molecular size range of the extract, with very profound inhibitory effects observed in the < 5 kDa range. Further characterization of the \textit{P. gingivalis} extracts revealed that both proteinaceous and non-proteinaceous products, including lipopolysaccharide, were able to inhibit osteogenesis. \textit{P. gingivalis} extract-mediated inhibition of osteogenesis in CPO cultures was blocked by indomethacin implicating prostaglandins in the regulation of the bacterial effects. The bacterial extracts had either reversible or irreversible inhibitory effects on osteogenesis when added after differentiation or before/during differentiation of bone cells, respectively. The data suggest that periodontopathogens such as \textit{P. gingivalis} might contribute to the bone loss in periodontal diseases not only by stimulating resorption but possibly by inhibiting bone formation directly.

Since the process of bone formation \textit{in vivo} does not occur in isolation but in combination with that of bone resorption, I next examined the simultaneous effects of \textit{P. gingivalis} on both
aspects of bone metabolism. This first required the development and validation of a co-culture model system that permitted communication between cells involved in individual processes of bone formation (osteoblasts) and resorption (osteoclasts), but that also facilitated quantification of the effects of an agent on each process individually. With regard to the effects of \textit{P. gingivalis} on bone metabolism in co-culture, inhibition of osteogenesis was significantly less than in mono-culture while mineral resorption was significantly increased. No direct effects of \textit{P. gingivalis} on resorption were observed in mono-culture. These results suggest that \textit{P. gingivalis} stimulation of osteoclastic mineral resorption may be regulated by osteoblasts. Taken together, the data show that extracts derived from \textit{P. gingivalis} are capable of modulating bone formation and mineral resorption \textit{in vitro}, which is consistent with their effects on the periodontium \textit{in vivo}. 
CHAPTER I

LITERATURE REVIEW
I.1. **Periodontal Diseases: General Introduction.**

The periodontium, the investing and supporting tissues of the teeth, is comprised of the gingiva, periodontal ligament, cementum and alveolar bone. Diseases of the periodontium are defined by destruction of the tooth’s connective tissue attachment to the root surface and of the surrounding supporting alveolar bone (Carranza, 1996). Clinically, these diseases are characterized by pathologic changes in the colour and texture of the gingivae (redness, edema, loss of stippling, blunting of interdental papillae, etc.), bleeding upon periodontal probing, deep periodontal probing pocket depths, tissue recession, tooth mobility and migration, and radiographic evidence of bone loss (Wilson and Magnusson, 1996). Histologically, one observes the loss of connective tissue attachment, migration of junctional epithelium, epithelial and connective tissue inflammatory cell infiltration and resorption of alveolar bone (Holmstrup, 1996).

While there are some common clinical and histological features between periodontal diseases, there are also several dissimilarities necessitating their subclassification into different entities based on such considerations as the age of patient, rate of progression, disease distribution pattern within the dentition, and even response to appropriate therapy (Carranza, 1996). At present, the most widely accepted classification of periodontal diseases is that of the American Academy of Periodontology and includes: adult periodontitis, early onset periodontitis (pre-pubertal periodontitis, juvenile periodontitis, rapidly progressive adult periodontitis), refractory periodontitis, recurrent periodontitis, and systemic disease-associated periodontitis. Despite these differences, many etiological elements remain common to all types of periodontal diseases, and final clinical outcomes in untreated patients are virtually identical.

Clinical therapy for periodontal disease is directed not only towards stopping the progression of the disease, but increasingly towards prevention. With regard to treatment of
periodontal diseases, the major focus is on the eradication of agents implicated in their pathogenesis, such as specific oral microorganisms (see below, I.3.i., for more detailed discussion). Successful treatment includes the supression of these microorganisms to non-pathogenic and maintainable levels by combinations of mechanical tissue and tooth debridement, chemical treatment or surgical therapy. However, as the mechanisms through which microorganisms and other factors possibly involved in etiology and progression of periodontal diseases are only partly known and poorly understood, the currently available treatment modalities, while very effective for controlling the disease process in the vast majority of patients, are not cures for the disease and are less effective in the treatment of the more aggressive forms of the disease. In addition, some conventional therapies currently being employed, such as surgery, while successful, are also quite invasive and therefore may be less acceptable or even contraindicated in certain individuals. Hence, the need for the development of improved treatment modalities, diagnostic methods and preventive regimes for periodontal diseases is evident. In order to formulate them, however, a more thorough comprehension of the mechanisms involved in the etiology and progression of periodontal diseases is required. The roles that bacteria play in periodontal diseases will be discussed, with emphasis on the current understanding of their involvement with regards to the processes of bone formation, bone resorption and remodelling.

I.2. Osseous Structures of the Periodontium.

I.2.i. Introduction.

Bone is a complex connective tissue composed primarily of mineral and type I collagen, and formed by multiple cell types. It is often referred to as an "organ" system rather than a "tissue" type because it is a specialized connective tissue that together with cartilage comprises the skeletal
system (Baron, 1993). It is a dynamic tissue, in a constant state of remodelling, whose main functions are the maintenance of blood levels of calcium and phosphate, mechanical support of the body, and physical protection of other vital organ systems (Puzas and Ishibe, 1991). The mechanisms that regulate the formation of bone, in addition to resorption and remodelling, are part of a complex process which involves multiple cellular functions that co-ordinate resorption of existing bone and formation of new bone. They are controlled by numerous factors that have yet to be fully elucidated, but are currently under in depth examination.

I.2.ii. **Anatomy of the Osseous Structures of the Periodontium.**

The osseous structures that are affected by periodontal diseases are the alveolar processes of the mandible and the maxilla. The alveolar processes are the anatomical portions of the mandible and the maxilla that form the tooth socket and support the dentition.

The alveolar process is composed of 3 layers: (i) an external plate of cortical bone formed by haversian bone and compacted bone lamellae, (ii) an inner socket wall of thin compact bone (lamina dura), and (iii) cancellous bone between these two layers. All layers function together as a single unit. The outer surface of the bone is lined with a non-mineralized area of tissue which is osteoid tissue. The periosteum contains the bone forming and bone resorbing cells, and is comprised primarily of type I collagen. The marrow spaces within bone are lined with endosteum, which has many features in common with the periosteum.

I.2.iii. **Constitution of Bone.**

Bone is composed of extracellular matrix and cells. The extracellular matrix of bone is comprised of two thirds inorganic material and one third organic material. The inorganic material
consists mainly of calcium and phosphate mineral, along with hydroxyl, carbonates, citrates and some trace elements such as fluoride (Glimcher, 1990). These minerals are organized into hydroxyapatite crystal-like structures. The organic material in bone is made up of mostly type I collagen (>90%), with much smaller amounts of noncollagenous proteins (including osteopontin, osteocalcin, osteonectin, phosphoproteins and bone morphogenetic proteins) and proteoglycans (Miller, 1973; Raisz and Rodan, 1990). Some of these noncollagenous proteins are considered to be growth factors, which are involved in the regulation of bone cell metabolism.

The major cell types of bone are osteoblasts and osteoclasts. Osteoblasts, bone forming cells, are derived from a heterogeneous population of local precursor cells from bone marrow (Long et al., 1995). While several aspects of the cellular origins of osteoblasts are not known, they are widely believed to be derived from stromal cells differentiating from early mesenchymal progenitors (Grigoriadis et al., 1988). For a complete review on the theories of the cellular origins and differentiation of osteoblasts, see Triffitt (1996) and Aubin and Liu (1996). Fully differentiated osteoblasts are responsible for the production of bone matrix, and the regulation of its mineralization. They are cuboidal cells found lining bone osteoid and have the morphology of a typical protein-producing cell, i.e. prominent Golgi and well-developed endoplasmic reticulum.

Osteoclasts, bone resorbing cells, are also derived from precursor cells from bone marrow, as are osteoblasts, but these cells being of haematopoietic origin, are widely believed to be derived from the monocyte-macrophage lineage having a common differentiation pathway with macrophages until the final differentiation steps (Alvarez et al., 1991). For a complete review on the theories of the cellular origins and differentiation of osteoclasts, see Väänänen (1996). They are large multinucleated cells, formed by the fusion of pre-osteoclastic tartrate resistant acid phosphatase-positive mononuclear cells, although little is known regarding the mechanisms regulating the fusion process (Bianco et al., 1987). In an active state, they are found on the bone
surface (retraction of bone lining cells must occur prior to osteoclast cell attachment), attached by means of a clear zone within their plasma membrane surrounding a highly infolded central area called a ruffled border. It is within this area that bone resorption occurs.

A third major cell type found in bone tissue, is the osteocyte. These cells are mature osteoblast cells that have become surrounded by matrix during bone formation. They are the most abundant cell type within bone, and their main functions are not completely understood, but believed to be involved in adaptation to and remodelling of bone in response to mechanical stress (Frost, 1985; Skerry et al., 1987). Osteocytes occupy spaces (lacunae) within bone in isolation, but maintain contact with other like cells by means of cell processes connected via gap junctions. These permit not only communication between cells, but also exchange of nutrients and waste products.

I.2.iv. **Formation of Bone.**

Bone formation begins with the migration and proliferation of osteoblastic precursor cells and subsequent differentiation of mature osteoblasts to a site of remodelling. Functional osteoblasts are found lining the surface of bone on a layer of uncalcified bone matrix which they produce (osteoid). They do not function in isolation, but are always present as clusters of cuboidal cells (Baron, 1993). Calcification of this unmineralized bone matrix occurs some time after its laying down, which varies between organism and model system (approximately 10 days in humans) (Maniatopoulos et al., 1988). Behind the row of fully differentiated osteoblast cells are several layers of pre-osteoblastic cells. These cells, although not functional, are committed to become true osteoblasts, and will do so when the adjacent osteoblast becomes incorporated into mineralized matrix graduating into the role of an osteocyte. Mineralization of the bone matrix is regulated, in part, by the osteoblast. In woven bone, it is believed to be initiated in matrix vesicles
that bud away from the plasma membrane of osteoblasts. In contrast, in lamellar bone mineralization is thought to occur in a gap region between overlapped collagen molecules (Landis et al., 1993).

Regulation of bone formation is highly complex and is controlled by a multitude of local and systemic factors. These may not only be involved in directing bone matrix production by osteoblast cells, but may also govern recruitment, differentiation and expression of precursor cells. In addition, they may have dissimilar effects on cells within the same lineage but of different degrees of differentiation (Ohishi et al., 1994). As well, these factors may also yield different effects on the processes of bone formation when tested in multiple combinations in contrast to in isolation, although this property may be more a function of the in vitro model systems used (Chaudhary and Avioli, 1994).

I.2.v. Resorption of Bone.

Bone resorption is a function of osteoclast cells. While other cells such as macrophages have been shown to resorb bone in vitro, this has not been confirmed in vivo and rather may be the result of phagocytosis of bone particles in contrast to true resorption (Väänänen, 1996). Therefore, it appears likely that only osteoclasts are truly capable of resorbing bone.

Both inorganic and organic components of bone are resorbed by the osteoclast, using acid and enzyme secretions to accomplish this, respectively (Fallon et al., 1984; Baron et al., 1985; Delaisse et al., 1993; Okamura et al., 1993). The process of resorption occurs after the osteoclast has attached to surface of the bone at the so-called clear zone of its plasma membrane (Reinholt et al., 1990). This attachment, believed to be mediated through specific integrins, results in a seal and permits acidification of the local environment to facilitate solubilization of hydroxyapatite crystals of the bone (Fallon et al., 1984). The delivery of acid (i.e. hydrogen ions) into the sealed
compartment occurs mainly through V-type ATPase proton pumps located in the ruffled border of osteoclasts (Bekker and Gay, 1990). After dissolution of mineral, the organic matrix is then degraded. This process, although not fully understood, may involve several host proteolytic enzymes including lysozomal cysteine proteases and matrix metalloproteinases produced by both osteoblasts and osteoclasts (Väänänen, 1996). Organic bone degradation products may be removed by leakage from the sealing zone into the local environment, or by continuous transcytosis through osteoclast cells (Salo et al., 1994).

I.2.vi. **Osteoblast-Osteoclast Interactions.**

Although osteoblasts, which make bone, and osteoclasts, which resorb it, may act independently, it is clear from the literature that, when isolated, bone cells may not behave as those in a mixed cell milieu. Aside from spatial interactions, such as bone lining osteoblast cells displacing themselves from their location prior to bone resorption to permit direct access to bone surfaces for osteoclast cells (Ferrier et al., 1994), there is also evidence suggesting that these cells may interact directly in the regulation of each other's activities. For example, osteoblastic cells have been shown to influence osteoclastic recruitment, differentiation and function via the release of paracrine factors from the former cell type (Takahashi et al., 1988; Udagawa et al., 1989; Hattersley et al., 1991; Weir et al., 1993). Similarly, osteoclasts also may modulate osteoblastic function through paracrine activity, or more indirectly by the release of factors from resorbing bone (Sugimoto et al., 1993; Galvin et al., 1994). Thus, the aspects of bone formation and resorption in the remodelling process have been found to be co-ordinated both spatially and temporally, a phenomenon that has been termed "coupling" (Baylink et al., 1982).

As suggested above, in addition to direct (i.e. paracrine) influence osteoblasts and osteoclasts have on one another, bone tissue itself is also a rich source of growth factors which,
when made available to target cell, could contribute significantly to the regulation of bone formation and bone resorption (Canalis 1983). These locally released factors may exert their influence by direct impact on individual cell types, or on coupling itself. It is also probable that while factors released from bone and paracrine factors may act independently, they may also act in concert on resident target cells of bone and surrounding connective tissues. In addition to local regulation, systemic factors, such as hormones, may have both direct and indirect actions on bone metabolism. These may be exerted on initial recruitment of cells, the differentiation of precursor cells, the function of mature cells, or a combination of the above (Canalis et al. 1991; Canalis, 1993).


I.3.i Putative Periodontopathogenic Bacteria.

As suggested above (see I.1), the association between the community of bacterial species that colonize gingival crevices and the onset and progression of periodontal diseases has been well established (Zambon, 1990; Socransky and Haffajee, 1992). Clinically healthy sites have been shown to be colonized by a sparse plaque consisting of mostly Gram-positive aerobic cocci, while periodontally diseased sites harbour a mainly Gram-negative, anaerobic microbiota (Genco et al., 1988). Studies analyzing the microbiota of diseased sites, while difficult to directly compare because of wide variations in sampling and cultivating techniques used, have demonstrated overall trends that implicate a limited number of microbial species that function as major contributors in the periodontal disease process. It is these findings upon which the specific plaque hypothesis is based (Loesche, 1975). Dominant organisms found in diseased sites include: Porphyromonas gingivalis, Prevotella intermedia, Actinobacillus actinomycetemcomitans, Eikenella corrodens,
Fusobacterium nucleatum and spirochaetal organisms (Loesche and Laughon, 1981; Zambon, 1985; Van Winkelhoff et al., 1988; Chen and Wilson, 1992). In contrast, members of the Actinomyces and Streptococcus genera tend to predominate in healthy sites. Despite the knowledge of microbial specificity in periodontal diseases, the complex mechanisms by which these bacteria cause connective tissue destruction have not yet been fully elucidated.

I.3.ii. **Virulence of Periodontopathogenic Bacteria.**

Virulent bacteria associated with the destruction of the periodontium, or "periodontopathogens", participate in the destruction of host periodontal tissues by two different mechanisms: (i) the direct action of their products on host connective tissue stromal cells, and (ii) the induction of complex inflammatory responses leading to alterations in connective tissue cell metabolism (Page and Schroeder, 1981). The property that enable the bacteria to cause disease is known as virulence. It is a function of the microhabitat of the bacteria, various host properties, and the inherent pathogenic potential of the bacteria themselves (Slots and Rams, 1992). Many of the bacterial virulence determinants have been identified and their mechanisms of action are well understood. They have been classified into one of two broad categories: (i) those that enable a pathogen to colonize and invade host tissues, and (ii) those that enable a pathogen to cause host tissue damage either directly or indirectly. Generally, the more of these factors possessed by a bacterial species, the more virulent it may be (Socransky and Haffajee, 1991).

Pathogenic bacterial colonization in the periodontal environment is mediated through bacterial adhesins. These include: fimbriae, fibrils, capsule, outer membrane proteins and surface associated material (Lantz et al., 1990). Individual bacteria, including *P. gingivalis*, may possess one or more of these characteristics.
Virulence factors involved in helping bacteria, including *P. gingivalis*, neutralize or evade host mechanisms involved in bacterial removal and killing include: the production of leukotoxins, immunoglobulin and complement degrading proteases, cytotoxins and capsule mediated inhibition of phagocytosis (Socransky and Haffajee, 1991).

With regard to the destruction of host tissue, bacterial virulence factors may act by one of two modes of action: (i) direct degradation of host tissues, and, (ii) elicitation of tissue-damaging biological responses by host cells. Factors involved in host tissue degradation include: tissue and intercellular matrix degrading enzymes (including collagenase, hyaluronidase, trypsin-like enzymes, keratinase, arylsulphatase, neuraminidase, fibronectin-degrading enzymes, phospholipase, fibrinolysin, acid phosphatases), metabolic by-products (volatile sulphur compounds, ammonia, non-volatile organic acids), endotoxins (lipopolysaccharides), epithelial cell toxin and endothelial cell toxin. (Mayrand et al., 1980; Loesche, 1993). Many of these factors have both direct and indirect effects on host tissue.

**I.3.iii. Bacterial Invasion into Tissue.**

On the basis of the foregoing, it is clear that bacteria associated with periodontal diseases may possess a number of features which render them pathogenic. However, although a particular microbe may make products which could conceivably induce damage (directly or indirectly) to the host, it is also essential to know whether that particular bacterial cell can actually mediate its potential effects by growing in close association (via invasion) with the targeted host tissues or whether its effects are mediated from a distance. There is some uncertainty as to whether periodontopathogens invade host tissues or if they do, to what depth the invasion occurs. In fact, it is not known for certain whether in all cases, deeper periodontal structures, including alveolar bone, have direct physical contact with bacteria and/or their products. However, invasion of
bacteria from the gingival sulcus into the underlying connective tissue has been clearly
demonstrated histologically to occur in most forms of periodontal disease including that of chronic
adult periodontitis (Listgarten, 1965). Although not fully understood, this process may be
mediated through a number of mechanisms including bacterial proteases that degrade host tissues,
thus enabling easy access of deeper tissue for the bacteria (Frank, 1980; Saiglie et al., 1982).
Species that have been shown to have the capability for tissue invasion include: fusiform bacilli,
oral spirochaetes, and *P. intermedia*. In addition, several studies have detected *P. gingivalis*
intragingivally in biopsies, although these findings could have been the result of translocation of
the bacteria as a result of the sampling process rather than true invasion (Pekovic and Fillery, 1984;
Zambon et al., 1990). Importantly, bacteria have been detected histologically at the surface of deep
structures, in particular at the bony surface of the alveolar process, in sites of advanced periodontal
destruction (Frank et al., 1978; Frank and Voegel, 1980). The frequency of such occurrences at
less severely diseased sites is unknown, as is the depth of penetration of bacterial products.

I.4. **Effects of Periodontopathogenic Bacteria on Bone.**

I.4.i. **Introduction.**

One of the major consequences of periodontal diseases is the loss of alveolar bone (see
sections I.1 and I.2). It has been previously demonstrated, with the use of various *in vitro* and *in
vivo* model systems, that extracts and metabolic products derived from bacteria associated with
periodontal disease, can induce and promote resorption of bone or bone-like tissue. However the
effects of such bacterial products on bone formation, on osteoblasts and osteoblast-like cells are
not well known. In health, bone formation and bone resorption are in balance and are said to be
coupled. In periodontal disease, however, there is an alteration of the physiological balance
between the formative and resorptive processes of bone metabolism most probably as a result of an increase in local factors, in particular those that cause gingival inflammation (Hausman, 1974). Net alveolar bone loss likely results from the stimulation of bone resorption, the inhibition of bone formation, or a combination of both. Thus, an understanding how bone formation may be regulated under the influence of microbial products or associated inflammatory reactions is of equal importance as an understanding of the resorptive mechanisms.

Several pathways have been proposed by which bacteria and their products may cause alveolar bone loss (as distinct from soft connective tissue breakdown as discussed in I.3.i) in periodontal diseases: (i) direct action of bacterial products on bone cells and tissue, (ii) direct action of bacterial products on osteoprogenitor cells (i.e. promotion of differentiation into osteoclasts), (iii) bacterial stimulation of host connective tissue cells to release mediators for induction of progenitor cells into osteoclasts, (iv) bacterial stimulation of resident connective tissue cells to release products that function in collaboration with bacterial products to stimulate bone resorption, and (v) bacterial stimulation of connective tissue cells to release products that act directly upon bone tissue (Hausmann, 1974). While the exact mechanisms causing bone loss in periodontal diseases are not known, it is likely they include a combination of several or all of the above pathways.


