The role of the reactive oxygen species-generating enzyme, xanthine oxidoreductase, in cytokine- and hormone-induced bone resorption

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THE ROLE OF THE REACTIVE OXYGEN SPECIES-GENERATING ENZYME, XANTHINE OXIDOREDUCTASE, IN CYTOKINE- AND HORMONE-INDUCED BONE RESORPTION.

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Dedication.

To my Mother, Father, Sister

and Fiona.

Abstract.

Bone resorption underlies the pathology of many disabling diseases, ranging from the inflammatory condition, rheumatoid arthritis to the hormonally controlled, metabolic disorder, osteoporosis. It is known that various agents, including cytokines, prostaglandins, parathyroid hormone (PTH), 1α,25 dihydroxyvitamin D_3 and free radicals influence the bone resorptive process. Pro-inflammatory cytokines such as TNFα and IL-1β have been ascribed a pivotal role in rheumatoid arthritis (RA).

Bone resorptive mechanisms employ complex cellular signalling pathways between osteoblasts and osteoclasts that involve reactive oxygen (ROS) and nitrogen species (RNS). Recent studies have demonstrated that one of these species, hydrogen peroxide (H_2O_2) can increase osteoclast differentiation and bone resorption. One possible source of these ROS is xanthine oxidase. This thesis explores the role of XO-derived ROS directly and as mediators of cytokine- and hormone-induced bone resorption *in vitro.*

The results of this thesis demonstrated that calvarial osteoblasts contain XO, which can be upregulated by TNFα and IL-1β . Consequently, induction of XO by these cytokines led to the generation of hydrogen peroxide. TNFα and IL-1β caused a dose-related increase in resorption of mouse calvariae in culture, which was inhibited by 10U/ml catalase. The competitive inhibitor of XO, allopurinol, also caused a dose related (0.05-50μM) inhibition of TNF-α (0.1-10μM) and IL-1β-induced resorption respectively. Additionally, IFN-γ inhibition of bone resorption could be reversed by the addition of superoxide dismutase to the cultures. PTH- and 1α,25 $(OH)_2$ D_3-induced bone resorption could be inhibited by catalase (10U/ml) but was unaffected by allopurinol, implying that a different mediator, other than XO, is required for hormone-induced bone resorption.

In conclusion, this thesis demonstrates that modulation of the redox balance in the bone microenvironment can have profound effects on the bone resorbing process. Our results show that XO may play a pivotal role in affecting this redox balance and if manipulated appropriately, could be used to have a therapeutic benefit in inflammatory bone disorders such as RA.

Abbreviations and acronyms.

ABC.	Avidin biotin complex.
1α,25-$(OH)_2D_3$.	1α, 25$(OH)_2$ vitamin D_3 (calcitriol).
ALP.	Alkaline phosphatase.
AMPS.	Ammonium persulphate.
AP-1.	Activator protein-1.
BMP.	Bone morphogenic protein.
BOF-4272.	Sodium-8-(3-methoxy-4-phenylsulfynilphenyl) pyrzolo[1,5,-a]-1,3,5-tria-zine-4-olate monohydrate.
Ca^{2+}.	Calcium.
$Ca_{10}(PO_4)_6(OH)_2$.	Hydroxyapatite.
ECL.	Enhanced chemiluminescense.
CO_2.	Carbon dioxide.
COX.	Cyclooxygenase.
CT.	Calcitonin.
DDW.	Distilled deionised water.
DMEM.	Dulbecco's modified eagles medium.
DPI.	Diphenyliodonium chloride
DTT.	Dl-Dithioreitol.
ECACC.	European Collection of Animal Cell Culture.
ECM.	Extracellular matrix.
EDTA.	Ethylenediamine tetracetic acid disodium salt.
EGF.	Epidermal growth factor.
FAD.	Flavin adenine dinucleotide.
FCS.	Foetal calf serum.
Fe-S.	Iron-Sulphur.
FGF.	Fibroblast growth factor.
GAGs.	Glycosaminoglycans.
GI.	Gastrointestinal.
GM-CSF.	Granulocyte/Macrophage-colony stimulating factor.
H_