Histological analysis of the periodontium at various stages of progression of periodontal disease has lead to a greater understanding of the disease process at the cellular level (Page et al., 1975). Initial lesions begin in the gingival tissues ("gingivitis") in response to bacterial plaque challenge. These lesions are characterized by a connective tissue infiltrate of fibroblasts and polymorphonuclear leukocytes, in conjunction with vascular dilation, exudation of serum proteins
and perivascular loss of collagen. With time, plasma cells tend to be the predominant immune cell type, and loss of collagen continues in combination with increased vascular proliferation. When the inflammatory process, vis a vis infiltrating inflammatory cells, extends from the superficial gingival tissues into the deeper supporting periodontal tissues, alveolar bone (and periodontal ligament attachment) loss occurs (Page et al., 1975). Chronic inflammation is the most common cause of bone loss in periodontal disease, with the degree of inflammation directly related to the amount of infiltrate. Even though the inflammatory infiltrate has a tendency to concentrate along the peripheral aspects of the marginal periodontium, the inflammatory response is much more diffuse. In fact, elicitation of a response in the osseous tissues may be evident even prior to evidence of crestal resorption or loss of attachment (Moskow and Polson, 1991). The distance from the apical border of the inflammatory infiltrate to the alveolar bone crest correlates with the number of osteoclasts present on the bone surface (Rowe and Bradley, 1981). However, the pathogenic potential of specific bacteria within plaque, in conjunction with the resistance of the host, have the capacity to modify the pattern and spread of the inflammatory periodontal destruction, and as a result the degree of bone resorption (Frank and Voegel, 1978). It has been postulated that the effective range within which bacteria can exert an effect on bone is 1.5 to 2.5 mm, although inflammatory mediators can extend this range considerably (Page and Schroeder, 1982; Tal, 1984).


The animal model that has most frequently been employed in the study of the immunopathogenesis of periodontal diseases is the gnotobiotic rodent. These animals are obtained germ-free at birth by surgical intervention of pregnancy at term (caesarian section) and subsequently infected with a known microflora (Heneghan, 1973; Foster, 1980). This model system facilitates evaluation of defined microbial modification of an otherwise germ-free host,
where the pure actions of such modification are of interest (Gordon and Pesti, 1971; Taubman et al., 1989). It does not mimic, however, what may occur when a specific microbe is introduced into an already established oral microbiological community of a normal host.

Despite some differences from humans in the immunopathology of periodontal diseases in rats (the first line of defence in rats is mainly lymphocytic with minimal infiltration of leukocytes, followed by B-lymphocytes and plasma cells becoming more abundant at later stages), studies using germ-free rats have provided some useful findings with regards to bacterial induction and ensuing immune response of periodontal disease (Irving et al., 1975; Guggenheim, 1980; Taubman et al., 1984). Monoinfection with periodontopathogenic organisms including Capnocytophaga, F. nucleatum, Bacteroides, E. corrodens and A. actinimycetemcomitans produced significant inflammatory and immune responses leading to periodontal bone loss in gnotobiotic rats (Irving et al., 1978; Johnson et al., 1978; Listgarten et al., 1978). In contrast, immunization of germfree rats with heat-killed P. gingivalis or E. corrodens completely or partially inhibited periodontal bone loss when monoinfected with the respective organism (Behling et al., 1981; Klausen et al, 1991). With regard to bacterial products, periodontal tissue injections of LPS markedly increased alveolar resorption, while the addition of indomethacin (a prostaglandin synthesis inhibitor) blocked the resorptive effect of LPS (Umeza et al., 1989).

These studies have helped establish a positive link, beyond that of epidemiological based human association studies, between the presence of certain types of bacteria or their products and periodontal bone loss. However, these models did not permit the study of the effects of bacteria on the individual processes of either bone formation or resorption, and the mechanisms by which they are mediated. To address this, in vitro studies have been utilized more successfully.

I.4.i.v. In vitro Studies.
Studies designed to examine the effects of bacteria and their products on bone loss during periodontal disease have focussed upon either bone resorption or bone formation, or the overall effects on both. As there is a shift in the physiologic balance between bone resorption and formation to favour the former, most investigations of periodontal bone loss have been directed towards the study of resorption, which have been facilitated by the use of a variety of in vitro models.

(a) Bone Resorption.

The effects of bacteria and their products on bone resorption in culture have been studied rather extensively owing to the relative importance of bone resorption in periodontal disease as noted above. While the majority of these studies have concentrated upon the effects of LPS on bone resorption, other bacterial products have also been examined. Early observation on the resorption-inducing effects of bacterial LPS on bone in vitro was provided by Hausmann et al. (1970). Many other studies, using various model systems of bone resorption, have corroborated these findings (Daly et al., 1980; Sveen and Skaug, 1980; Raisz et al., 1982; Iano and Hopps, 1984; Millar et al., 1986; Sisme-Durrant and Hopps, 1987; Saiglie et al., 1990; Ishihara et al., 1991).

More recent studies have tried to identify the specific bone cells which are affected by bacteria or their products. For example, LPS from P. gingivalis did not activate osteoclasts directly (as assayed by the release of tartrate resistant acid phosphatase), nor could it induce bone resorption by osteoclasts in the absence of osteoblasts (Sisme-Durrant and Hopps, 1987). In contrast, LPS could stimulate collagenase release from mouse osteoblasts (Sisme-Durrant and Hopps, 1987). Therefore, these studies suggest that osteoblasts, rather than osteoclasts, may be
the target cells for LPS. Other studies have suggested an LPS-mediated stimulation of osteoblastic production of prostaglandins (in particular prostaglandin E2) and interleukin-1β (Dewhirst et al., 1985; Millar et al., 1986), both of which are considered important local mediators of bone resorption. Macrophages have also been shown to respond to LPS by major shifts in gene expression which include induction of bone cytokines and matrix metalloproteinases such as collagenases (Wahl et al., 1974; Welgus et al., 1990). In contrast, fibroblasts have not been demonstrated to undergo LPS-induced direct effects, but may be indirectly stimulated to upregulate metalloproteinase production through cytokine mediators released by LPS-activated macrophages (Hanazawa et al., 1985; Takada et al., 1991).

Other bacterial factors can also induce bone resorption in vitro, although their mechanisms of action have not yet been clearly defined. These factors include: lipoteichoic acids and peptidoglycans (Lensgraf et al.; 1979), muramyl dipeptides (Dewhirst, 1982), outer membranes and isolated lipoproteins (Millar et al., 1986), fimbriae (which are able to trigger interleukin-1β production in monocytes and macrophages) (Hanazawa et al., 1995), capsule (Wilson et al., 1988), P. gingivalis -derived fibroblast-activating factor (which was shown to stimulate both resorption and the formation of osteoclast-like cells in a rat long-bone assay) (Mihara et al., 1993), proteinaceous surface-associated material (Wilson et al., 1993) and outer membrane (sheath) (Gopalsami et al., 1993).

Proteolytic enzymes elaborated from putative periodontopathogens, including P. gingivalis and A. actinomycetemcomitans, may also contribute to bone resorptive process by playing direct roles in cleavage of major structural components of bone including, type I collagen (Grenier, 1992; Lawson and Meyer, 1992). In addition, these proteinases can also activate mucosal fibroblasts and keratinocytes, increasing their expression and/or activation of various matrix metalloproteinases (Birkedal-Hansen et al., 1984). In such a manner, the destructive potential of bacterial enzymes
may be greatly amplified over just that of their direct degradative capacity. Other mechanisms by which bacterial enzymes may act to mediate host tissue destruction in periodontal diseases include interference with host defensive functions, such as IgA cleavage (Grenier et al., 1989; Kilian, 1989), complement cleavage (Sundqvist et al., 1985), and plasma proteinase and proteinase inhibitor inactivation (Nilsson et al., 1985).

(b) Bone Formation.

Although less is known about the effects of bacteria or bacterial products on osteogenesis, there have been a variety of relevant studies. The direct inhibitory effects of various bacterial products including bacterial plaque on bone derived cells or tissues has been shown by several researchers. Norton et al. (1970) demonstrated that LPS from an enteropathogen (Eschericia coli) inhibited in vitro bone growth (rat forepaw). Multanen et al. (1985) reported that low concentrations of dental plaque could inhibit type I collagen synthesis (but not alter the hydroxylation stage of collagen) in cultured fetal rat calvariae, but the cellular source of collagen, whether osteoblastic or fibroblastic, was not known. Denatured plaque extracts were shown to be equally effective, suggesting that some component other than protein was likely the causative agent. Others have shown that culture media filtrates from P. gingivalis can inhibit matrix production by chick embryo cartilage cells in vitro (Touw et al., 1982). When the filtrates were heated at 100°C the inhibitory activity was not abolished, again suggesting that the inhibitory factors were not proteinaceous.

In another investigation (Millar et al., 1986) the effects of two separate species (based on molecular weight and carbohydrate to fatty acid ratio) of P. gingivalis 381 LPS on bone formation in fetal rat long-bones were evaluated. Both LPSs induced a 30 to 40% reduction in net collagen formation at a concentration of 10 μg/mL. However, resorption and low levels of bone deposition
occur simultaneously in the long-bone model, making it difficult to discriminate between agent-mediated effects on resorption and/or formation.

In an attempt to identify the bacterial products that might influence osteogenesis directly or indirectly, Bom-van Noorloos et al. (1989) tested the direct and immune-cell-mediated effects of *P. gingivalis* on bone metabolism in fetal mouse long-bone rudiments *in vitro*. Metabolic products from spent medium, with a molecular size under 1 kDa, not only induced bone resorption but also appeared to inhibit mineralization. Conditioned media of bacteria-activated spleen cells strongly enhanced bone resorption and increased osteoclast numbers while inhibiting mineral formation. Metabolically inactivated bacteria had no effect when added directly to the cultures (Bom-van Noorloos et al., 1989). From this it was concluded that bacteria and their products do not have direct effects on bone metabolism but that their effects must be mediated through the immune system.

Meghji et al. (1992), using a murine calvarial assay, demonstrated that surface associated material isolated from *P. gingivalis* had a dose-dependant inhibitory effect on both DNA and collagen synthesis. They also noted evidence of osteolysis with presence of osteoclasts in areas of resorption in the calvaria. The addition of indomethacin reversed these effects, again suggesting prostaglandins may play a role as a mediator by which bacterial products may cause their adverse effects on bone metabolism.

Previous investigations have led to a greater understanding of the more direct effects of microbial products and extracts on bone cell metabolism. However, their effects on osteodifferentiation and osteogenesis cannot be ascertained with certainty due to some inherent deficiencies of the model systems used. In many of these model systems resorption and deposition occur simultaneously, thus making it difficult to discriminate between the effects of agents on the
individual processes of resorption or formation. Furthermore, only one or two parameters of osteogenesis, or non-specific parameters of cellular proliferation, were often measured, making it difficult to draw definitive conclusions regarding bone formation itself. Therefore, a model in which formation occurs only and which permits the analysis of multiple parameters of osteogenesis should be used to assess the effects of bacteria purely on bone formation. For this study, such a type of in vitro model system was employed, using chick periosteal tissues (described below).

I.5. **In vitro Bone Cell Culture Model Systems.**

I.5.i. **Models of Bone Formation.**

In order to acquire a more precise understanding of bone and bone cell metabolism, several model systems of bone formation have been developed. These have included: bone cell lines (clonal, tumour), primary osteogenic cell cultures (stromal, calvarial, enzymatic digestion released), and bone organ cultures (long bones, periosteal).

Isolated osteoblast-like cells, derived from frontal and parietal bones of fetal or neonatal rat calvaria by collagenase digestion from surrounding uncalcified matrix (Peck et al., 1964), or released after sequential digestion of mouse calvaria (Wong and Cohen, 1974), have been used to study the behaviour of bone cells *in vitro*. While these cells exhibit many properties characteristic of osteoblasts (i.e. high levels of alkaline phosphatase, ability to synthesize type I collagen, responsiveness to calcium-regulating hormones, etc.), they lack the ability to form histologically discernible bone (Jones and Boyde, 1984). However, newer models (described below) do form bone nodules.
Osteoblast-like transformed tumour cell lines, such as human osteoblastic SaOS-2 cells or rat osteosarcoma ROS 17/2.8 cells, have also been utilized for in vitro study of bone (Majeska et al., 1980). While these cells possess many properties in common with normal osteoblasts, these cell lines are transformed and as such may yield findings that may or may not be relevant with regards to the function of normal cells. Similarly, non-transformed clonal cell lines, such as neonatal rat calvarial UMR cells or newborn mouse calvarial MC3T3-E1 cells, also express a number of osteoblastic properties but not all. However, ease of use has made cell lines the choice of model system for studying bone for many researchers. Moreover, in situations where homogeneous cell lines are needed (e.g. receptor binding studies), such models have proved to be invaluable.

Model systems of bone formation have been developed using primary cultures of bone cells. These can be cells isolated from marrow which contain osteoprogenitor cells that are able to divide and differentiate into colonies containing differentiated osteoblasts and their associated matrices. Two mammalian models have been developed, fetal rat calvarial cells and rat bone marrow stromal cells (Escarot-Charrier et al., 1983; Nefussi et al., 1985; Bellows et al., 1986; Maniatopoulos et al., 1988). These model systems have a number of advantages over clonal or transformed cell lines: bone formation occurs much in the same fashion as it does in vivo, morphologic and structurally normal bone is produced, and enzymatic extraction of the bone cells from bone tissue is not required (Aubin et al., 1990). These bone forming model systems have been demonstrated to be appropriate for in vitro study of temporal and spatial expression of bone-related proteins during osteogenesis and formation, mineralization, and maturation of bone (Malaval et al., 1994). Further, these models recapitulate the same sequence of events which occurs during bone formation such that various stages of osteogenesis including differentiation, matrix production and maturation, and mineralization are replicated. Thus it is possible to perturb these models experimentally at various stages of bone development thereby permitting the
assessment of the effects of various factors on any one or all of the stages of osteogenesis.

An avian model using embryonic chick periosteum, the CPO model, has also been employed for the study of bone formation (Tenenbaum and Heersche, 1982). It is unique in that it employs solely the osteogenic layer of periosteum which is devoid of differentiated osteoblasts. Thus, bone formation in this model is reliant on the differentiation of osteoprogenitor cells into functional osteoblasts. This system has been shown to have distinct advantages over most other in vitro models in that bone cells are not removed from their normal physiologic environment but form bone with normal morphology and possessing all the characteristics of normal bone. The CPO model was the first in vitro model system to reliably demonstrate osteogenic cell differentiation and mineralized bone formation (Tenenbaum and Heersche, 1982; Tenenbaum and Heersche, 1985; Bruder and Caplan, 1990). In fact, the bone formed in this model has been shown to be morphologically (at both the light and electron microscopic level) indistinguishable from bone synthesized in vivo on the basis of ultrastructural criteria (Tenenbaum et al., 1986). Although it is an avian system, there is substantial evidence that issues addressed with this model are relevant to mammalian systems. For example, glucocorticoid effects in this model have been replicated in various mammalian bone cell models (Meghji et al., 1992; Tenenbaum et al., 1993). In addition, the necessity for the presence of an organic phosphate source for the induction of morphotypic mineralization in vitro was first demonstrated with the CPO model, and this has now been replicated in cell lines and osteogenic models derived from mammalian species (Escarot-Charrier et al., 1983; Whitson et al., 1984; Bellows et al., 1986; Ibaraki et al., 1992; Notoya et al., 1992) including human (Francis and Martodam, 1983; Gehron-Robey and Termine, 1985; Nefussi et al., 1985). Furthermore, the CPO model is amenable to biochemical assessment of a large number of osteogenic parameters, and can also be exploited temporally (Loomer et al., 1994). Therefore, findings obtained using this model with regard to bacterial regulation of osteogenesis would be relevant to what may be expected using mammalian systems. For these
reasons, the CPO model was selected as the model system for evaluating the effects of bacterial products on osteogenesis *in vitro*.

Mineralization in the CPO model system appears to follow that which occurs in intramembranous ossification. Osteoid begins to mineralize after 4 days of growth, with initial mineral deposits heterodispersed and not necessarily associated with collagen fibrils (which have been laid down in a dense and highly regular arrangement) or matrix vesicles (Tenenbaum et al., 1986). The growth of mineral, which was demonstrated by electron diffraction to be hydroxyapatite, continues spherically (Tenenbaum et al., 1986).

**I.5.ii. Models of Bone Resorption.**

As transformed or clonal cell lines for osteoclast cells have not yet been developed, each experiment using osteoclasts requires newly isolated fully differentiated cells or their mononuclear precursors. Osteoclasts, haematopoietic in origin, may be isolated from bone marrow cells. The earliest experiments generating multinucleated cells were in long-term cultures of feline marrow cells (Testa et al., 1981). They have since been isolated from other animal species including, rabbit (Fuller and Chambers, 1988), canine (Ibbotson et al., 1987), baboon (Roodman et al., 1985) and human (Takahashi et al., 1986). The multinucleated cells formed in these cultures possess many properties characteristic of osteoclasts, including: multinuclearity, tartrate-resistant acid phosphatase, calcitonin receptors and responsiveness to hormones such as 1,25-dihydroxyvitamin D₃ and parathyroid hormone. However, since only very low numbers of osteoclast cells may be obtained from the bone marrow of mammalian species, these sources of osteoclasts are not suitable for experimentation requiring larger numbers of cells.
When large numbers of cells are required, the best source for isolation is from the laying Leghorn hen placed on a calcium deficient diet (<0.1%) for a sufficient duration of time (Alvarez et al., 1991). Sustained sub-optimal dietary intake of calcium in the hen results in the formation of large numbers of osteoclast cells, required to mobilize calcium from the skeletal structures necessary for proper egg shell formation. Criticism of the use of an avian source for osteoclasts is based on differences in cellular responses to hormones (e.g. parathyroid hormone) of avian cells from those of mammalian sources. However, osteoclasts, or more specifically osteoclastic multinucleated cells, when isolated from some mammalian sources may also lack some properties classically associated with osteoclasts *in vivo*. For example, osteoclast cells isolated from some human bone marrow cultures were shown not to possess calcitonin receptors and were unable to resorb bone (MacDonald et al., 1987). Osteoclast cells from avian sources, however, were demonstrated to possess all of the properties listed above (with the exception of parathyroid hormone responsiveness) in addition to the ability to resorb bone and dentin (Alvarez et al., 1991). Thus, an avian source of osteoclast cells would appear to be well suited for *in vitro* experimentation as compared to mammalian cells.

In order to study osteoclast cell mediated resorption, the cells must be plated onto a resorbable substrate. Thin bone and dentine slices have been used successfully to quantify resorption. Alternatively, radiolabelled (with $^{45}$Ca) bone chips have also proved useful for the quantification of resorption. However, all of these substrates have several disadvantages including, commercial availability and a lack of standardization of substrate between researchers. In an attempt to solve these problems, a synthetic resorbable mineral substrate which can be used to quantify resorption and is commercially available was recently developed (Davies et al., 1993). This system comprises a uniform quantity (amount, surface area) of calcium phosphate thin film which is scintered onto the surfaces of quartz discs or glass slides upon which osteoclasts may be plated. At the termination of the cell incubation period, resorption can be easily assessed using a
variety of methods. While simplifying quantification of resorption as a result of its uniformity, a possible drawback of these mineral thin films is the absence of the organic matrix of bone, although this may also be employed to advantage. This system will be discussed in greater detail in Chapter V.

I.5.iii. Co-culture Osteoblast-Osteoclast Model Systems.

The processes of bone formation and bone resorption, while quantifiable in vitro on an individual basis, do not occur in isolation in vivo. Osteoclasts in vivo do not act in isolation from other cell types, in particular, osteoblasts, and vice versa. In healthy adults, bone is constantly being remodelled to maintain bone formation and resorption in balance with one another. Thus, cellular responses of isolated osteoclasts may be quite different from those in a mixed cell environment. Several investigators have tried to circumvent these problems by developing models that allow co-culturing of bone cells or their products. One such "co-culture" model uses conditioned media derived from one or the other cell-type which is supplemented into the growth medium of the other (Greenfield et al., 1992; Osdoby-Collins et al., 1994). Although this approach is useful, this does not permit simultaneous osteoblast/osteoclast interaction. In this regard, osteoblast or osteoclast by-products are produced and released without any influence from osteoclast cells or that of bone resorption, and thus real-time "cross-talk" between the cells is not permitted, which could be a crucial issue relating to their regulation of one another's activities. Similarly, other authors have utilized co-cultures but when osteoclasts and osteoblasts are grown directly together, the subsequent assessment of their respective activities (resorption for osteoclasts and bone formation for osteoblasts) is difficult at best (Morita et al., 1992). Therefore, I elected to develop a novel osteoclast/osteoblast co-culture model which would be used ultimately to test the effects of the putative periodontopathogens on these cells' activities and which would circumvent many of the problems alluded to above.
CHAPTER II

STATEMENTS OF THE PROBLEMS
II.1.i. **Statement of the First Problem.**

Previous studies have established the essential role that specific microorganisms, such as *P. gingivalis*, play in the etiology and progression of periodontal diseases which are manifested largely by bone loss. As a loss of alveolar bone support is observed during periodontal disease, appropriately it has been shown by others that products isolated from this bacterium have the capacity to enhance bone resorption *in vitro* and *in vivo*. This may represent one method by which bone loss might occur during the disease process.

In periodontal diseases, net alveolar bone loss likely results from abrogation of the balance between bone formation and resorption which normally occurs in health (Hopps and Sisme-Durrant, 1991). While the effects of microbial products and extracts on bone resorption have been studied rather extensively, their effects on bone formation (osteogenesis) have not been examined in as much detail. This is likely due in part to the lack of reliable *in vitro* model systems in which mineralized bone is produced. Most model systems involving the use of whole calvarial explants may not be useful to study factors regulating formation, as little active bone formation actually occurs (McCulloch et al., 1989). There have also been studies assessing the effects of various bacterial factors on bone-derived cells in culture (see I.4). However, these investigations measured relatively nonspecific parameters such as cellular proliferation in isolation rather than a spectrum of parameters which might reflect bone metabolism more holistically, like alkaline phosphatase activity, calcium uptake, and alpha-1-type-1 collagen production. Accordingly, I undertook to investigate the direct effects of products isolated from *P. gingivalis* on bone formation *in vitro*. The CPO model system, a valid and reliable model for bone formation (see I.5.i), was used for the quantification of these effects.
II.1.ii. **Hypothesis of the First Problem.**

Products derived from *P. gingivalis* inhibit osteogenesis *in vitro*.

II.2.i. **Statement of the Second Problem.**

It is conceivable that any bacterial extract, if added directly to an *in vitro* bone culture model, might potentially inhibit the functions of or be cytotoxic towards bone cells. Therefore, before the inhibitory effects on bone formation can be assigned exclusively to a periodontopathogenic bacterium, such as *P. gingivalis*, or other periodontopathogenic bacteria, the effects of products from other pathogenic and non-pathogenic bacteria on bone formation need to be examined.

II.2.ii. **Hypothesis of the Second Problem.**

Inhibition of osteogenesis *in vitro* is a property shared by a limited number of other oral bacterial species, namely periodontopathogens. Products derived from oral bacteria not correlated with disease do not inhibit osteogenesis *in vitro*.

II.3.i. **Statement of the Third Problem.**

In addition to the possibility that bacterial products may inhibit bone formation, there is a large body of evidence indicating that bone resorption may be stimulated by these same products (see I.4.iv). As stated above (I.5.ii) previous *in vitro* model systems for osteoclastic bone resorption have employed whole bone explants or isolated osteoclast-like cells (Bonucci et al.,
1992; Lowik et al., 1994). This made it difficult to determine the direct effects of such bacterial products on any given cell type as in whole explants. Moreover, bone cells in vivo do not act in isolation from other cell types and the responses of isolated bone cells to bacterial products may be quite different than those in an environment which may be more akin to in vivo. Although some investigators have tried to circumvent these problems by the use of conditioned media or growing bone cells in layered tiers, these co-cultures have proved to be unsatisfactory for the reasons previously outlined (see I.5.iii). Therefore, I elected to develop a novel osteoclast/osteoblast co-culture model which would be used ultimately to test the effects of the putative periodontopathogens on these cells' activities and which would circumvent many of the problems alluded to above.

II.3.ii. **Hypothesis of the Third Problem.**

To find answers to these problems above, a model system needed to be developed which would allow osteoblasts and osteoclasts to grow and function within a common cultural environment, thus permitting intercellular communication through the release of soluble factors. Once designed, various hypotheses may be tested. In view of the above, I have tested the hypothesis stating that the activities of osteoblasts and/or osteoclasts will be co-regulated by one another in vitro via the production of soluble factors. Accordingly, products from *P. gingivalis* will have differential effects on bone formation and resorption in co-culture in comparison to mono-culture.
CHAPTER III

THE DIRECT EFFECTS OF METABOLIC PRODUCTS AND SONICATED EXTRACTS OF *PORPHYROMONAS GINGIVALIS* 2651 ON OSTEOGENESIS IN VITRO.
III.1. **INTRODUCTION.**

*Porphyromonas gingivalis* has been implicated in the etiology and progression of periodontal diseases (see I.3). *P. gingivalis* likely causes tissue destruction by the direct action of its products on host connective tissue stromal cells and by the induction of complex inflammatory responses leading to alterations in connective tissue cell metabolism (Page and Schroeder, 1981). Although various microbial virulence factors have been identified, the complex mechanisms by which *P. gingivalis* may cause connective tissue destruction, in particular alveolar bone loss, has not been elucidated fully.

Net loss of bone during periodontal disease is likely a consequence of not only enhanced bone resorption but may also be caused by a concomitant decrease in bone formation or some combination thereof. Although there is considerable knowledge with respect to the mechanisms through which bacterial products influence bone resorption (see I.4.iv.a), little is known regarding their effects on bone formation. Previous investigations into the effects of microbial products and extracts on bone formation have provided at best restricted information, due to inherent deficiencies of the model systems used (see I.4.iv.b). In this chapter is a description of the direct effects of metabolic products and extracts of *Porphyromonas gingivalis* 2561 on osteogenesis *in vitro* using the chick periostal osteogenesis (CPO) model (Tenenbaum and Heersche, 1982; Tenenbaum and Heersche, 1985) in which a combination of biochemical parameters of osteogenesis (described below) can be quantified.
III.2 METHODS AND MATERIALS

III.2.i. Bacterial Culture Conditions.

*Porphyromonas gingivalis* strain 2561 (ATCC 33277) was originally supplied by J. Slots, State University of New York, Buffalo (currently at University of Southern California) and added to the frozen culture collection at the University of Toronto. Working stocks were grown on blood agar plates at 37°C in an anaerobic chamber in an atmosphere containing 80% N₂, 10% H₂, and 10% CO₂. After 5 to 7 days growth, bacterial inocula were added to trypticase yeast extract broth (TYB) which contained per litre, 17 g trypticase peptone (Becton Dickenson Microbiological Systems, Cockeysville, MD), 3 g yeast extract (Difco, Detroit, MI), 5 g NaCl, 2.5 g K₂HPO₄, 0.084 g NaHCO₃ and 2.5 g glucose and supplemented with 5 g hemin and 0.5 mg menadione. The bacteria were grown in TYB for a further three days. The purity of cultures was verified by phase-contrast microscopy, Gram stain and subculture on blood agar plates. Bacteria were harvested by centrifugation at 10,000 x g for 15 minutes at 4°C. The supernatant medium was sterilized by filtration through a 0.45 μm pore size filter (Millipore®) and stored at -20°C. The bacterial cell pellet was washed in phosphate-buffered saline, pH 7, and resuspended in BGJb tissue culture medium (GIBCO, Grand Island, NY) to optical density 1.0 at 690 nm (Model 350 spectrophotometer, G. K. Turner Associates, Palo Alto, CA). The bacterial suspensions were then sonicated at maximum power output using a Biosonik IV sonicator (Bronwill Co., Rochester, NY). The insoluble debris was removed by centrifugation at 10,000 x g for 30 minutes at 4°C, and the medium supernatant was filter sterilized.

A portion of the sonicated bacterial extract was then subjected to ultrafiltration (Amicon®) to
produce five fractions spanning the following molecular size ranges: 1) <5 kDa, 2) 5-10 kDa, 3) 10-50 kDa, 4) 50-100 kDa, and 5) >100 kDa. All fractions were stored at -20°C.

III.2.ii. **CPG Culture System.**

This osteogenic model system has been described in detail previously (McCulloch et al., 1989; Tenenbaum et al., 1992). Ectocranial periosteal tissues were removed from calvariae derived from 17-day-old embryonic chicks after excision of most of the fibrous tissue. Removal of the fibrous tissues by microdissection significantly reduces non-osteogenic cell content of these cultures thereby enhancing osteogenic "signal" for biochemical assessments of cultures (Sveen and Skaug, 1986; McCulloch et al., 1989; Tenenbaum et al., 1992). The periostea were then folded with the side originally facing bone (i.e. the osteogenic layer of cells) in apposition. The explants, supported on a Millipore® filter (HA 0.45 μm), were held at the gas/liquid interface of the culture medium on a stainless-steel grid over the centre well of an organ-culture dish (Falcon Plastics, Lincoln Park, NJ, USA) and incubated for up to six days at 37°C in a humidified atmosphere containing 5% CO₂ in air.

III.2.iii. **P. gingivalis** Extract Conditions.

(a) Control medium.

The control culture medium consisted of BGY medium supplemented with 10% fetal calf serum (Gibco), 10⁻⁷M dexamethasone (Sigma, St. Louis, MO, USA) (McCulloch and Tenenbaum, 1986), 10 mM β-glycerophosphate (Sigma) (Tenenbaum et al., 1992), and 300 μg L-ascorbate (Gibco) (Tenenbaum et al., 1986) per ml.
(b) *P. gingivalis* Metabolic Products.

To test the effects of bacterial metabolic products on osteogenic cultures, the control medium (described above) was supplemented with varying proportions (v/v) of either uninoculated TYB growth medium (25%) or sterile conditioned (spent) TYB growth medium (12.5%, 25%).

(c) *P. gingivalis* Sonicated Extracts.

To test the effects of sonicated bacterial extracts on osteogenic cultures, they were added in varying proportions (10%, 20%, and 40% [v/v]) to the control medium. The extract fractions derived by ultrafiltration were added directly to control culture medium in concentrations of 20%, 40% and 60% (v/v). Media in all cultures were changed every 48 hours.

### III.2.iv. Analysis of Osteogenesis.

(a) Biochemical Measurements.

Single CPO explants were homogenized in 1 ml of a bicarbonate buffer (3 mM NaHCO₃ in 15.0 mM NaCl pH 7.4) using a Polytron homogenizer (Kinematica GMBH, Switzerland). The homogenate was transferred to glass test tubes and centrifuged at 3180 x g for 10 minutes at 6°C. The supernatant fraction was assayed for soluble protein content to estimate culture size (Bradford, 1976), as well as alkaline phosphatase activity, colourimetrically using an enzyme-substrate assay, to assess osteoblastic differentiation and activity (Tenenbaum and Heersche, 1982; Tenenbaum and Heersche, 1985). Mineralization was measured by estimating the amount of acid-extractable calcium and phosphate from the pellet after overnight hydrolysis in 0.5 N HCl (Tenenbaum and
Palangio, 1987; Tenenbaum et al., 1992). All colorimetric assays (used for determination of acid and alkaline phosphatase activity, inorganic phosphate and protein content) were carried out in Titertek 96 well plates, and optical density was measured with a Titertek Multiskan MC Spectrophotometer (Flow Laboratories, Mississauga, Ontario, Canada). The calcium extracted from the pellets was measured by atomic absorption spectrophotometry (Perkin-Elmer, Norwalk, CT, USA).

(b) Measurement of alpha-1 type-1 Collagen Content and Synthesis.

Newly synthesized collagen was labelled for a 48-hour period, at 4-6 days, by the addition of $^{14}$C-glycine (Amersham, Arlington Heights, Ill.; 10 μCi/ml; 59 mCi/mMole) to the culture medium. Following incubation, the cultures were harvested and frozen at -20°C.

Whole radiolabelled explants were demineralized by washing briefly in 0.1N HCl. Total protein content of the acid extract was determined by the method described by Smith et al. (1985) using bicinchonic acid in a buffer containing 0.2 N NaOH and 4% copper sulphate, and read colorimetrically at 562 nm on a Titertek spectrophotometer.

To measure collagen, the explant was digested at 150°C for four hours in pepsin (100 μl; 50 μg/ml in 1.67 x 10⁻⁵ M acetic acid), and the digest was pelleted in the microfuge at 12,000 x g for 10 minutes. The pepsin extract was freeze-dried and reconstituted in SDS-PAGE sample buffer (Tris/glycine) for separation on Phast-Gels® (Pharmacia), and then stained with coomassie blue. The alpha-1 bands were scanned on an E.C. 910 densitometer (Pharmacia) and quantified using an integration program (GelScan®, Pharmacia, Pharmacia-LKB, Uppsala, Sweden). Fluorographs were made from the same gels, and assessed densitometrically using the same scanner and
integration program. This approach allowed for simultaneous measurement of both total and newly synthesized alpha-1 type 1 collagen.

(c) Histology.

To confirm that osteogenesis occurred and that cultures were indeed viable, five to eight cultures per group were fixed in neutral buffered formalin and processed for routine paraffin sections (5 μm). They were subsequently stained with H and E and by the von Kossa method to demonstrate mineral deposits.


Values for the various biochemical parameters were used to calculate a mean and standard error for each group (8 - 10 cultures), and the differences between means were evaluated using Student's t-test for comparisons between specific experimental groups and control, or ANOVA for multiple comparisons. Significance was assigned at the $p < 0.05$ level. All values in tables and figures represent the mean ± the standard error of the mean.
III.3. RESULTS

III.3.i. *P. gingivalis* Metabolic Products.

Growing CPO cultures in BGJ_B medium containing dilutions of *P. gingivalis* growth medium yielded biochemical parameters of osteogenesis that were reduced in a dose dependent pattern. Alkaline phosphatase activity was reduced and acid phosphatase activity increased significantly in the presence of 12.5% and 25% concentrations of *P. gingivalis* conditioned medium (Figure III.1). The non-conditioned bacterial medium also produced some reduction of osteogenesis in CPO cultures, but inhibition of alkaline phosphatase activity and mineralization (Figure III.2) by the bacterially conditioned medium were more profound.

III.3.ii. *P. gingivalis* Whole Sonicated Extracts.

The addition of sonicated extracts from pellets of *P. gingivalis* to CPO cultures also yielded a reduction in most parameters of osteogenesis (Figures III.3 and III.4). Statistically significant reductions in alkaline phosphatase activity, calcium, and phosphate were observed. In addition, there was at least a 40% reduction in total and newly synthesized-radiolabelled alpha-1 collagen as assessed by densitometry of Coomassie stained gels and fluorographs respectively. Although there was an apparently dose-dependent relationship between concentration of extract and inhibition of parameters of osteogenesis, the relationship was not entirely linear, probably as a result of the heterogeneous nature of the extracts.

Cultures grown in the presence of the various whole extracts appeared to maintain viability, as it was observed that histological sections of all cultures contained mineralized bone (Figure
III.3.iii. Effects of Various Molecular Sized Fractions on Osteogenesis.

Whole sonicated extracts were separated by ultrafiltration into different molecular size fractions. The most significant decrease in alkaline phosphatase activity was observed in CPO cultures grown in the presence of the 60% concentration in all five fractions (Table III.1). Yet, significant decreases were observed even at lower extract concentrations. Alkaline phosphatase activity was reduced most dramatically (greater than 8-fold, \( p < 0.001 \)) and almost to the detection threshold in the presence of the < 5 kDa fraction, regardless of the concentration used.

Calcium and phosphate accumulation levels were reduced significantly in all five fractions at concentrations of 40% and 60% (Table III.2). For both parameters, greater effects were observed at the higher extract concentration. Calcium and inorganic phosphate were below the detection threshold for all < 5 kDa fraction treatment groups.

Both total and radiolabelled alpha-1 collagen content extracted by pepsin digestion were reduced in the presence of all sonicated extracts (Table III.3). Inhibition of collagen production was greater at higher extract concentrations for most of the fractions. There was at least a 10-fold decrease in collagen production by CPO cultures grown in the presence of the < 5 kDa fraction as compared with controls (\( p < 0.001 \)). The absence 1/4 and 3/4 fragments on the gels indicated that degradation products of collagen were not observed, although it is likely that some turnover of collagen may have been occurring in the cultures.

Non-collagenous protein content was reduced significantly in all fractions at most extract concentrations, with the exception of the < 5 kDa fraction where values did not differ significantly
from the control cultures (data not shown).

Histological observation by light microscopy demonstrated apparently normal bone formation in all extract fraction groups except the < 5 kDa group. The latter cultures contained extensive areas of nonmineralized fibrous tissue and thin seams of osteoid, but little or no mineralized bone (Figure III.5). However, the appearance of the cells and tissues was within normal limits, and there was no evidence for toxicity. The bone matrix in the other extract fraction groups appeared to be highly cellular and remarkably similar in appearance with that of chondroid bone (Beresford, 1981).
III.4. DISCUSSION.

I have found that products derived from conditioned media or extracts of *P. gingivalis* have the capacity to inhibit bone formation directly. All measured parameters of osteogenesis were reduced. These findings suggest the possibility that the bone loss observed in periodontitis might be related not only to increased resorption but also to decreased bone formation.

There have been numerous investigations of bacterially stimulated bone resorption, as discussed earlier in Chapter I. For example, *P. gingivalis* elaborates several extracellular and cell-associated products which have been reported to stimulate bone resorption *in vitro* (Millar et al., 1986; Sveen and Skaug, 1986; Bom-van Noorloos et al., 1989). Lipopolysaccharide (LPS) isolated from *P. gingivalis* 381 has also been found to stimulate resorption in fetal rat bone cultures (Nair et al., 1983). Fimbriae derived from *P. gingivalis* have been shown to trigger IL-18, an important local mediator of bone resorption, production by monocytes/macrophages (Hanazawa et al., 1991).

In this study, I used the CPO model, a bone formation system in which osteoclasts have not been demonstrated. Thus, reductions of mineral or collagen content observed in the CPO cultures grown in the presence of bacterial metabolites are in all probability not related to induction of osteoclast differentiation or activity.

Apart from their apparent ability to stimulate osteoclast-mediated bone resorption, there is ample evidence indicating that various periodontal pathogens produce proteases which may degrade extracellular matrices in bone and soft connective tissues (Touw et al., 1982; Fujimura and Nakamura, 1987; Grenier and Mayrand, 1987; Hanazawa et al., 1991; Grenier, 1992). Thus,
apart from mediating effects on osteoblastic differentiation or function, it is conceivable that bacterial proteases might also degrade extracellular products of osteoblasts, leading to the decreases in net bone matrix formation observed in vivo, or in vitro as in this study. However, in this investigation, the presence of serum in the culture medium makes this less likely, due to its collagenase-inhibitory factors. Moreover, diffusion of large proteolytic molecules through the multilayer of osteoblasts surrounding bone (Brommage and Neuman, 1979; Neuman and Neuman, 1981) would not be very efficient, diminishing the effects that these could have on measurements of collagen synthesis in bone tissues. In addition, it has shown previously that the majority of collagen synthesis measured in the modified micro-dissected CPO model is attributable to osteoblasts (McCulloch et al., 1989). Therefore, it is unlikely that changes observed in radiolabelled collagen synthesis are due to degradation of collagen produced in the more external fibroblastic layers which might be more accessible to the proteases.

Although, there is less known about the direct effects of bacteria or bacterial products on osteogenesis, there have been a few relevant reports. For example, IL-1β has also been shown to inhibit DNA synthesis and proliferation of osteoblast-like cells, which might have implications with respect to inhibition of osteogenesis (Hanazawa et al., 1991). In addition, Norton et al. (1970) reported that bone formation in the rat forepaw was inhibited in vitro in the presence of LPS derived from Escherichia coli. However, this could have been due to stimulation of resorption, inhibition of bone formation, or possibly even cartilage formation (i.e. endochondral ossification).

Others have shown direct inhibitory effects of various bacterial products including bacterial plaque on bone derived cells or tissues. Multanen et al. (1985) reported that low concentrations of dental plaque could inhibit type I collagen synthesis in cultured fetal rat calvariae, but the cellular source of collagen, whether osteoblastic or fibroblastic, was not known. Denatured plaque extracts were shown to be equally effective, suggesting that some component other than protein was likely
the causative agent. Others have shown that culture media filtrates from *P. gingivalis* can inhibit matrix production by chick embryo cartilage cells *in vitro* (Touw et al., 1982). When the filtrates were heated at 100°C the inhibitory activity was not abolished, again suggesting that the inhibitory factors were not proteinaceous.

In another investigation (Millar et al., 1986), the effects of two separate species (based on molecular weight and carbohydrate/fatty acid ratios) of *P. gingivalis* 381 LPS on bone formation in fetal rat long-bones were evaluated. LPS induced a 30 to 40% reduction in net collagen formation at a concentration of 10 μg/mL. However, resorption and low levels of bone deposition occur simultaneously in the long-bone model, making it difficult to discriminate agents affecting resorption and formation.

In an attempt to identify the bacterial products that might influence osteogenesis directly or indirectly, Bom-van Noorloos et al. (1989) tested the direct and immune-cell-mediated effects of *P. gingivalis* on bone metabolism in fetal long-bone rudiments *in vitro*. Metabolic products from spent medium, with a molecular size under 1 kDa, not only induced bone resorption but also appeared to inhibit mineralization. Conditioned media of bacteria-activated spleen cells strongly enhanced bone resorption and increased osteoclast numbers while inhibiting mineral formation. Metabolically inactivated bacteria had no effect when added directly to the cultures (Bom-van Noorloos et al., 1989). From this it was concluded that bacteria and their products do not have direct effects on bone metabolism but that their effects must be mediated through stimulating the immune system. Clearly, the results reported in this investigation do not support this contention.

Previous investigations have led to a clearer understanding of the more direct effects of microbial products and extracts on bone cell metabolism. However, the effects of these factors on osteodifferentiation and osteogenesis cannot be ascertained with certainty due to some inherent
deficiencies of the model systems used. While all model systems have drawbacks, the CPO model has been demonstrated clearly to be reliable for osteogenic cell differentiation and mineralized bone formation (see I.5.i). Thus, it would seem appropriate that findings obtained with this model with respect to bacterial regulation of osteogenesis are relevant to what might be expected in mammalian systems under the same conditions. However, the actual level of bacterial products at the bone surface in vivo is not known, and thus the concentrations used in this study may under or over-represent what amount reaches osteoblasts in vivo.

The results obtained in these experiments demonstrate clear and direct inhibition of osteogenesis. Although a linear concentration-dependent relationship between extracts/products and inhibition of osteogenesis was not observed, inhibition was greatest at the higher concentrations for every parameter of osteogenesis tested. The nonlinear findings are most likely due to the rather crude nature of the extracts.

To begin identification of inhibitory factors, sonicated extracts were fractionated into five molecular size ranges. While inhibition of osteogenesis appeared to follow similar trends for the fractions greater than 5 kDa, there were some noteworthy differences. For example, pronounced inhibitory effects for all parameters were observed at lower concentrations for the 10-50 kDa fraction than for the other three fractions. This might be due to the multitude of proteolytic enzymes, fimbrial, and other outer membrane and extracellular vesicle proteins contained within this fraction (Grenier and Mayrand, 1987; Papaioannou et al., 1991; Smalley and Birss, 1991 Grenier, 1992; Lawson and Meyer, 1992).

The results obtained with the less than 5 kDa fraction were unique in that virtually complete inhibition of osteogenesis was observed at all tested concentrations. It did not appear that the cultures were killed by the extract, as collagen production still continued, as did alkaline
phosphatase production, albeit at a lower level than that observed for the other fractions. In addition, histological observations suggested the presence of healthy cells without the presence of cellular debris, and noncollagenous protein synthesis was not inhibited in CPO cultures treated with the less than 5 kDa fraction. These findings indicate that although bone formation was inhibited profoundly, the cultures were able to maintain their viability. Nonetheless, these results do suggest inhibition of osteogenic cell differentiation. The active compounds contained in this fraction were probably neither protein nor intact LPS, as their molecular weights of these compounds would likely be too great. However, this fraction may contain high levels of organic acids. Production of nonvolatile organic acids, for example phenylacetic acid and others, by P. gingivalis had been found to be exceedingly high (Mayrand, 1979; Mayrand and Bourgeau, 1982; Mayrand and Holt, 1988), and likely has the potential to effect osteoprogenitor cell differentiation profoundly.

In conclusion, the data indicate that metabolic products and sonicated extracts of P. gingivalis directly inhibit osteogenesis in vitro. This might have implications for the progression of periodontitis-associated bone loss if these factors were to possess similar activity in vivo. Such bone loss may not only be related to increased levels of resorption but also to direct inhibition of bone formation. Initial characterization of the inhibitory constituents of the extract fractions was carried out, but will be further examined in greater depth and described in the next chapter.

Finally, despite the determination that P. gingivalis extracts caused osteogenic inhibition, the possibility remained that an extract from any bacterium might also do the same. Thus, inhibition would be not a property attributable to a periodontopathogenic nature of the bacterium, but rather a function of the direct addition of a bacterial extract to an in vitro bone model such as the CPO. Therefore, to ascertain if this in fact was the case or if osteogenic inhibition is a characteristic limited to periodontopathogenic bacteria, the effects of extracts from
several other bacteria (both pathogenic and non-pathogenic) on bone formation \textit{in vitro also} requires examination.
III.5. **FIGURES, TABLES AND LEGENDS.**

Figure 1. **Alkaline and acid phosphatase activities for control. 25% unconditioned (uncultured) bacterial medium, 12.5% conditioned (cultured) bacterial medium, and 25% conditioned medium groups.**

Alkaline phosphatase (AP) activity is significantly different (*p<0.05) between control and all other treatment groups. There was a similar statistically significant difference in AP activity between the unconditioned control group and 25% conditioned medium (#p<0.05). Acid phosphatase activity was increased in the three treatment groups over the control (*p<0.05), but was much lower than AP activity within the same group. Each bar represents a mean of eight to ten cultures and the vertical line represents the SEM.
Figure 2. Inorganic phosphate levels for control, 25% unconditioned (uncultured) bacterial medium, 12.5% conditioned (cultured) bacterial medium, and 25% conditioned medium groups.

A significant decrease from control levels is seen in all treatment groups (*p<0.05). Each bar represents a mean of eight to ten cultures and the vertical line represents the SEM.
Figure 3. Biochemical parameters for control (100% plain BG.Ib media), and 10%, 20% and 40% concentrations of BG.Ib media sonicated extracts of *P. gingivalis*.

(a) Alkaline and acid alkaline phosphatase activities. A significant difference in AP activity is seen between control and all treatment concentrations (*p < 0.05). Acid phosphatase activity did not differ significantly between control and test groups.

(b) Inorganic phosphate levels. A significant decrease from control levels is seen in both the 10% and 40% concentrations (*p < 0.05).

(c) Calcium levels. A significant decrease from control levels is seen at all treatment concentrations (*p < 0.05). Each bar represents a mean of eight to ten cultures and the vertical line represents the SEM.
Figure 4. Collagen and Non-collagenous protein evaluations for control (100% plain BG.Ib media), and 10%, 20% and 40% concentrations of BG.Ib media sonicated extracts of P. gingivalis.

(a) Densitometric evaluation of Coomassie-blue stained mini-gels run on SDS-PAGE for control. A significant decrease from control levels is seen at all treatment concentrations (*p<0.05).

(b) Densitometric evaluation of fluorographs of SDS-PAGE gels. A significant decrease from control levels is seen in both the 20% and 40% concentrations (*p<0.05).

(c) Non-collagenous protein levels. A significant decrease from control levels is seen at the 40% concentration (*p<0.05). Each bar represents a mean of eight to ten cultures and the vertical line represents the SEM.
Figure 5. Light microscopc photomicrographs of CPO cultures grown in control medium (a, b), in medium containing 40% 50-100 kDa sonicated ultrafiltered extract (c, d), or in medium containing 40% <5 kDa sonicated ultrafiltered extract (e, f).

Sections from each test group were stained with H and E (a, c, e) and by the von Kossa method (b, d, f). The control culture appears to contain fully mineralized bone (B), as shown by the black von Kossa staining, surrounded by a thin seam of osteoid (O) and osteoblast-like cells (closed arrow). Fibrous tissue (F), seen in all cultures, is found peripheral to the osteoblast layers. The culture grown in 50-100 kDa extract (d) has a bone matrix that appears to be extremely cellular with high numbers of large-sized osteocytes (open arrow), and a larger than average osteoid seam indicating that a greater proportion of the osteoid had yet to mineralize. The culture grown in <5 kDa extract appeared to be minimally mineralized, with only small "spotty" areas of von Kossa positive material (f). Magnification X570.
Table 1. Effects of Fractionated *P. gingivalis* Sonicated Extracts On Alkaline and Acid Phosphatase Activity Measured in CPO Cultures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%</th>
<th>Alkaline Phosphatase (nmol pNP/µg Protein/hr)</th>
<th>Acid Phosphatase (nmol pNP/µg Protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>4.2 ± 0.6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>&lt; 5 kDa</td>
<td>20</td>
<td>0.5 ± 0.3*</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.5 ± 0.9*</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.6 ± 0.4*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>5 - 10 kDa</td>
<td>20</td>
<td>4.1 ± 1.0</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.6 ± 0.9</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.9 ± 0.6*</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>10 - 50 kDa</td>
<td>20</td>
<td>3.8 ± 0.7</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.4 ± 0.8</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.3 ± 1.1*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>50 - 100 kDa</td>
<td>20</td>
<td>2.1 ± 0.7*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2.6 ± 0.6*</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>1.7 ± 0.6*</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>&gt; 100 kDa</td>
<td>20</td>
<td>4.0 ± 0.9</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.0 ± 0.6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2.1 ± 0.7*</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

A significant decrease in alkaline phosphatase activity in the treatment groups in comparison to the control is noted by the * (p < 0.05). Each value represents a mean of eight to ten cultures ± the standard error of the mean.
Table 2. **Effects of Fractionated *P. gingivalis* Sonicated Extracts On Calcium and Inorganic Phosphate Measured in CPO Cultures.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%</th>
<th>Calcium (μmol Ca²⁺/μg Protein)</th>
<th>Inorganic Phosphate (nmol Pi/μg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>98.56 ± 10.49</td>
<td>134.0 ± 0.0050</td>
</tr>
<tr>
<td>&lt; 5 kDa</td>
<td>20</td>
<td>BDT</td>
<td>BDT</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>BDT</td>
<td>BDT</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>BDT</td>
<td>BDT</td>
</tr>
<tr>
<td>5 - 10 kDa</td>
<td>20</td>
<td>85.98 ± 6.20</td>
<td>134.9 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>70.23 ± 6.40*</td>
<td>98.1 ± 4.7*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>70.63 ± 7.82*</td>
<td>14.8 ± 4.9*</td>
</tr>
<tr>
<td>10 - 50 kDa</td>
<td>20</td>
<td>83.00 ± 5.10</td>
<td>78.9 ± 17.4*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>71.60 ± 8.61*</td>
<td>45.9 ± 6.1*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>60.71 ± 8.55*</td>
<td>26.4 ± 9.3*</td>
</tr>
<tr>
<td>50 - 100 kDa</td>
<td>20</td>
<td>71.81 ± 8.66*</td>
<td>52.0 ± 11.0*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>66.83 ± 5.15*</td>
<td>38.9 ± 7.8*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>51.74 ± 8.50*</td>
<td>35.6 ± 7.2*</td>
</tr>
<tr>
<td>&gt; 100 kDa</td>
<td>20</td>
<td>94.76 ± 11.10</td>
<td>125.0 ± 15.0</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>77.12 ± 7.45*</td>
<td>110.0 ± 22.0</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>65.66 ± 4.53*</td>
<td>38.0 ± 18.0*</td>
</tr>
</tbody>
</table>

A significant decrease in calcium and inorganic phosphate levels in the treatment groups in comparison to the control is noted by the * (p < 0.05). Each value represents a mean of eight to ten cultures ± the standard error of the mean. BDT indicates a below detection threshold levels of calcium and inorganic phosphate (in the < 5 kDa fraction group).
Table 3.  **Effects of Fractionated *P. gingivalis* Sonicated Extracts On Total Collagen and Collagen Synthesis From Day 4 to 6 Measured in CPO Cultures.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%</th>
<th>Total Collagen (Coomasie Density)</th>
<th>Collagen Synthesis (Fluorograph Density)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.0780 ± 0.0094</td>
<td>0.211 ± 0.032</td>
</tr>
<tr>
<td>&lt; 5 kDa</td>
<td>20</td>
<td>0.0014 ± 0.0003*</td>
<td>0.037 ± 0.007*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.0012 ± 0.0002*</td>
<td>0.027 ± 0.006*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.0010 ± 0.0002*</td>
<td>0.032 ± 0.006*</td>
</tr>
<tr>
<td>5 - 10 kDa</td>
<td>20</td>
<td>0.0490 ± 0.0044</td>
<td>0.233 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.0294 ± 0.0031</td>
<td>0.092 ± 0.019*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.0208 ± 0.0040*</td>
<td>0.086 ± 0.014*</td>
</tr>
<tr>
<td>10 - 50 kDa</td>
<td>20</td>
<td>0.0525 ± 0.0038</td>
<td>0.142 ± 0.032*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.0311 ± 0.0027</td>
<td>0.089 ± 0.010*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.0204 ± 0.0030*</td>
<td>0.069 ± 0.013*</td>
</tr>
<tr>
<td>50 - 100 kDa</td>
<td>20</td>
<td>0.0560 ± 0.0010*</td>
<td>0.099 ± 0.008*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.0297 ± 0.0023*</td>
<td>0.063 ± 0.007*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.0210 ± 0.0028*</td>
<td>0.031 ± 0.003*</td>
</tr>
<tr>
<td>&gt; 100 kDa</td>
<td>20</td>
<td>0.0551 ± 0.0073</td>
<td>0.185 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.0362 ± 0.0041</td>
<td>0.124 ± 0.007*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.0277 ± 0.0034*</td>
<td>0.084 ± 0.010*</td>
</tr>
</tbody>
</table>

A significant decrease in total collagen and collagen synthesis from day 4 to day 6 in the treatment groups in comparison to the control is noted by the * (*p < 0.05*). Each value represents a mean of eight to ten cultures ± the standard error of the mean.
CHAPTER IV

CHARACTERIZATION OF THE INHIBITORY EFFECTS OF
SUSPECTED PERIODONTOPATHOGENS ON
OSTEOGENESIS IN VITRO.
IV.1. INTRODUCTION.

Sonicated extracts of various molecular sizes and metabolic products from *P. gingivalis* 2561 were shown to have the capacity to inhibit osteogenesis *in vitro* directly (see Chapter III). This osteogenic inhibition was most probably caused by any number of different bacterial products, i.e. virulence factors (Robertson et al., 1982; Sveen and Skaug, 1986; Birkedal-Hansen et al., 1988; Lawson and Meyer, 1992; Sojar et al., 1993), contained within the extract fractions. However, characterization of the products which might be involved in osteogenic down-regulation, as well as the mechanisms by which these products mediate it, had only been examined on a cursory level.

In addition, inhibition of bone formation, although produced using *P. gingivalis* extracts, might not be a property specific to this or other species of periodontopathogenic bacteria. In fact, it may be merely a consequence of the direct addition of extracts from any oral species culture medium on the CPO cultures.

To address these issues, the next set of experiments were carried out to 1) further characterize the nature of the inhibitory effects, and 2) to determine if inhibition of osteogenesis is restricted to putative periodontopathogenic bacteria (e.g. *Actinobacillus actinomycetemcomitans* and *Prevotella intermedia* as well as *P. gingivalis*), or whether any oral bacterium, even those not generally associated with periodontitis (e.g. *Streptococcus sanguis*, *Prevotella denticola* and *Veillonella atypica*), might mediate such effects. Further, demonstration of whether bacterial products mediated their effects strictly by influencing phenotypic expression of osteoblasts or whether these products also had effects on osteoblast cell differentiation was also determined. Finally, additional characterization of the nature of the bacterial products and their effects was
studied, including determination of whether the observed inhibition of osteogenesis was mediated by prostaglandins.
IV.2. METHODS AND MATERIALS.

IV.2.i. Bacterial Culture Conditions.

*P. gingivalis* strain 2561 was grown and sonicated extracts prepared as described above in III.2.i. *P. intermedia* 2561 and *P. denticola* 33184 stocks were grown in the same type of medium as *P. gingivalis*, and sonicated extracts prepared in a like fashion.

*Actinobacillus actinomycetemcomitans* 652 was supplied by J. DiRienzo, University of Pennsylvania. Working stocks were grown on blood agar plates. Following 5 to 7 days, bacterial inocula were added to trypticase soy broth (Difco, Detroit, Mich.) which was supplemented with 0.6% yeast extract (Difco) and 0.04% sodium bicarbonate. The bacteria were grown for a further three days prior to harvest, and sonicated extracts were prepared in the same manner as for *P. gingivalis*.

*Streptococcus sanguis* 49295 stocks were grown in tryptic soy broth (Difco) containing 5% defibrinated sheep blood. *Veillonella atypica* 17744 stocks were grown on *Veillonella* agar (Difco) plates. After 5 to 7 days of growth, these strains were harvested and sonicated extracts prepared in the same way as for *P. gingivalis*.

Extracts of *P. gingivalis* were subjected to ultrafiltration in order to establish molecular weight ranges for the various factors which might influence osteogenesis (Loomer et al., 1994). The methodology used was as described in II.2.iii.
IV.2.ii. **CPO Culture System.**

The CPO culture system, as described in III.2.ii, was used to assess the effects of the bacterial extracts on osteogenesis *in vitro*.

IV.2.iii. **Bacterial Extract Conditions**

(a) Control Medium.

The control culture medium consisted of BGJ_B medium supplemented with 10% fetal calf serum (Gibco), 10-7M dexamethasone (Sigma, St. Louis, Mo.), 10 mM β-glycerophosphate (Sigma), and 300 μg/ml L-ascorbate (Gibco) (Tenenbaum and Heersche, 1985; Tenenbaum et al., 1992).

(b) Effects of Bacterial Sonicated Extracts.

To test the effects of sonicated bacterial extracts on osteogenic cultures, extracts from *P. intermedia, A. actinomycetemcomitans, P. denticola, S. sanguis* and *V. atypica* were added in varying proportions (20%, 40%, and 60% [v/v]) to the control medium throughout the incubation period in separate experiments. Since the bacterial extracts were prepared in BGJ_B medium, there would be no dilution of essential nutrients contained in this medium when the dilutions were prepared. Media in all cultures were changed every 48 hours.

(c) Effects of Temporal Addition of *P. gingivalis* Extracts.

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Bone formation by intramembranous ossification in the CPO model may be divided into three dominant phases (with some overlap) including: 1) differentiation of osteoprogenitors into osteoblasts (days 0-2), 2) osteoid matrix production (days 2-4), and 3) mineralization of the osteoid matrix (days 4-6). It is possible to test the effects of various factors on relatively specific stages of osteogenesis by adding (or removing) them at various times of incubation. To extend previous findings obtained using *P. gingivalis* (Loomer et al., 1994), whole unfractionated and <5 kDa sonicated *P. gingivalis* extracts at a concentration of 40% (v/v) were added to CPO culture media on days 0-6, 0-2, 0-4, 2-4, 2-6, and 4-6 (Figure 1) in CPO cultures grown for a total of 6 days. These cultures were subjected to the various biochemical analyses (see below) at the end of the 6 day culture period. As some biochemical measurements might not reflect immediate effects of sequential incubation (for example, mineral content at day 6 essentially represents total mineral accumulation and might not reflect the immediate effects of a factor on active mineral deposition if added at an earlier time, e.g. over days 2-4), some cultures were stopped at the end of the respective extract challenge period. Thus, cultures (with appropriate controls) were also terminated after two or four days.

(d) Effect of Heat Treatment.

Further characterization of osteogenic inhibitory factors from *P. gingivalis* 2561 sonicates was carried out. Sonicated extracts from *P. gingivalis* 2561 fractionated on the basis of molecular size were previously shown to possess different degrees of inhibition of osteogenesis (Loomer et al., 1994). To determine whether inhibition was mediated by protein-like factors, the five extracts were subjected to heat treatment sufficient to denature and thereby inactivate all proteinaceous components, including various enzymes (Grenier et al., 1987). The extracts were heated to 90°C for 30 minutes. They were added to culture medium of CPO cultures in concentrations of 20%, 40% and 60% (v/v). Cultures were analyzed at the end of the 6 day culture period.
(e) Effect of *P. gingivalis* Lipopolysaccharide.

The effects of *P. gingivalis* lipopolysaccharide (LPS) on osteogenesis was also tested. LPS from *P. gingivalis* 2561 was extracted using phenol-water extraction techniques as described by Westphal and Jann (1965), and was added to the culture medium for final concentrations of 0.1, 1 and 10 μg/ml for a period of 6 days. LPS was analyzed by the SDS-PAGE procedure of Laemmli (1970), by biochemical analysis for protein, carbohydrate and nucleic acid content, and morphologically by transmission electron microscopy.

(f) Effects of Indomethacin.

Since factors contained in the extracts might mediate their effects indirectly through the generation of prostaglandins, some cultures treated with extracts or fractions were co-incubated with indomethacin, a cyclooxygenase inhibitor (Howell and Williams, 1993). Indomethacin (Sigma, St. Louis, Mo.) was added at a concentration of 1 μM to control culture medium, as well as to media supplemented with either whole bacterial extract obtained from *P. gingivalis* (40% (v/v) unfractionated), or with the <5 kDa fraction (40%; v/v) also obtained from *P. gingivalis*.


The effects of the various extracts on osteogenesis were assessed by measuring multiple parameters of bone cell activity and bone formation including alkaline phosphatase activity, calcium and phosphate accumulation (mineralization), and collagen synthesis (osteoid matrix production), as described in III.2.iv.
IV.2.v. **Statistical Analysis.**

Values for the various parameters were used to calculate a mean and standard error for each group (8 - 10 CPO cultures per experimental group) and the differences between means were evaluated using Student's *t*-test for comparisons between specific experimental groups and control. Significance was assigned at the *P* < 0.05 level. All values in tables and figures represent the mean ± the standard error of the mean. All values have been presented as a percentage of the control value for the respective experiment (control = 100 ± SEM) to facilitate comparisons between different experiments.
IV.3. RESULTS.

IV.3.i. Effects of Sonicated Extracts from Oral Microorganisms on Osteogenesis.

One of the goals was to determine whether sonicated extracts from oral bacteria other than *P. gingivalis* affected osteogenesis. Alkaline phosphatase was reduced by up to 60% in CPO cultures grown for six days in the presence of 20, 40 and 60% concentrations of sonicated extracts derived from *P. intermedia* and *A. actinomycetemcomitans*, putative periodontal pathogens (Table IV.1). In contrast, there was virtually no reduction in alkaline phosphatase activity in CPO cultures grown in the presence of extracts derived from the species considered nonpathogenic with the exception of the lowest dose of *P. denticola* (notably, there were no reductions in any of the other parameters of osteogenesis at this dose of *P. denticola*, see below) (Table IV.1).

Calcium accumulation was significantly reduced for cultures grown in the presence of most concentrations of extracts derived from suspected periodontopathogens, as was collagen accumulation (0-6 days) and newly synthesized collagen content (labelled over days 4-6) (Table IV.1). No effects were observed in cultures grown in the presence of the extracts derived from any of the nonpathogenic bacteria (Table IV.1).

IV.3.ii. Effect of Temporal Addition of *P. gingivalis* Extracts to the CPO Cultures.

The effects of the bacterial extracts on the different phases of osteogenesis were examined
by treating the cultures with these extracts at various time periods (Figure IV.1). All measured parameters of osteogenesis (i.e. alkaline phosphatase activity, inorganic phosphate and calcium accumulation) were significantly reduced in 2 day cultures treated continuously with both the whole extract and the <5 kDa fraction-treated groups (Figure IV.2), when compared to controls.

Cultures grown for four days were incubated with unfractionated (whole) _P. gingivalis_ extract or the <5 kDa fraction over days 0-4 or 2-4. The results were different for each of the tested extracts. When CPO cultures were grown in the presence of whole extracts, significant decreases in all parameters were found only when the bacterial extract was present for the entire four days (Figure IV.3). When the extract was first added on day 2 of culture (i.e. just after the differentiation stage), no differences in alkaline phosphatase levels were observed. However, significant decreases in initial mineral deposition were noted. In contrast, when the <5 kDa fraction was tested, addition of the extract at either the start of culture or at day 2 caused significant reduction in parameters of osteogenesis (Figure IV.3).

A significant decrease in the measured parameters of osteogenesis was demonstrated in cultures grown for six days in the presence of the <5 kDa _P. gingivalis_ extract (Figure IV.4). When the extract was present only on days 2 to 4, the cultures recovered, and by day 6 there were no significant differences compared with controls. In contrast, complete recovery was not found when cultures were incubated with this extract during days 0-2 or 0-4, with the exception of radiolabelled collagen over days 0-4. Furthermore, measures of bone formation were reduced when the cultures were exposed to this bacterial extract during days 2-6 or 4-6. Similar findings were obtained with cultures grown in the presence of the whole bacterial extract (Figure IV.5).
IV.3.iii. Further Characterization of *P. gingivalis* Extracts.

(a) Effect of Heat Treatment of *P. gingivalis* Sonicated Extracts.

Heat denaturation either eliminated (50-100 and >100 kDa fractions), reduced (<5 and 5-10 kDa fractions), or had no effect (10-50 kDa fraction) on the osteogenic inhibitory effects of the various fractions. For example, alkaline phosphatase activity remained significantly reduced when cultures were grown in higher concentrations of both heated <5 or 5-10 kDa extract fractions and at all concentrations of the heated 10-50 kDa extract fraction (Table IV.2). No reductions in alkaline phosphatase activity were found at any tested concentration of the heated 50-100 and >100 kDa extract fractions, suggesting complete abrogation of the inhibitory factor effects in the latter fractions by heating. Unlike alkaline phosphatase, mineral accumulation, as measured by calcium and phosphate accumulation, remained significantly reduced in all five heated fractions (data not shown).

Both total and newly synthesized radiolabelled alpha-1-type-1 collagen content were reduced in the presence of all heated extracts, with the exception of the 50-100 kDa fraction (Table IV.2). However, in most cases, higher concentrations of most heated extract fractions were required to inhibit collagen synthesis and accumulation. Degradation products of collagen were not detected.

(b) Effect of *P. gingivalis* LPS.

Significant reductions in all measured parameters of osteogenesis were observed in CPO cultures incubated continuously with 1 and 10 μg/ml LPS (Table IV.3). There were no detectable effects at 0.1 μg/ml. There were no significant differences in acid extracted non-collagenous
protein levels in any of the culture groups.

(c) Effects of Indomethacin.

Indomethacin alone had no effect on osteogenesis (Figure IV.6). However the inhibitory effects of the bacterial extracts on alkaline phosphatase activity, calcium and inorganic phosphate accumulation, and collagen synthesis (both unlabelled and labelled) were blocked by indomethacin (Figure IV.6).
It was shown previously that *P. gingivalis* has the capacity to inhibit bone formation directly (Chapter III). As there was some question as to whether inhibition of bone formation could be mediated by any oral species, the effects of extracts from putative periodontal pathogens (e.g. *A. actinomycetemcomitans* and *P. intermedia* as well as *P. gingivalis*), and organisms not associated with periodontal diseases (e.g. *S. sanguis*, *V. atypica*, *P. denticola*) were tested. The data suggest that among the strains tested, the ability to inhibit bone formation was restricted to the suspected periodontal pathogens tested here. Thus, it appears that these bacteria have the capacity to contribute to the bone loss observed in periodontitis by inhibiting bone formation in addition to stimulating bone resorption (Sveen and Skaug, 1986; Born-van Noorloos et al., 1989; Hopps and Sismey-Durrant, 1991). These findings are consistent with results derived from clinical and animal investigations that have suggested that similar bacteria play a major role in the initiation and progression of periodontal diseases (Zambon, 1990; Moore et al., 1991; Socransky and Haffajee, 1992).

Bone formation in the CPO model system can be divided into three relatively discrete phases. By testing the various extracts at different times in culture it was possible to determine their effects on different stages of bone formation such as differentiation (days 0 to 2), matrix production (days 2-4), and mineralization (days 4-6). Due to the large number of possible permutations and combinations (i.e. with various extracts and time points/periods) this aspect of our investigation was restricted to studying only the unfractionated extract and the <5 kDa fraction derived from *P. gingivalis*, as these have been shown to possess the most profound inhibitory effects in comparison to other periodontal extracts studied (Chapter III). The results revealed that osteogenesis was inhibited irreversibly when either extract or fraction was added during the
differentiation stage. In contrast, the inhibitory effects of the extracts appeared to be reversible when cultures were exposed during the matrix production stage (i.e. after differentiation). Finally, the extracts directly inhibited mineralization, but due to the length of the culture period, it was not determined whether such inhibition was reversible. Taken together, the findings suggest that the factors contained in the *P. gingivalis* extract as well as the <5 kDa fraction may inhibit osteodifferentiation and therefore the development of functional osteoblasts. When this occurs, the inhibitory effects on osteogenesis are largely irreversible. Alternatively, the extracts also seem to inhibit phenotypic expression by differentiated osteoblasts (e.g. matrix formation, alkaline phosphatase activity, and mineralization), but this effect is essentially reversible as long as the inhibitory agent is removed.

Heat treatment caused virtually complete loss of the inhibitory properties in the 50-100 kDa fraction, implying that heat-labile compounds might be responsible for some of the observed reductions in bone formation by CPO cultures treated with its unheated counterpart. *P. gingivalis* produces various proteolytic enzymes in this molecular size range (George et al., 1994). It has also been shown by others (Mihara et al., 1993) that heat treatment of a 24-kDa protein isolated from the outer membrane of *P. gingivalis* W50 caused loss of the ability to induce bone resorption *in vitro*. In other fractions, notably the >100 kDa fraction, only incomplete abrogation of the inhibitory effects was observed suggesting that proteinaceous factors might only be partially responsible for inhibition of osteogenesis by this fraction. In contrast, heat treatment did not inactivate the inhibitory potential in the <5 kDa *P. gingivalis* extract fractions. Thus, the factor(s) in this fraction were not likely proteinaceous and yet had rather profound effects on differentiation and phenotypic expression of bone cells. These findings seem to parallel other reported results indicating that low concentrations of dental plaque can inhibit type I collagen synthesis in cultured fetal rat calvariae, and that heat denatured plaque extracts are equally as effective (Multanen et al., 1985). This suggests that some component other than protein is likely the causative agent.
Similarly, the inhibition of matrix production by chick embryo cartilage cells in vitro was not abolished when culture media filtrates from P. gingivalis were heated to 100°C (Touw et al., 1982). Of further interest was the finding showing that various parameters of osteogenesis were affected differently and at different doses (e.g. heat treated extract effects on alkaline phosphatase activity versus collagen synthesis). This underscores the need to rely on more than one single parameter of bone cell function when assessing the effects of a particular factor on osteogenesis.

Inhibitory activity was not completely eliminated by heating and therefore other bacterial factors, such as LPS, must also be considered. The results indicated that all parameters of osteogenesis were reduced significantly at LPS concentrations of 1 and 10 µg/ml. No decreases were evident when a very low concentration was used (i.e. 0.1 µg/ml). Extensive research has been performed on the potential inhibitory effects of LPS on periodontal tissues, including bone. LPS isolated from P. gingivalis 381 and W83 has been found to stimulate resorption in foetal rat bone cultures (Nair et al., 1983; Iino and Hopps, 1984). Millar et al. (1986) found that two separate species (based on molecular weight and carbohydrate/fatty acid ratios) of P. gingivalis 381 LPS induced a 30 to 40% reduction in net collagen formation at a concentration of 10 µg/ml using a long-bone model. However, this long-bone model cannot be used to differentiate between the effects on bone deposition or resorption as both occur simultaneously.

Although LPS may have direct effects on osteoblastic phenotypic expression (and possibly differentiation), there is evidence which may suggest a more indirect effect. LPS extracted from P. gingivalis W83 and other pathogenic bacteria have been shown to stimulate human fibroblast and monocyte/macrophage production of PGE2 and IL-1β (Heath et al., 1987; Bramati et al., 1989; Sismey-Durrant and Hopps, 1991; Yamazaki et al., 1992). In addition, LPS from either invasive or noninvasive strains of P. gingivalis was able to activate the alternate complement pathway (Schifferle et al., 1993). It is conceivable that LPS may exert its inhibitory effects on bone
formation through its ability to stimulate cytokine production by target cells other than osteoblasts. This could be mediated through fibroblast products, such as PGE₂, since the outer cell layers of the folded periosteal cultures contain large numbers of fibroblasts (even after micro-dissection) (Tenenbaum et al., 1986).

Although inhibition of osteogenesis by the bacterial extracts was probably not due to direct toxicity, this is still a possibility. The findings showing that bone forming cultures could recover from the inhibitory bacterial effects when the extracts were added after differentiation tend to support the notion that toxicity is not involved. This finding, in addition to the lack of effect on non-collagenous protein levels and our previous histological analysis (Chapter III), implies that the inhibitory effects of the \textit{P. gingivalis} extracts on CPO osteogenesis were likely cytopathic rather than cytotoxic.

In conclusion, the data indicate that sonicated extracts derived from bacteria implicated in the pathogenesis of periodontal diseases were capable of inhibiting bone formation. Conversely, bacteria which are not associated with periodontal diseases did not have this property, which would lend support to the concept that infection with specific bacteria is required to initiate or at least aggravate bone loss associated with periodontal disease. These bacteria may not only contribute to progression of periodontal diseases by stimulating bone resorption as shown by others (Millar et al., 1986; Nair et al., 1983; Hopps and Sismey-Durrant, 1991), but might also inhibit bone formation itself thus upsetting the balance in favour of bone loss. In addition, there are also data implicating these same species in infective failures of osseointegrated dental implants, a therapy that relies on osteogenesis at the interface with biomaterials for success (Grenier and McBride, 1987; Becker et al., 1990; Quirynen and Listgarten, 1990). Furthermore, there is reason to believe that these organisms might have a negative impact on treatment outcome for the so-called guided tissue regeneration procedures (GTR) in periodontal therapy (Selvig et al., 1990; Grevstad
and Leknes, 1993; Guillemin et al., 1993). Inasmuch as these bacteria seem to have an, in essence, direct inhibitory effect on bone formation, the latter findings should not be surprising as both osseointegration and GTR procedures are reliant upon bone formation either in whole (osseointegration) or in part (GTR). If regeneration of resorbed bone is to be a realistic goal of therapy, then it would seem prudent that this be attempted in the absence of bacteria which have profound inhibitory effects on osteogenesis.

While insights into the mechanisms by which periodontopathogenic bacteria influence bone formation may help to further our understanding of the disease processes involved in periodontal diseases (in an end to formulating more efficacious treatment therapies), in addition to having direct clinical relevance with respect to GTR, we know that in vivo, bone formation does not occur in isolation but in combination with bone resorption. The mechanisms regulating formation, in addition to resorption and remodelling, of bone are part of a complex process which involves multiple cellular functions that co-ordinate resorption of existing bone and formation of new bone. Therefore, it is also likely that influences exerted by periodontopathogenic bacteria, which have been shown to affect many of these cell types and their processes on an individual basis, will be modified by tissue cell interactions. Consequently, the final outcomes of bacterial effects on the formative and resorptive processes of bone metabolism may be quite different when examined in a mixed cell environment (i.e. more similar to what occurs in vivo ) than in one where only one phase of bone metabolism is dominant.

Accordingly, I wished to quantify the effects of extracts from P. gingivalis in such a milieu, e.g. in co-cultures of osteogenic (osteoblast-like) and osteoclast cells. However, a system which would enable me to do this was not in existence. Therefore, a co-culture model which permitted communication between bone cells types and which the effects of an added agent, such
as a bacterial extract, on bone formation and resorption could be easily quantified needed to first be developed (Chapter V).
IV.5. **FIGURES, TABLES AND LEGENDS.**

Figure 1. *Schematic representation of the timing and sequence of the three principal stages of osteogenesis (cell differentiation, matrix production and mineralization) during the six day CPO culture period.*

The black bars demonstrate the various times during the six days of the cultures that the cultures were grown in the presence of the bacterial extracts.
Cell differentiation, matrix production, mineralization

CULTURE PERIOD (days)
Figure 2. Biochemical parameters of two day-old cultures for control (C: 100% plain BG1b media), and 40% concentration of BG1b media sonicated extracts of P. gingivalis (<5kDa fraction and whole (unfractionated) sonicates).

(a) Alkaline phosphatase activity. Alkaline phosphatase activity was significantly different ($P<0.05$) (*) between treatments and control.

(b) Inorganic phosphate level. Significant decreases from control levels were seen in both extract treatment groups ($P<0.05$) for inorganic phosphate accumulation. Calcium accumulation levels (data not shown) paralleled that of inorganic phosphate.

The data are means and SEM for 8 to 10 cultures. All numerical values have been expressed as a % of control values.
Figure 3. Biochemical parameters of four day-old cultures for control (C: 100% plain BG.Ib media), and 40% concentration of BG.Ib media sonicated extracts of <5kDa fraction and of unfractionated (whole) P. gingivalis.

(a) Alkaline phosphatase activity. Alkaline phosphatase activity was significantly reduced (P<0.05) (*) when incubated with either extract for the entire four day period.

(b) Inorganic phosphate level.

(c) Calcium level. Significant (P<0.05) decreases from control levels were seen for both treatment periods for inorganic phosphate and calcium accumulation.

The data are means and SEM for 8 to 10 cultures. All numerical values have been expressed as a % of control values.
Figure 4. Osteogenic parameters of six day-old cultures for control (C: 100% plain BGJb media), and 40% concentration of BGJb media sonicated extracts of <5 kDa of P. gingivalis.

(a) Alkaline phosphatase activity.
(b) Calcium level.
(c) Densitometric evaluation of Coomassie-blue stained mini-gels run on SDS-PAGE for control.
(d) Densitometric evaluation of fluorographs of SDS-PAGE gels.

Significant ($P<0.05$) (*) decreases from control levels were seen for most treatment periods for all measured parameters. Notably, when the extract was present only on days 2 through 4, all parameters returned to control levels. The data are means and SEM for 8 to 10 cultures. All numerical values have been expressed as a % of control values.
Figure 5. Osteogenic parameters of six day-old cultures for control (C: 100% plain BG.Ib media), and 40% concentration of BG.Ib media sonicated extracts of unfractionated of *P. gingivalis*.

(a) Alkaline phosphatase activity.
(b) Calcium level.
(c) Densitometric evaluation of Coomassie-blue stained mini-gels run on SDS-PAGE for control.
(d) Densitometric evaluation of fluorographs of SDS-PAGE gels.

A significant ($P<0.05$) (*) decreases from control levels were seen for most treatment periods for all measured parameters. The presence of the extract on days 2 through 4 did not cause any significant reductions in any measured biochemical parameter. The data are means and SEM for 8 to 10 cultures. All numerical values have been expressed as a % of control values.
UNLABELLED COLLAGEN

ALKALINE PHOSPHATASE ACTIVITY

RADIO-LABELLED COLLAGEN

CALCIUM LEVELS

ALKALINE PHOSPHATASE ACTIVITY

(% of control)

(% of control)

(% of control)

(% of control)
Figure 6. Osteogenic parameters for control (C: 100% plain BG.Ib media), and 40% concentrations of BG.Ib media sonicated extracts of *P. gingivalis* (<5 kDa fraction (<5) and unfractionated (W) sonicates) incubated in the presence or absence of 1 μM indomethacin (I).

(a) Alkaline phosphatase activity.

(b) Calcium level.

(c) Densitometric evaluation of Coomassie-blue stained mini-gels run on SDS-PAGE for control.

(d) Densitometric evaluation of fluorographs of SDS-PAGE gels.

Significant (*P*<0.05) (*) decreases from control levels were seen for both tested extracts for all measured parameters of osteogenesis activity. The addition of indomethacin blocked the inhibitory activity of the bacterial extracts. Indomethacin alone had no effect on osteogenesis. The data are means and SEM for 8 to 10 cultures. All numerical values have been expressed as a % of control values.
Table 1. Effects of oral bacterial sonicated extracts on parameters of osteogenesis of CPO cultures.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Conc (%)</th>
<th>A. P. activity(^b)</th>
<th>Calcium(^c)</th>
<th>Pi(^d)</th>
<th>Total Collagen(^e)</th>
<th>Collagen Synthesis(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. intermedia</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>69 ± 9</td>
<td>50 ± 8(^*)</td>
<td>63 ±16(^*)</td>
<td>69 ± 9</td>
<td>33 ± 9</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>45 ± 8(^*)</td>
<td>55 ± 7(^*)</td>
<td>72 ± 12</td>
<td>45 ± 8(^*)</td>
<td>12 ± 3(^*)</td>
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<tr>
<td>60</td>
<td>40 ± 9(^*)</td>
<td>20 ± 3(^*)</td>
<td>31 ± 4(^*)</td>
<td>40 ± 9(^*)</td>
<td>17 ± 6(^*)</td>
<td></td>
</tr>
<tr>
<td><em>A.aJ</em></td>
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<td></td>
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<td></td>
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<tr>
<td>20</td>
<td>77 ± 8</td>
<td>47 ± 12(^*)</td>
<td>58 ± 13</td>
<td>51 ± 14(^*)</td>
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<td>52 ± 4(^*)</td>
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<td>28 ± 5(^*)</td>
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<tr>
<td>60</td>
<td>36 ± 4(^*)</td>
<td>22 ± 4(^*)</td>
<td>31 ± 5(^*)</td>
<td>16 ± 3(^*)</td>
<td>16 ± 3(^*)</td>
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<tr>
<td><em>S. sanguis</em></td>
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</tr>
<tr>
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<tr>
<td>40</td>
<td>83 ± 11</td>
<td>110 ±10</td>
<td>95 ± 16</td>
<td>80 ± 13</td>
<td>102 ± 24</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>109± 11</td>
<td>114± 7</td>
<td>121± 20</td>
<td>85 ± 9</td>
<td>81 ± 15</td>
<td></td>
</tr>
<tr>
<td><em>V. atypica</em></td>
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<tr>
<td>20</td>
<td>81 ± 4</td>
<td>73 ± 17</td>
<td>66 ± 19</td>
<td>95 ± 18</td>
<td>52 ± 13(^*)</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>88 ± 5</td>
<td>90 ± 13</td>
<td>82 ± 3</td>
<td>88 ± 12</td>
<td>92 ± 14</td>
<td></td>
</tr>
<tr>
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<td>92 ± 4</td>
<td>70 ± 19</td>
<td>63 ± 13</td>
<td>72 ± 6</td>
<td>95 ± 24</td>
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<td><em>P. denticola</em></td>
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<td></td>
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<td></td>
</tr>
<tr>
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<td>119± 47</td>
<td>78 ± 19</td>
<td>79 ± 12</td>
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<tr>
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<td>66 ± 13</td>
<td>69 ± 7</td>
<td>87 ± 10</td>
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</tr>
<tr>
<td>60</td>
<td>106± 7</td>
<td>105± 15</td>
<td>100± 16</td>
<td>72 ± 11</td>
<td>69 ± 36</td>
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</tr>
</tbody>
</table>

\(^a\)Mean ± SEM for 8 to 10 cultures. \(^*\), significantly different than the value for the control of the respective experiment \((P < 0.05)\). Values for each bacterial group \(^b\)For A. P. (alkaline phosphatase) activity all values originally expressed in nmol pNp/μg of protein/hr are expressed as % of control values. \(^c\)All values originally expressed in μmol of Ca\(^{2+}\)/μg of protein have been normalized to a control value of 100. \(^d\)All values originally expressed in nmol of Pi/μg of protein are expressed as % of control values. \(^e\)All values originally expressed in absorbance units x mm are expressed as percentage of control values.

\(^f\) A.a., Actinobacillus actinomycetemcomitans; pNp, paranitrophenol.
Table 2. Effects of fractionated *P. gingivalis* heat treated sonicated extracts on parameters of osteogenesis of CPO cultures.

<table>
<thead>
<tr>
<th>Extract (kDa)</th>
<th>Concentration (%)</th>
<th>A.P. Activity</th>
<th>Total collagen</th>
<th>Collagen synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100 ± 6</td>
<td>100 ± 18</td>
<td>100 ±12</td>
</tr>
<tr>
<td>&lt; 5</td>
<td>20</td>
<td>99 ± 13</td>
<td>33 ± 4*</td>
<td>27 ± 6*</td>
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<td></td>
<td>40</td>
<td>56 ± 17*</td>
<td>31 ± 4*</td>
<td>49 ± 5*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>13 ± 10*</td>
<td>29 ± 4*</td>
<td>16 ± 4*</td>
</tr>
<tr>
<td>5-10</td>
<td>20</td>
<td>83 ± 11</td>
<td>51 ± 10*</td>
<td>52 ± 18</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>57 ± 7*</td>
<td>49 ± 6*</td>
<td>48 ± 11*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>42 ± 12*</td>
<td>29 ± 4*</td>
<td>18 ± 3*</td>
</tr>
<tr>
<td>10-50</td>
<td>20</td>
<td>44 ± 17*</td>
<td>57 ± 6*</td>
<td>35 ± 4*</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>36 ± 8*</td>
<td>51 ± 14*</td>
<td>25 ± 4*</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>36 ± 9*</td>
<td>25 ± 5*</td>
<td>14 ± 2*</td>
</tr>
<tr>
<td>50-100</td>
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<td>117 ± 10</td>
<td>93 ± 10</td>
<td>63 ± 19</td>
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<tr>
<td></td>
<td>40</td>
<td>70 ± 32</td>
<td>104 ± 6</td>
<td>60 ± 13*</td>
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<tr>
<td></td>
<td>60</td>
<td>112 ± 17</td>
<td>110 ± 8</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>&gt;100</td>
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<td>102 ± 8</td>
<td>46 ± 4*</td>
</tr>
<tr>
<td></td>
<td>40</td>
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<td>60</td>
<td>105 ± 11</td>
<td>59 ± 8*</td>
<td>15 ± 3*</td>
</tr>
</tbody>
</table>

*Mean ± SEM for 8 to 10 cultures. *, significantly different than the value for the control (P < 0.05). All values originally expressed in absorbance units x mm are expressed as % of control values.
**Table 3. Effects of *P. gingivalis* LPS on osteogenic parameters in CPO cultures**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th><em>P. gingivalis</em> LPS (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>Alkaline Phosphatase(^b)</td>
<td>100 ± 3</td>
<td>85 ± 11</td>
</tr>
<tr>
<td>Acid Phosphatase(^b)</td>
<td>100 ± 13</td>
<td>113 ± 10</td>
</tr>
<tr>
<td>Calcium(^c)</td>
<td>100 ± 7</td>
<td>101 ± 24</td>
</tr>
<tr>
<td>Inorganic Phosphate(^d)</td>
<td>100 ± 6</td>
<td>104 ± 6</td>
</tr>
<tr>
<td>Total Collagen(^e)</td>
<td>100 ± 20</td>
<td>107 ± 18</td>
</tr>
<tr>
<td>Collagen Synth. (from day 4 to 6)(^e)</td>
<td>100 ± 15</td>
<td>110 ± 13</td>
</tr>
<tr>
<td>Noncollagenous Proteins(^f)</td>
<td>100 ± 9</td>
<td>129 ± 12</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SEM for 8 to 10 cultures. \(^b\)All values originally expressed in nmol pNp/µg of protein/hr have been normalized to a control value of 1.00. pNp, paranitrophenol. \(^c\)All values originally expressed in µmol of Ca2+/µg of protein have been normalized to a control value of 100. \(^d\)All values originally expressed in nmol of Pi/µg of protein have been normalized to a control value of 100. \(^e\)All values originally expressed in absorbance units x mm have been normalized to a control value of 100. \(^f\)All values originally expressed in µg/ml are expressed as % of control values. *, significantly different than the value for the control (P < 0.05).
CHAPTER V

DEVELOPMENT OF AN IN VITRO CO-CULTURE MODEL SYSTEM FOR STUDYING OSTEOGENIC AND OSTEOCLAST CELLULAR INTERACTIONS.
The major cell types involved in bone metabolism are osteoblasts and osteoclasts. Although osteoblasts, which make bone, and osteoclasts, which resorb it, may act independently, it is clear from the literature that when isolated, bone cells may not behave as those in a mixed cell milieu. An ever growing body of evidence suggests that these cells may interact directly. For example, osteoblastic cells have been demonstrated to influence osteoclastic recruitment, differentiation and function via the release of paracrine factors from the former cell type (Takahashi et al., 1988; Udagawa et al., 1989; Hattersley et al., 1991; Takahashi et al., 1991; Weir et al., 1993). Similarly, osteoclasts may modulate osteoblast function by paracrine activity, or more indirectly through the release of factors from resorbing bone (Sugimoto et al., 1993; Galvin et al., 1994). Thus, the processes of bone formation and resorption have been demonstrated to be interrelated, a phenomenon that has been termed "coupling" (Baylink et al., 1982).

As suggested above, in addition to direct influences (i.e. paracrine) osteoblasts and osteoclasts have on one another, bone tissue itself is also a rich source of growth factors which when made available to target cell types could contribute significantly to the regulation of bone formation and bone resorption (Canalis, 1983). These locally released factors may exert their influence by direct impact on individual cell types, or on coupling itself. It is also probable that while factors released from bone and paracrine factors may act independently, they may also act in concert on resident target cells of bone and surrounding connective tissues. In addition to local regulation, systemic factors, such as hormones, may have both direct and indirect actions on bone metabolism. These may be exerted on initial recruitment of cells, the differentiation of precursor cells, the functioning and expression of mature cells, or a combination of the above (Canalis et al., 1991; Canalis, 1993).
While it is of value to study the influence of various biological factors on individual or isolated cell types in vitro, we know that in vivo, such agents act concurrently on multiple cell types present in the bone remodelling microenvironment. Although studying the influence of cell by-products found in conditioned media on other target cells may yield interesting findings, this arrangement does not permit simultaneous cell “cross-talk”. Intercellular communication may have a profound influence on how cells regulate one another in addition to their own self-regulatory activities. Thus, an examination of the effects of biologically active agents on co-cultured osteoblasts and osteoclasts might generate information which more closely mimics that which occurs in vivo.

To further understand the nature of bone cell interaction, I developed a novel co-culture model system which permits simultaneous assessment of both osteogenic (osteoblast-like) cell and osteoclast cell activity (i.e. bone nodule formation and mineral resorption respectively). Thus, the objectives of this part of the thesis were: first, to determine whether bone forming stromal cells derived from marrow could influence mineral resorption by osteoclast cells; second, to determine if mineral resorbing osteoclast cells had any effect on bone nodule formation and other aspects of osteogenic function. Finally, to demonstrate the validity of the model, I also determined whether cells in co-culture would respond differently to pharmacological perturbation than cells in monoculture. To test this, a bisphosphonate, disodium-1-hydroxy-1-aminopropylidene-1,1-diphosphate (Pamidronate; APD), was selected as a pharmacological probe since it is a known inhibitor of osteoclastic resorption (Francis and Martodam, 1983; Kanis et al., 1995).
V.2. METHODS AND MATERIALS.

V.2.i. Osteogenic Culture Model and Conditions.

Osteogenic (bone nodule forming) cells were derived from bone marrow stromal cells obtained from the tibiae of 18-day-old embryonic chickens (Brampton Chick Hatchery, Brampton, Canada), using modifications to the method for fetal rat marrow-derived stromal cells described by Maniatopoulos and co-workers (1988) and the method for embryonic chick cells described by Kamalia and co-workers (1992). Briefly, the marrow was obtained by resecting the tibiae and flushing each marrow cavity with 5 ml of supplemented medium, which consisted of alpha-MEM (minimum essential medium) supplemented with 15% fetal calf serum, 10-8M dexamethasone, 1% \( \beta \)-glycerophosphate, and 15 \( \mu \)g/ml ascorbic acid. The chick bone marrow stromal cells were grown in 150 mm culture plates (Sarstedt, Newton, USA) at 37°C in 5% CO\(_2\) in air in the above medium. Nonadherent cells were removed after 24 hours at the first medium change. The medium was subsequently changed every 48 hours. The adherent cells were grown for a further six days, after which they were subcultured into 24-well plates (Sarstedt), as described below.

V.2.ii. Harvest of Osteoclast Cells.

(a) Laying Hens.

Putative osteoclast precursor cells were obtained using a modification of the technique described by Alvarez and co-workers (1991). Osteoclast precursor cells were obtained from the
marrow longbones of six month old white Leghorn laying hens maintained on calcium-deficient (<0.1%) diets (Ren's Feed and Supplies, Oakville, Canada) for a minimum of 28 days prior to sacrifice. Calcium- and magnesium-free phosphate buffered saline (PBS) was used to flush the marrow from tibiae and femora of the hens. Cells were filtered twice through 100 μm nylon filters (Shandon Scientific, Pittsburgh, PA) and then pelleted at 1500 rpm for 7 minutes at 4°C.

The pellet of cells was resuspended into 20 mls of PBS, and 5 ml aliquots were further diluted with 15 ml of PBS and placed in 50 ml conical centrifuge tubes. Subsequently, 15 mls of Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) was introduced below the cells. The Ficoll-Hypaque gradients were centrifuged at 1400 rpm for 45 minutes. Partially purified mononuclear cells were recovered from the interface of Ficoll-Paque gradients, and washed with PBS. The osteoclast cells were grown at 37°C in 5% CO₂ in air in a culture medium that consisted of alpha-MEM supplemented with 5% fetal calf serum (Gibco BRL, Grand Island, NY, USA), and 5% chicken serum (Gibco BRL). As putative osteoclast precursors have been demonstrated to fuse reliably between days 3 and 6 of culture (Greenfield et al. 1992), highly purified preparations of osteoclast cells were harvested after 4 days, after the removal of nonadherent cells.

Previous studies by Oursler and co-workers (1992) had demonstrated differences in chicken osteoclasts' responsiveness to exogenous oestrogen dependent on the age of chicken from which the cells were derived. To explore this issue further, we examined co-culture effects using osteoclast cells from chickens at several stages of maturity, including embryonic chickens and chick hatchlings.
(b) Embryonic Chickens.

Putative osteoclast cell precursors were obtained from the tibiae of 18 day-old embryonic chickens, in a similar manner as described for the laying hens. Partially purified mononuclear cells were recovered from the interface of Ficoll-Hypaque gradients. These primary cultures were grown at 37°C in 5% CO₂ in air in the osteoclast culture medium.

(c) Chicken Hatchlings.

Putative osteoclast cell precursors were obtained from the femora and tibiae of white Leghorn chicks that had been maintained on a calcium-deficient diet for a minimum of 28 days. The method of cell isolation used was as described above for the laying hens.

V.2.iii. Assessment of Osteoclast Purity.

Several criteria were used to confirm that osteoclast precursor cells had been isolated using the procedure described above (Alvarez et al., 1991). Specifically, isolated osteoclast cells had to demonstrate the presence of multiple nuclei, ability to resorb bone, presence of specialized membrane structures (including ruffled borders, clear zones, etc.), and high levels of tartrate resistant acid phosphatase (TRAP). Levels of TRAP were determined by histochemical staining by flooding prepared representative aliquots of cells with hexazotized pararosaniline azo-coupled dye and counting the number of positively stained cells (Anderson, 1984).

Cells derived using the isolation method described were determined both by Alvarez and co-workers (1991) and our laboratory (unpublished data) to fulfill the above criteria. However, to assess the homogeneity of the putative osteoclasts further, flow cytometric methods were used.
By exploiting the fluorescent nature of the TRAP histochemical stain product, the percentage of TRAP positive cells was quantified by determination at the wave length corresponding to the product colour. Similarly, multinuclearity was assessed by staining the cells with a fluorescent nuclear stain, DAPI, and quantification at the appropriate wave length. Viability was assessed by dye exclusion using 0.4% trypan blue (Gibco).

V.2.iv. **Substrates For Osteoclast Cell-Mediated Resorption.**

(a) **Mineral Thin Films.**

Calcium phosphate ceramic thin film coated quartz discs (Millenium Biologix, Kingston, Canada) were used for most experiments to quantify osteoclast cell-mediated mineral resorption. Mammalian osteoclast cells have been shown to behave similarly on these thin films as on other bone or dentine slices (Davies et al., 1993).

Osteoclast cells \((5 \times 10^5)\) were overlaid on top of each disc. Wells containing either marrow-derived stromal cells or osteoclast cells grown under identical experimental conditions, described below, served as controls.

(b) **Bone Slices.**

To confirm the osteoclastic nature of the isolated multinucleated cells, it was necessary to show that these avian cells could actually resorb bone as opposed to the more "simple" mineral film on the quartz discs. Accordingly, osteoclast-like cells \((5 \times 10^5)\) were also grown on bovine bone slices (kindly provided by J. N. M. Heersche, University of Toronto, Toronto, Canada).
Resorption of bone was assessed morphologically, ultrastructurally, and biochemically through measurement of calcium release.

V.2.v. Co-culture Model System.

The co-culture was carried out in 24-well plates as shown in Figure V.1. Stromal cells were plated (10,000 cells per well) on the bottoms of each well. These cells were allowed to continue to grow for a further 6 to 8 days, after which osteoclast cells were introduced into the co-culture system. Directly above the plate well bottoms, a metal mesh platform of 0.5 mm height was placed to support a calcium phosphate ceramic thin film coated quartz disc onto which 5 X 10^5 osteoclast cells were overlaid. Wells containing either bone forming or osteoclast cells grown under identical experimental conditions, described below, served as controls. The cultures were incubated at 37°C for 4 days after which the experiments were terminated. Unless otherwise noted, the cultures were grown in the medium used for the osteogenic cultures (pilot studies had previously demonstrated that osteoclast cells grew and functioned normally in such a medium).

The number of osteoclast cells used for experimentation was determined by titration from 5 x 10^4 to 1 x 10^6 cells per disc (Figure V.2). Maximal resorption occurred at 5 x 10^5 osteoclast cells per disc.

V.2.vi. Analysis of Osteogenesis.
The effects of the various experimental conditions on osteogenesis in the chick bone marrow-derived stromal cell (CBC) cultures were assessed by measuring parameters of bone cell activity and bone formation including alkaline phosphatase activity, calcium and phosphate accumulation (mineralization), and collagen synthesis (osteoid matrix production).

(a) Biochemical Measurements.

Individual CBC cultures were removed from the bottoms of 24-well plates by scraping and were then homogenized in 1 ml of a bicarbonate buffer (3 mM NaHCO₃ in 15.0 mM NaCl pH 7.4) using a Polytron homogenizer (Kinematica GMBH, Switzerland). The homogenate was transferred to polyethylene test tubes and centrifuged at 3180 x g for 10 minutes at 4°C. The supernatant fraction was assayed for soluble protein content, as well as alkaline phosphatase activity to assess osteoblastic differentiation and activity, as described in III.2. Mineralization was measured by estimating the amount of acid-extractable calcium and phosphate from the pellet, also described in III.2.

(b) Measurement of Total alpha-1 type-1 Collagen Content.

Collagen content was quantified using the same method as described in III.2.


After completion of each experiment, adherent osteoclast cells were removed from the film
surfaces by brief washing with 5% sodium hypochlorite in distilled water. The films were rinsed further in distilled water and then air-dried. Complete removal of cells and debris was assessed by light and electron microscopy. Three methods were used to quantify osteoclast cell mediated mineral resorption of the thin films, as described below.

(a) Computer-assisted Morphometry.

To quantify the surface area resorbed by osteoclast cells, air-dried discs were stained by the von Kossa method and subsequently analyzed morphometrically using a Bioquant image analyzer.

(b) Acid Hydrolysis of the Residual Ceramic Film Coating.

The remaining mineral film coating was quantified after being released by placing the disc in a 6N HCl solution for 24 hours. Residual calcium and phosphate were quantified as using the methods previously described above.

(c) Mineral Released into Culture Medium During Resorption.

Calcium and inorganic phosphate released into the culture medium during resorption were determined as a measure of osteoclast cell mediated resorption. Calcium was measured by atomic absorption spectrophotometry, and phosphate was determined using a colourimetric assay, as described above.
V.2.viii. **Microscopic Assessment of Resorption.**

Selected discs and bone slices, with and without cells, were processed for scanning electron microscopy. Some discs were also examined, after being stained by the von Kossa method, by light microscopy.

V.2.ix. **Co-culture Experimental Conditions.**

(a) **Co-culture Time Course.**

The effects of co-culture on osteogenic and osteoclast cells were examined over time by culturing those cells together for 2, 3 and 4 day periods. Outcome parameters were compared to those of respective mono-cultures.

(b) **Replacement of Osteogenic Cultures with Non-Bone Forming Cultures.**

To determine if only the presence of an osteogenic type cell would be able to modify osteoclast cell behaviour, an alternate cell line, LLC-PK1, was substituted as a control for the CBC cultures (courtesy of M. Z. Hui, Mt. Sinai Hospital, Toronto, Canada). This epithelial cell line expresses alkaline phosphatase as do bone cells, but is incapable of making bone.

(c) **Replacement of Osteoclast Cells with Non-Mineral / Bone Resorbing Cells.**

To assess whether osteoclast cells alone are able to modify the osteogenic activity of the
CBC cultures, the chick osteoclast cells were replaced with non-mineral/bone resorbing cells in co-culture. A fibroblastic cell line, R2 (courtesy of M. Z. Hui, Mt. Sinai Hospital, Toronto, Canada), was tested in these experiments.

(d) Effect of Osteoclast Cell Conditioned Medium on Osteogenic Cultures.

As indicated above, it is possible that cells grown in co-culture will produce different factors and/or respond differently than cells treated only with conditioned medium. In order to confirm this supposition, it was essential to incubate osteoclast cells or osteogenic cells in monoculture using conditioned medium from the respective cells. Conditioned medium was collected from osteoclast cell cultures between days 1 and 4 (i.e. after removal of non-adherent cells and prior to use of attached cells for co-culture experiments), and filter-sterilized before further use. Concentrations of 10% up to 50% of conditioned medium were added to fresh alpha-MEM medium, after which the medium was supplemented as previously described. The medium was changed after 48 hours. CBC cultures of varying ages were tested: 12, 21 and 56 day-old. Osteogenic parameters were assessed after 4 days.

(e) Effect of Osteogenic Cell Conditioned Medium on Osteoclast Cell Mediated Mineral Resorption.

Filter-sterilized medium from CBC cultures (grown over days 6 to 14) was added in concentrations of 10% up to 50% to alpha-MEM medium, and supplemented as described earlier. Osteoclast cell mediated resorption of mineral thin films was assessed after a culture period of 4 days.
(f) Sensitivity of Co-culture Model to Drug Mediated Perturbation.

In order for the co-culture model to be valid, it was necessary to show that it is not only sensitive to experimental perturbation but that the observed responses are measurably different in co-culture as compared to mono-culture. This was accomplished by the addition of a bisphosphonate, Pamidronate (APD), a known inhibitor of resorption mediated by osteoclast cells.

The effects of APD were examined in several experiments: (i) by its addition to the culture medium in concentrations of 0.01, 0.1, 1 and 10 µM (in all cases, osteoclasts were allowed to attach to the mineral film prior to the addition of APD to the medium); and (ii) by pretreatment of the thin film discs by soaking overnight in PBS containing APD in the concentrations indicated above. Appropriate control experiments were conducted in parallel.

V.2.x. Statistical Analysis.

Values for the various parameters were used to calculate a mean and standard error for each group (4 to 6 cultures per experimental group, with each experiment being performed a minimum of three times) and the differences between means were evaluated using Student's t-test for comparisons between specific experimental groups and control. Significance was assigned at the $P < 0.05$ level. All values in tables and figures represent the mean ± the standard error of the mean. All values have been presented as a percentage of the control value for the respective experiment (control = 100 ± SEM) to facilitate comparisons between different experiments.
V.3. RESULTS.

V.3.i. Osteoclast Cell Culture Purity.

The percentage of osteoclast cells in culture was determined by staining for tartrate-resistant acid phosphatase (TRAP), and counting for the percentage of TRAP positive cells using flow cytometry. On day 4, the percentage of cells that were determined to be TRAP positive was 93.4 ± 3.2. Viability, as assessed by trypan blue stain exclusion, on day 4 was found to be 87.4% ± 8.2%. Multinucularity and cell morphology was assessed by light microscopy. Flow cytometry using DAPI and analyzing fluorescence to confirm the multinucularity of these cells (82.4% ± 11.4%) was also used.


(a) Effects of Co-culture as a Function of Time.

The bone cell co-culture was examined over 2, 3 and 4 day incubation periods. A significant reduction (up to 3-fold; P<0.05) in osteoclast cell mediated resorption (as determined by area of resorption of mineral thin film coating) was observed for all incubation periods in co-cultures, in comparison to osteoclast cell mono-cultures (Figure V.3). For both, resorption of mineral thin films increased over time, with near complete resorption of the disc coating by day 4 in osteoclast cell mono-cultures (Figure V.5). Data obtained with computer morphometry (Figure V.5), acid hydrolysis (data not shown), and released mineral in culture supernatant (data not shown) all yielded complementary findings. With respect to osteogenesis, significant reductions (up to 5-fold, P<0.05) in the measured parameters of osteogenesis (alkaline phosphatase, calcium
and inorganic phosphate accumulation, and type I collagen formation) were observed in CBCs grown under co-culture conditions, in contrast to those in CBC mono-cultures ($P<0.05$) (Table V.1). This trend was consistent for all culture time periods. Notably, in parallel experiments carried out with bone slices instead of mineral thin films, analogous results were obtained for both osteoclast and osteogenic cells (Table V.2).

Scanning electron micrographs revealed areas of resorption of mineral thin film on the quartz disc surfaces which were reminiscent of resorption lacunae seen on the surfaces of bone slices (Figure V.4.a). Prior to the removal, cells were observed to be in close juxtaposition to the areas of resorption, with pseudopod-like structures at the advancing mineral resorption fronts (Figure V.4.b).

(b) Replacement of Bone-derived Cells with LLC-PK1 and R2 Cells.

When the alkaline phosphatase positive epithelial cell line, LLC-PK1, was substituted for CBC cultures, no decreases in osteoclast cell mediated mineral resorption in co-culture were observed (data not shown). Similarly, R2 cells, when grown on the thin films, neither resorbed the mineral layer nor had any effects on the subjacent CBC cultures (data not shown).

(c) Effect of Osteoclast Cell Conditioned Medium on Osteogenic Cultures.

Conditioned medium from laying hen osteoclast cell cultures was used to study the influence of osteoclast products, released in isolation (i.e. without the influence or presence of other bone cells), on osteogenic activity of CBC cultures. No statistically significant differences
were seen at any tested concentration for alkaline phosphatase activity, bone nodule calcium accumulation and type I collagen formation for 12 day old cultures (Figure V.6 a, b, c). However, significant reductions in all osteogenic parameters were observed on older (i.e. 21 and 56 day-old) cultures, with the most profound suppression noted in the 21 day-old osteogenic CBC cultures (Figure V.6 a, b, c).

(d) Effect of Osteogenic Conditioned Medium on Osteoclast Mineral Resorption.

Conditioned medium from CBC cultures, in concentrations of up to 50%, was used to determine if products generated from isolated osteogenic mono-cultures could influence osteoclast cell activity in the same manner as in co-culture. There were no statistically significant differences in osteoclast cell mediated resorption of mineral thin films at any concentration of added CBC conditioned medium (Figure V.7).

(e) Drug-mediated Perturbation of the Co-culture Model System.

When APD was added to the culture medium, there were no significant decreases in osteoclast cell activity in either co-culture or in mono-culture (Figure V.8). However, pretreatment of the mineral thin films caused a complete inhibition of osteoclast cell mediated mineral resorption at concentrations of APD of 0.1, 1 and 10 μM (Figure V.8).

In comparison to controls (i.e. no thin film pre-treatment), a significant decrease in osteogenic activity was observed in co-culture using pre-treated thin films. In contrast, when APD was added to the culture medium, no decreases were observed in co-culture (Figure V.9 a, b, c).
(f) Relationship Between Cell Donor Age and Co-culture Effects on Mineral Resorption or Bone Formation.

(i) Embryonic chick-derived osteoclast cells. Osteoclast cells were derived from the tibiae of 18-day old embryonic chickens. The experiment was carried out for a four day period. In co-culture over that observed in osteoclast cell mono-cultures, a significant increase in osteoclast cell mediated resorption was found (Figure V.10). This is also in contrast to the findings observed in co-culture using laying hen-derived osteoclast cells. With respect to osteogenesis, the findings were mixed. No increase in alkaline phosphatase activity was noted, however, there was an increase in mineralization of the bone nodules.

(ii) Chick hatchling-derived osteoclast cells. Osteoclast cells were derived from tibiae and femora of chick hatchlings that had been placed on a low calcium diet for a minimum of 28 days. A significant decrease in osteoclast cell mediated mineral resorption was observed in co-culture, while co-culturing had no effects at all on the measured osteogenic parameters (Figure V.11).
V.4. DISCUSSION.

The data revealed that osteogenic cells and osteoclast cells behave differently in isolation than in co-culture. Communication, possibly via the release of soluble factors, exists between the cell types and may regulate their respective activities. Importantly, identical experiments carried out using conditioned medium, failed to reproduce the findings in co-culture depending on the age of the cells used. The stage of development of the animal (and possibly diet since the laying hens and chick hatchlings were on a low calcium diet while the embryonic donors were not), may play roles in determining the outcomes of the cell communication experiments.

At present, the study of bone cell interactions has been largely restricted to examining the effects of osteoblastic cells or their by-products on osteoclast cell mediated bone resorption. The majority of these studies were conducted using by-products, released from bone derived osteoblastic cells into growth medium (Greenfield et al., 1992). Other studies have utilized by-products from osteoblast-like transformed cell lines, which express many osteoblastic features with the exception of bone formation (Yamashita et al., 1990; Kukita et al., 1993; Kuroki et al., 1994). Although the use of metabolic by-products provides interesting and highly useful information regarding some aspects of bone cell interactions, this approach does not permit simultaneous live osteoblast and osteoclast communication (i.e. real-time “cross-talk”). In this regard, different osteoblast or osteoclast by-products may be synthesized and released in the absence of the other cell type, as compared to factors produced when the cells can communicate in a simultaneous fashion. Thus intercellular communication is not necessarily mimicked under mono-culture conditions. Accordingly, it may be surmised that the products produced by isolated cells, and concentrated in conditioned media, may be quite different from products produced by cells responding to factors released by co-regulatory cell types. In addition, short-lived paracrine
factors (such as prostaglandins) may be absent in conditioned medium by the time it is used for experimentation, but would be present in a co-cultured cell environment. Hence, it may be expected that cellular behaviour in co-culture would be quite different than that observed when using only conditioned media.

Other types of in vitro co-culture model systems have been employed in the study of bone cell interactions. These have included: co-cultures of osteoblastic and osteoclast cells grown in intimate contact (usually layered) (Bonucci et al., 1992; Morita et al., 1992); and rat and other animal long-bone remodelling systems (Lowik et al., 1994). Although such models do permit intimate contact and real-time communication between osteoclast and osteogenic cells, analysis of the end-points may be confounded by the very nature of the mixed cell milieu. In thus regard it would be difficult to separate such cells at the end of culture and this might not permit unequivocal assessment of either osteogenic or osteoclast activities on an individual basis.

Alternatively, with the model described here, it is possible to co-culture osteogenic and osteoclast cells (albeit without cell-cell contact) and because the components can be disassembled at the end of the experiment, osteogenesis and mineral resorption can then be measured independently. Importantly, the results obtained using mineral thin film coated discs and bone slices yielded comparable findings. Osteoclast resorption of mineral thin films was found to be analogous to resorption of bone slices. For example, the osteoclast cells morphologically and biologically behave in a similar manner on discs as on bone, i.e. produce resorption areas and form tracts within the mineral substrate. Non-osteoclast cell types (epithelial, fibroblastic) were unable to resorb the mineral substrate or bone. Furthermore, despite the simple compositional nature of thin films, as compared to bone, the functional relationship between osteoclast and osteogenic cells in co-culture was demonstrated irrespective of whether osteoclast cells were grown on bone slices or the thin films. This suggests that, at least under the conditions used here, organic products
released from bone during osteoclast resorption may play a less significant role in regulating bone cell function than do soluble products directly released from bone cells themselves.

Although co-culture appeared to have profound effects on the cells, it is conceivable that similar results could have been obtained if conditioned medium had been used. If so, co-culture would not be necessary and medium filtrates could be substituted. This hypothesis was tested by examining the effect of culture medium filtrates from osteogenic cells and osteoclast cells on each other's respective activities. The results indicated that medium filtrates from osteogenic cells, in concentrations of up to 50% (v/v), had no effect on osteoclast cell activity. Similarly, osteoclast cell medium filtrates had no effect on 12 day-old CBC cultures. Therefore, products generated without the influence of other bone cell types did not appear to have similar effects on bone cell activity as in co-culture. This suggests that different factors are produced by cells grown under co-culture conditions than when grown in mono-culture. Alternatively, osteogenic activity in older cultures was significantly inhibited by osteoclast cell conditioned medium. Although this is consistent from studies with isolated cell types (Galvin et al., 1994), it raises the issue pertaining to the maturational stages of cells used in culture. Various studies have shown that the responsiveness of a cell, for example to parathyroid hormone, is dependent on its stage of maturation (Rouleau et al., 1986, 1990). Perhaps older CBC cultures responded differently than younger cultures because the osteogenic potential of older cultures was less than younger cultures (i.e. fewer osteoblast and progenitor cells in older cultures) (Stein et al., 1989; Heersche et al., 1992).

Similar to that observed with osteogenic cultures at different stages of development, the findings observed in co-culture varied depending on the source of the osteoclast cells. For example, the findings obtained using embryonic chick derived osteoclast cells paralleled those found in other co-culture systems (Greenfield et al., 1992) in that increases in osteoclast cell
mediated resorption occurred in the presence of osteoblast-derived products. On the other hand, mineral resorption produced by hatchling-derived osteoclast cells was similar to that produced by osteoclast cells derived from laying hens. As with osteoblasts, the notion that osteoclasts derived from donors of different ages may themselves be different is not new, and indeed others (Oursler et al., 1992) have already shown that osteoclast oestrogen receptor levels and oestrogen responsiveness varied according to the age or diet of the donor chicken as suggested here.

Although it was expected that osteoclasts and osteoblasts might regulate one another’s activities in co-culture (or even when conditioned media are used), it must be recognized that cells from disparate tissues or origins may also regulate one another’s activities. For example, epithelial cells are known to induce ectomesenchymal cell differentiation into osteoblasts (Hall, 1978; Hall et al., 1983). To investigate this further, one of the two cell populations was replaced by either an epithelial (LLC-PK1) or fibroblastic (R2) cell line. Notably, when either LLC-PK1 or R2 cells were substituted for osteogenic or osteoclast cells respectively, no effects on bone cell activity (i.e. either bone nodule formation or mineral resorption) were observed. While multiple cell types need to be tested before it can be stated conclusively that the co-culture effects observed here were bone cell specific, these findings do suggest that only certain types of cells can influence the activity of bone cells and that there is a strong inter-relationship between the two main bone cell populations studied here.

As noted above, in order for this co-culture model system to be valid, it was necessary to demonstrate that the two cell types used influenced one another’s activities under control co-culture conditions. However, it was also essential to show that cell responses to biological or pharmacological perturbation would be different in co-culture than mono-culture. For example, if a biological agent induces osteoblasts (or osteoclasts) to respond in one manner under mono-culture conditions and in a completely different manner under co-culture conditions, this would
then underscore the necessity for testing the actions of that agent under co-culture conditions (as well as under mono-culture conditions). We chose to use an agent with known effects on osteoclast mediated resorption, the bisphosphonate APD. While bisphosphonates are known inhibitors of osteoclast resorptive activity, and used in treatment of Paget's and hypercalcemia of malignancy, their exact mechanism of action is not fully understood (Kanis et al., 1995). The effects of APD on co-cultured cells were tested in two manners, either by adding the drug directly into the culture medium or by pretreatment of the mineral thin films with various concentrations of APD. When osteoclast cells were allowed to attach to the thin films, and subsequently incubated in medium containing various concentrations of APD, no effect was observed on the activity of either cell population in co-culture. This implies that APD had no effect on the behaviour of mature osteoclast cells that had already attached to their mineral substrate, and therefore no further effects were observed on the osteogenic cells. However, when the mineral thin films were pretreated with APD, osteoclast cell mediated resorption was completely inhibited, and further there was a concomitant and greater decrease in osteogenic activity than observed in control co-cultures. This might suggest the presence of a homeostatic relationship between the formative and resorptive processes in vitro.

Finally, another important consideration as to the cause of osteogenic suppression in co-culture was the effect of increased concentrations of calcium released from the disks into culture media. Recent studies have demonstrated that high concentrations of extracellular calcium had pronounced effects on DNA synthesis and alkaline phosphatase activity of osteoblastic MC3T3-E1 cells, and that these effects were stimulatory and possibly mediated through monocytes (Kanatani et al., 1991; Sugimoto et al., 1993). However, even if increased levels of calcium were generated in co-culture, inhibition rather than stimulation of osteogenic activity was observed. Nonetheless, it must be recognized that elevated medium calcium might have inhibitory rather than stimulatory effects in co-culture. However, an argument against this notion can be found in the APD
experiments using discs pre-treated with APD. In these experiments resorption by osteoclast cells was inhibited (i.e. no extra calcium or phosphate was released into culture media), and yet osteogenic activity was still down-regulated. With regard to resorption, it has been observed that high levels of extracellular calcium inhibit osteoclast attachment to bone slices, while no effects were noted on ongoing osteoclast resorption (Hall, 1994).

In conclusion, the findings demonstrate that communication between osteogenic and osteoclast cells occurs in co-culture. In this regard, osteogenic cells may modify osteoclast activity by the release of paracrine factors, the same may hold true for the effects of osteoclast cells on osteogenic cell activity. However, factors that could be released from bone during resorption, such as TGF-β, are not released in the co-culture model using mineral thin films as the osteoclast cell substrate. While this may be considered a limitation in its applicability to what occurs in vivo, this can also be used to advantage as specified amounts of bone factors may be selectively added back to the co-culture medium or onto the discs to test their effects on bone metabolism. This could also be used to eliminate confounding effects, should they occur, from the release, during resorption, of multiple factors from bone. The co-culture model developed for this study permits communication between functioning osteogenic and osteoclast cells, as well as independent analysis of both bone nodule formation and mineral resorption concurrently. Thus, using the co-culture model, the effects of extracts derived from P. gingivalis on bone cell metabolism could now be quantified. (Chapter VI).
Figure 1. Schematic Representation of the Co-culture System.

Osteogenic embryonic chick bone marrow stromal cells were plated on bottoms of 24-well tissue culture plates. Osteoclast cells, isolated from the tibiae and femora of chickens (either laying hen, hatchling or embryonic), were seeded onto mineral thin film coated quartz discs, suspended above the osteogenic cells. After adequate time or attachment of osteoclast cells, the wells were filled with culture medium (2 ml total volume).
Figure 2. Resorption of Mineral Thin Films as a Function of Osteoclast Cell Number.

Osteoclast cells, derived from laying hens on low calcium diets, were placed on mineral thin films. After a 4 day incubation period, resorption was assessed, as described earlier. Resorption increased in an almost linear fashion, with maximal activity at 4 to \(5 \times 10^5\) cells per disc. Each point represents the mean ± SEM for 4 cultures.
Figure 3. **Light Microscopic Appearance of Osteoclast Cell Mediated Mineral Resorption in Mono- and Co-culture Over 2, 3 and 4 day Incubation Periods.**

Upon termination of each experiment, remaining adherent osteoclast cells were removed from the quartz disc surface by brief washing with 5% sodium hypochlorite. The residual mineral film on the surface of the quartz disc surface was subsequently stained by the von Kossa method. Transparent, i.e. resorbed, areas were easily quantified using computer-assisted morphometry. Resorption was found to be suppressed in co-culture.
Osteoclast
day 2
day 3
day 4

Co-culture
day 2
day 3
day 4
Figure 4. Scanning Electron Photomicrographs of Osteoclast Cell Mediated Resorption of Thin Films.

(a) An area of mineral resorption with osteoclast cell extending out pseudopod-like structures in the formation of resorption tracts. Magnification, x3000.

(b) Morphologic appearance of a discrete area of resorption of the mineral thin film after removal of osteoclast cells. Magnification, x3750.
Osteoclast cells from laying hens \((4 \times 10^5)\) were seeded onto mineral thin films and grown in the presence or absence of osteogenic cells \((1 \times 10^4)\) which were cultured on the bottoms of plate wells. For all culture incubation periods, osteoclast cell mediated resorption of thin films was significantly inhibited in co-culture \((P<0.05)\)\((*)\). Mineral resorption was assessed morphometrically. Increased resorption was observed with longer incubation times. Each point represents the mean ± SEM for 4 cultures. All experiments were repeated a minimum of three times.
Bone marrow-derived stromal cells from 18-day old chick embryos were plated on the bottoms of 24-well tissue culture plates (1×10⁴ cells/well). In separate experiments, after 12, 21 or 56 days of growth, conditioned medium from 4-day old cultures of laying hen osteoclast cells was then added, in varying proportions (10%-50%), to the osteogenic cell culture medium, and the cells were incubated for a further 4 days.

(a) Calcium accumulation in bone nodules. No effects on mineralization were observed in 12-days old osteogenic cultures exposed to up to 50% (v/v) of osteoclast cell conditioned medium. However, significant decreases relative to control were found in 21 and 56 days-old cultures (P<0.05).

(b) Alkaline phosphatase activity. Alkaline phosphatase activity was significantly reduced, relative to control, in 21 and 56 days-old cultures (P<0.05). No effects were observed in 12 day-old cultures.

(c) Type I collagen. As shown for calcium accumulation and alkaline phosphatase activity, type I collagen production was not affected by the addition of osteoclast cell conditioned medium in 12 days-old cultures, but significantly reduced relative to control in 21 and 56 days-old cultures (P<0.05).

Each point represents the mean ± SEM for 4 cultures. All experiments were repeated a minimum of three times.
Figure 7. Effect of Osteogenic Cell Conditioned Medium on Osteoclast Cell Mediated Resorption of Thin Films.

Osteoclast cells from laying hens (4×10^5) were seeded onto mineral thin films and grown in the presence of increasing concentrations of conditioned medium derived from osteogenic cells (10% to 50%, and 0% controls; v/v). Experimentation was terminated after 4 days and osteoclast cell mediated mineral resorption was quantified. No effects were observed on osteoclast cell mediated mineral thin film resorption when the culture medium was supplemented with osteogenic cell conditioned medium, up to 50% (v/v). Each point represents the mean ± SEM for 8 cultures. All experiments were repeated three times.
Osteogenic Conditioned Medium

Mineral Resorption (% Control) vs. % Osteogenic Conditioned Medium
Osteoclast cells from laying hens (4x10^5) were seeded onto mineral thin films and grown in the presence or absence of osteogenic cells (1x10^4) which were cultured on the bottoms of plate wells. APD (0.01 - 10 μM, and 0 μM controls) was added either to the culture medium, or used to pretreat the mineral thin films for 12 hours prior to osteoclast attachment. The co-cultures were subsequently incubated for 4 days, after which the effects on resorption were assessed. The addition of APD to the culture medium had no effect on the resorptive activities of osteoclast cells that had pre-attached to mineral thin films. When thin films were pre-treated with APD (0.1, 1, 10 μM), resorption was completely inhibited (P<0.05). Each point represents the mean ± SEM for 8 cultures. All experiments were repeated three times.
Figure 9: Effect of APD on Osteogenic Activity of Chick Bone Marrow Stromal Cell Cultures.

Under the same conditions as described for figure 8, the effects of APD treatment on bone formation in co-culture was assessed.

(a) Calcium accumulation in bone nodules. No effects were observed on mineralization when APD was added to the culture medium. Significant decreases ($P<0.05$) relative to control were found when thin films were pre-treated with APD, when osteoclasts were present.

(b) Alkaline phosphatase activity. Alkaline phosphatase activity was significantly reduced ($P<0.05$) in co-culture when APD pre-treated thin film were used.

(c) Type I collagen. Type I collagen production was not affected by the addition of APD to the culture medium, but was reduced significantly when APD pre-treated thin films were used.

Each point represents the mean ± SEM for 8 cultures. All experiments were repeated a minimum of three times.
Figure 10. Use of Embryonic Chick Osteoclast Cells in Co-culture.

Osteoclast cells derived from 18 day-old embryonic chickens (4x10^5) were seeded onto mineral thin films and grown in the presence or absence of osteogenic cells (1x10^4) which were cultured on the bottoms of plate wells. Assessment of osteogenesis after a four day incubation period revealed no differences in the alkaline phosphatase activities of osteogenic cultures in co-culture when compared with those obtained in mono-culture. However, a significant increase in mineralization (calcium accumulation) and in mineral thin film resorption by embryonic osteoclast cells were found in co-culture (P<0.05). Each point represents the mean ± SEM for 4 cultures. All experiments were repeated three times.
The graph shows the comparison of "mono"-culture and co-culture in terms of % control for alkaline phosphatase, calcium, and mineral resorption.

- Alkaline phosphatase: No significant difference between "mono"-culture and co-culture.
- Calcium: Significant increase in the co-culture condition.
- Mineral resorption: Large increase in the co-culture condition compared to "mono"-culture.
Figure 11. Use of Chick Hatchling Osteoclast Cells in Co-culture.

Osteoclast cells derived from chick hatchlings (4x10^5) were seeded onto mineral thin films and grown in the presence or absence of osteogenic cells (1x10^4) which were cultured on the bottoms of plate wells. Assessment of osteogenesis after a four day incubation period, revealed no significant differences in the alkaline phosphatase activities or calcium accumulation of osteogenic cells in co-culture, in comparison to the values obtained in osteogenic cells in mono-cultures. In contrast, significant decreases in mineral thin film resorption by hatchling osteoclast cells were found in co-culture (P<0.05). Each point represents the mean ± SEM for 4 cultures. All experiments were repeated three times.


% Control

ALKALINE PHOSPHATASE
CALCIUM
MINERAL RESORPTION

"MONO"-CULTURE
CO-CULTURE

150
100
50
0

*
<table>
<thead>
<tr>
<th>Day</th>
<th>cc</th>
<th>mono</th>
<th>Alkaline Phosphatase* (µmol pNp/hr/µg protein)</th>
<th>Calcium* (x10 µmol Ca++/ µg protein)</th>
<th>Type I Collagen* (area units)</th>
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<td>2</td>
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<td>1.85±0.38*</td>
<td>0.75±0.09*</td>
<td>2.45±0.23*</td>
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<td>4.66±0.23</td>
<td>1.31±0.17</td>
<td>4.65±0.34</td>
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<td></td>
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<td>3.93±0.51*</td>
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<td>7.56±0.63</td>
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<td>4</td>
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<td></td>
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<td>2.74±0.21*</td>
<td>5.36±0.41*</td>
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<td>4.97±0.28</td>
<td>4.12±0.25</td>
<td>8.86±0.96</td>
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</table>

*Mean ± SEM for 4 cultures. Each experiment repeated a minimum of four times. cc, co-culture. mono, mono-culture. pNp, paranitrophenol. *, significantly lower than the value for control (P<0.05).
Table 2. Evaluation of Various Parameters in Osteogenic Cultures and of Osteoclast Cell Mediated Mineral Resorption in Mono- and Co-culture: Use of Bovine Bone Slices.

<table>
<thead>
<tr>
<th>Day 4</th>
<th>Alkaline Phosphatase* (µmol pNp/hr/µg protein)</th>
<th>Calcium* (x10 µmol Ca++/µg protein)</th>
<th>Type I Collagen* (area units)</th>
<th>Mineral Resorption* (µmol Ca++/ml medium)</th>
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</thead>
<tbody>
<tr>
<td>cc</td>
<td>0.41±0.08*</td>
<td>1.42±0.11*</td>
<td>3.04±0.31*</td>
<td>141.0±4.8*</td>
</tr>
<tr>
<td>mono</td>
<td>1.46±0.21</td>
<td>2.96±0.32</td>
<td>5.68±0.63</td>
<td>206.1±7.40</td>
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</tbody>
</table>

*Mean ± SEM for 4 cultures. Each experiment repeated a minimum of four times. cc, co-culture. mono, mono-culture. pNp, paranitrophenol. *, significantly lower than the value for the control (P<0.05).
CHAPTER VI

MODULATION OF CO-CULTURED OSTEOGENIC AND OSTEOCLAST CELLULAR ACTIVITIES BY SONICATED EXTRACTS FROM PORPHYROMONAS GINGIVALIS 2561.
VI.1. INTRODUCTION.

I had previously shown that extracts derived from *P. gingivalis* and other periodontopathogenic bacteria can inhibit osteogenesis *in vitro*. Others have shown that specific products of *P. gingivalis* have the capacity to stimulate bone resorption *in vitro* (Sveen and Skaug, 1986; Born-van Noorloos et al., 1989). *P. gingivalis*, and other periodontopathogenic bacteria, are believed to evoke bone destruction in periodontal diseases in part through the direct action of their products and by-products on host tissue and cells (Page and Schroeder, 1981).

Since the cellular activities of osteoblasts and osteoclasts have been demonstrated to be influenced by one another (Takahashi et al., 1988; Udagawa et al., 1989; Hattersley et al., 1991; Takahashi et al., 1991; Weir et al., 1993; see V.1 and V.5), products from *P. gingivalis* may play a role in altering intercellular communication in bone that ultimately results in alveolar bone loss. In addition, the behaviour of bacterial extracts may be different in a heterogeneous bone cell population than in a homogeneous one, and most probably would generate information which more closely mimics that which occurs *in vivo*. In this chapter I will describe the effects of sonicated products from *P. gingivalis* 2561 on the cellular activities of both osteogenic and osteoclast cells in bone cell co-culture. The co-culture model system that I have previously described (chapter V) was used.
VI.2. METHODS AND MATERIALS.

VI.2.i. Bacterial Culture Conditions.

Bacterial culture conditions used were the same as those described previously for *P. gingivalis* strain 2561 in III.2. Bacterial extracts were prepared by sonication of bacterial cells at maximum power using a Biosonik IV sonicator (Bronwill Co., Rochester, NY), removal of insoluble debris by centrifugation at 10,000 x g for 30 minutes at 4°C, and subsequent freeze-drying of the filter-sterilized supernatant.

VI.2.ii. In vitro Co-Culture Model System.

(a). Osteogenic Culture Model and Conditions.

Osteogenic (bone nodule forming) cells were derived from the long bones of 18 day-old embryonic chickens. The technique and conditions used was as described in V.2.

(b). Harvest of Osteoclast Cells.

Putative osteoclast precursor cells were obtained from Leghorn laying hens that had been placed on a low calcium diet for a minimum of 28 days. The technique and conditions used was as described in V.2.
VI.2.iii. **Substrate For Osteoclast Cell Mediated Resorption.**

Calcium phosphate ceramic thin film coated quartz discs (Millenium Biologix, Kingston, Canada) were used to quantify osteoclast cell-mediated mineral resorption. Osteoclast cells (4 X 10^5) were overlaid on top of each disc. Wells containing either marrow-derived stromal cells or osteoclast cells grown under identical experimental conditions, described below, served as controls.

VI.2.iv. **Co-culture Model System.**

The co-culture was carried out in 24-well plates, under the conditions as described in V.2.

VI.2.v. **Analysis of Osteogenesis.**

The effects of the various experimental conditions on osteogenesis in the CBC cultures were assessed by measuring parameters of bone cell activity and bone formation including alkaline phosphatase activity, calcium and phosphate accumulation (mineralization), and collagen synthesis (osteoid matrix production). Methods for analysis were as described in V.2.

VI.2.vi. **Analysis of Mineral Resorption.**

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After completion of each experiment, adherent osteoclast cells were removed and the film surfaces were washed then air-dried. Complete removal of cells and debris was assessed by light and electron microscopy. Two methods were used to quantify osteoclast-mediated mineral resorption of the thin films, computer-assisted morphometry (to quantify the surface area of mineral thin film resorbed) and atomic absorption (to measure mineral released in the culture medium), as detailed in V.2.


(a) Effect of P. gingivalis Sonicates.

The effects of whole sonicated extracts from P. gingivalis on the processes of bone formation and mineral resorption in co-culture was examined over a 2 and 4 day culture period. Concentrations of 2.0 ng to 2.0 μg (dry weight) of extract per ml culture medium were tested. Outcome parameters in co-culture were also compared to those of respective mono-cultures.

(b) Effect of the Addition of Indomethacin.

As previous findings suggested that P.gingivalis extract-induced inhibition of osteogenesis was likely mediated by prostaglandins, this was also tested here. An inhibitor of prostaglandin synthesis, indomethacin, was added at a concentration of 1 μg/ml to the culture medium. Concentrations of P. gingivalis extract, culture periods and controls were as described above.

VI.2.viii. Statistical Analysis.
Values for the various parameters were used to calculate a mean and standard error for each group (6 to 8 cultures per experimental group, with each experiment being performed a minimum of three times) and the differences between means were evaluated using Student's t-test for comparisons between specific experimental groups and control. Significance was assigned at the \( P < 0.05 \) level. Where multiple comparisons were made, ANOVA was used as indicated in the text. All values in tables and figures represent the mean ± the standard error of the mean. All values have been presented as a percentage of the control value for the respective experiment (control = 100 ± SEM) to facilitate comparisons between different experiments.
VI.3. RESULTS.

VI.3.i. Effects of *P. gingivalis* Extracts on Osteogenic Cellular Activity.

The bacterial sonicated extracts induced significant inhibitory effects (*P* < 0.05) on the measured parameters of osteogenesis in isolated embryonic chick stromal cell mono-cultures. Alkaline phosphatase activity was reduced by as much as 45% over control (Figure VI.1a). In co-culture, significant reductions (up to 25% lower vs. controls; *P* < 0.05) in alkaline phosphatase activity of the osteogenic cultures were seen at all tested concentrations of *P. gingivalis* extract (Figure VI.1a). However, these reductions were markedly less profound than those observed in the CBC mono-cultures at most concentrations of *P. gingivalis* extract (*P* < 0.05, ANOVA).

Mineralization, as assessed by calcium and inorganic phosphate accumulation, was decreased up to 65% (*P* < 0.05) in mono-cultures of osteogenic cells when *P. gingivalis* extracts were added to the culture medium (Figures VI.1b and VI.1c). In contrast, there were no *P. gingivalis* induced decreases in calcium or inorganic phosphate in bone nodules formed in co-culture (Figure VI.1b and VI.1c).

Total alpha-I-type-I collagen content was reduced significantly (up to 45%; *P* < 0.05) in CBC mono-cultures grown in the presence of *P. gingivalis* sonicated extracts in concentrations of 0.02 μg/ml and above (Figure VI.1d). Small *P. gingivalis*-induced reductions in total alpha I type I collagen content were observed in comparable co-cultures (*P* < 0.05), but the magnitude of suppression was not as substantial as observed in mono-cultures (*P* < 0.05, ANOVA).
VI.3.ii. **Effects of *P. gingivalis* Extracts on Osteoclast Cell Mediated Mineral Resorption.**

*P. gingivalis* extracts had no effect on osteoclast cell mediated mineral resorption under monoculture conditions (Figures VI.2a and VI.2b). However, in co-culture significant increases in the resorptive capacity of the cells were observed (up to 70%; *P* < 0.05) (Figures VI.2a and VI.2b) when bacterial sonicate was present at all concentrations.

VI.3.iii. **Influence of Indomethacin.**

As shown earlier, indomethacin (1 μg/ml) blocked *P. gingivalis* -mediated inhibition of osteogenesis in the CPO model (see IV.3). Similarly, osteogenic inhibition by *P. gingivalis* sonicated extracts of CBC mono-cultures was also blocked by indomethacin. No significant bacterial-mediated decreases in alkaline phosphatase activity were observed in CBC mono-cultures (Figure VI.3). Similar results were obtained for the other measured parameters of osteogenesis (data not shown). However, under co-culture conditions, indomethacin was unable to alter the inhibitory effects of the *P. gingivalis* extracts on the various parameters of osteogenesis, which remained significantly suppressed over control values (*P* < 0.05; Figure VI.3).

The addition of indomethacin did not modify the effects of *P. gingivalis* extract induced up-regulation of osteoclast cell mediated resorption in mono- or in co-culture (data not shown).
VI.4. DISCUSSION.

I have shown that sonicated extracts derived from *P. gingivalis* 2561 inhibited osteogenic activity in bone nodule forming CBC cultures, which is in agreement with the trends found in the CPO model (Chapter III and IV). Moreover, similar results were also reported in earlier investigations using a variety of bone cell and whole bone model systems (Multanen et al., 1986; Meghji et al., 1992). In addition, the results of these experiments also show that although *P. gingivalis* extracts definitely suppress osteogenesis, their suppressive effects are reduced in the presence of viable osteoclasts. *P. gingivalis* extracts may induce osteoclasts to produce a factor (or factors) which subsequently attenuate *P. gingivalis* -mediated inhibition of osteogenesis (i.e. may even stimulate osteogenesis if *P. gingivalis* products were not also present). This might imply the presence of a homeostatic relationship between the two cell types as is suggested to occur in vivo and might imply the production of coupling factors (Baylink, 1982).

The data indicate that *P. gingivalis* extracts had no direct influence on osteoclast cell activity as measured by mineral resorption, in the absence of bone forming cells. In contrast, in co-culture, *P. gingivalis* extracts appeared to up-regulate resorption. This would indicate that *P. gingivalis* effects on osteoclasts are mediated via osteoblastic cells. This finding is consistent with previous data showing that osteoclast responses to a variety of biological agonists are dependent upon the presence of viable osteoblasts. For example, in this study, osteoblast and osteoclast interaction may be dependent on at least two mechanisms. First, *P. gingivalis* extracts may induce osteoblasts to produce factors which stimulate osteoclast activity. Second, inasmuch as osteoblasts, under normal co-culture conditions, appear to down-regulate osteoclast activity as compared to mono-culture, *P. gingivalis* may suppress osteoblastic production of factors which would otherwise inhibit osteoclast activity in co-culture. Indomethacin effectively abrogates *P.
gingivalis' inhibitory effects on osteoblastic cells suggesting a prostaglandin-mediated phenomenon as shown by others (Meghji et al., 1992). However, indomethacin in itself had no effect on osteoclast activity in either mono- or co-culture, and so it is probable that P. gingivalis might induce the production of a factor or factors which up-regulate osteoclast activity rather than suppressing the production of osteoclast inhibitory factors. In fact, P. gingivalis fimbriae have demonstrated up regulation production of some cytokines by monocytes, fibroblasts and epithelial cells (Hanazawa et al., 1985). Further in regard to the above, it seems clear that while P. gingivalis effects on osteoblasts may be mediated by prostaglandins, this is not the case for osteoclasts.

The exact factors contained within the P. gingivalis extracts which mediate the above-described effects are not known. One major component might be lipopolysaccharide (Millar et al., 1986; Branmati et al., 1989; Sisme-Durrant and Hopps, 1991), surface associated material (Meghji et al., 1992) or fimbrial-associated material (Hanazawa et al., 1985). While initial characterization of the bacterial factors that modulate bone formation has been carried out in mono-culture experiments (Chapter III and IV), this has not yet been done with the current model.

In conclusion, the data indicate that sonicated extracts derived from P. gingivalis stimulate osteoclast cell mediated mineral resorption in co-cultures with osteogenic (osteoblast-like) cells. The extracts did not stimulate osteoclast cells unless osteogenic cells were present. The differing effects of P. gingivalis extracts on mono- and co-cultures of bone cells highlights some of the problems involved in understanding the complexities of periodontopathogenic bacterial-mediated effects on bone metabolism. Defining the nature of these interactions will assist in the formulation of improved treatments for periodontal diseases which are targeted to block destructive bacterial-host interactions while allowing potentially beneficial or benign ones.
VI.5. FIGURES AND LEGENDS.

Figure 1. Effect of P. gingivalis Sonicated Extracts on the Osteogenic Activity of Chick Marrow Stromal Cells in Mono- and Co-Culture.

(a) Alkaline phosphatase activity. The addition of the sonicate to the culture medium resulted in significant ($P<0.05$) (*) reductions (up to 45%), from control levels, in alkaline phosphatase activity under both mono- and co-culture conditions. Suppression of alkaline phosphatase activity was, however, significantly ($P<0.05$) more profound under mono-culture conditions than those of co-culture.

(b) Calcium accumulation.

(c) Inorganic phosphate accumulation. The sonicate caused a decrease of up to 65% ($P<0.05$) in mineralization (calcium and inorganic phosphate accumulation) in bone nodules in mono-culture. No significant decreases were found in co-culture.

(d) Total alpha I type I collagen. Collagen content was reduced as much as 45% ($P<0.05$) in mono-culture grown in the presence of bacterial sonicate. Under co-culture conditions, significant decreases were also seen, although of a lesser magnitude than that observed in mono-culture.

The X-axis is displayed in a log scale. Each point represents the mean ± SEM for 6-8 cultures. All numerical values have been expressed as a % of control values.
P. gingivalis sonicate (µg/ml)

Alkaline Phosphatase (% control)

Calcium (% control)

Inorganic Phosphate (% control)

Type I Collagen (% control)

--- CBC culture

--- co-culture
Figure 2.  **Effect of P. gingivalis Sonicated Extracts on Osteoclast Cell Mediated Resorption of Thin Films in Mono- and Co-Culture.**

Osteoclast cells from laying hens (4x10^5) were seeded onto mineral thin films and grown in the presence of increasing concentrations of *P. gingivalis* sonicated extract. Osteoclast cell mediated mineral resorption (Ca released into culture medium) was quantified after 2 and 4 days of culture. For both culture periods, the addition of the bacterial extract significantly increased osteoclast resorption (up to 70%; *P*<0.05) under co-culture conditions. In contrast, no effects on resorption were observed in mono-culture. The X-axis is displayed in a log scale. Each point represents the mean ± SEM for 6-8 cultures. All numerical values have been expressed as a % of control values.
Mineral Resorption (days 0-2) (% control)

P. gingivalis sonicate (μg/ml)

Mineral Resorption (days 2-4) (% control)

P. gingivalis sonicate (μg/ml)

---osteoclast

---co-culture
The addition of indomethacin (1 μg/ml) to the culture medium blocked the inhibitory effects of the bacterial sonicate on osteogenesis in mono-culture. However, these effects were not blocked by indomethacin in co-culture.

The X-axis is displayed in a log scale. Each point represents the mean ± SEM for 6 -8 cultures. All numerical values have been expressed as a % of control values.
CO-culture --- mono-culture

Alkaline Phosphatase (% control)

P. gingivalis sonicate (μg/ml)

c--- co-culture
--- mono-culture
CHAPTER VII

SUMMARY AND CONCLUSIONS
Osteogenesis in vitro was inhibited significantly by metabolic products and sonicated extracts isolated from P. gingivalis 2561. An initial characterization of the inhibitory factors contained within the extracts was carried out. Whole extracts were segmented by ultrafiltration into five fractions based on molecular size (<5, 5-10, 10-50, 50-100 and >100 kDa). Differences between fractions in osteogenic inhibitory potential were observed, with the most profound noted with the <5 kDa fraction. Cytotoxic effects due to the presence of bacterial extract were not found with any fraction. The bacterial inhibition of osteogenesis was blocked with the addition of indomethacin (1 μg/ml), suggesting a role for prostaglandins in the mediation of the bacterial effects. Heat treatment of the fractions resulted in either complete, partial or no alteration of the osteogenic inhibitory capacity of individual fractions, intimating that the inhibitory factors consist of a group of both heat-labile (e.g. proteinaceous) and not heat-labile (e.g. non-proteinaceous: LPS, organic acids, etc.) compounds. Finally, the individual extracts were determined to have either reversible or irreversible effects on osteogenesis when added after differentiation or before/during differentiation of bone cells, respectively.

To determine if inhibition of bone formation by P. gingivalis was limited to it and other periodontopathogenic bacterial species, the effects of extracts from several other oral bacterial species were examined in CPO cultures. The data revealed that extracts from bacterial species having strong associations with periodontal disease, A. actinomycetemcomitans and P. intermedia (in addition to P. gingivalis) caused significant osteogenic inhibition. In contrast, extracts from
species not correlated with periodontal disease, *S. sanguis, V. atypica* and *P. denticola*, had no effects on osteogenesis. These results suggest that the ability to inhibit osteogenesis *in vitro* may be a pathogenic property shared by a limited group of species.

VII.3. **Development and Validation of a Co-culture Model System for Studying Osteogenic and Osteoclast Cellular Interactions.**

As previous experiments had confirmed the inhibitory role of *P. gingivalis*, and other periodontopathogenic bacteria, in bone formation *in vitro*, and given the findings of others that suggest they may also have stimulatory effects on bone resorption, the aim of the next group of experiments was to determine if the effects would be similar in a bone cell co-culture environment (i.e. one that allows both bone formation and resorption to occur simultaneously). The co-culture model developed consisted of laying Leghorn hen osteoclast cells which were seeded onto mineral thin films suspended above osteogenic embryonic chick bone marrow stromal cells plated on the bottoms of tissue culture plate wells. The data revealed differences in both bone nodule formation and mineral resorption rates between co-culture and mono-culture. These results were not mimicked when (osteogenic/osteoclast cell) conditioned medium was used to replace either osteogenic or osteoclast cell cultures, respectively. The responses were shown to be dependent upon the source or the age of osteoclast cells. Co-culture also appeared to alter individual cellular responses to a known anti-resorptive agent. The results indicated that the co-culture model, which permits communication between osteogenic and osteoclast cells via the release of soluble factors, may be useful in the study of bone cell interactions.
VII.4. Modulation of Co-cultured Osteogenic and Osteoclast Cellular Activities by Sonicated Extracts from P. gingivalis 2561.

Bone cell co-cultures and their respective mono-culture controls were grown in the presence of P. gingivalis sonicated extracts. The experiments demonstrated significant up-regulation of osteoclast mineral resorption in co-cultures. In contrast, the bacterial extracts had no effects on resorption in osteoclast mono-cultures. Bone formation was inhibited in both mono- and co-culture, although to a significantly lesser degree in the latter. Indomethacin (1 μg/ml), although it could block P. gingivalis induced osteogenic inhibition in mono-cultures, had no effects on co-cultures or on osteoclast mono-cultures. These results suggest that P. gingivalis extract up-regulation of mineral resorption may be mediated via osteogenic cells. In addition, differences in bacterial-mediated effects on bone formation observed between co-culture and mono-culture implies regulation between osteoblasts and osteoclasts in an attempt to maintain the balance between resorption and formation.

VIII.5. Final Conclusions.

In conclusion, the in vitro effects of extracts derived from P. gingivalis on bone formation and mineral resorption were described. A novel co-culture model system was developed to study how bone cell communication may influence bacterial-mediated outcomes. This further understanding of the mechanisms by which periodontopathogenic bacteria may elicit destruction of the periodontium will ultimately lead to the development of more efficacious treatment therapies, including those which take advantage of the relationship between bone cells. In addition, the co-culture model system may be employed not only to advance our knowledge of the periodontal disease process, but also to examine the effects of various agents in an in vitro bone cell
environment which, at present, most closely resembles that which occurs *in vivo*.

VIII.6. **Long Term Aims.**

Long term aims of this research include using the described model systems to determine the cellular mechanisms by which putative periodontopathogenic bacteria elicit their effects on bone metabolism. Markers, such as gene expression of bone specific proteins, will be determined. In addition, the co-culture model will be exploited to isolate factor(s) released (into the cell culture medium) by one bone cell type that mediate effects on the other (i.e. isolation of so-called "bone coupling factors"), and how exogenous agents, such as bacterial products or drugs, may alter this.
LITERATURE CITED


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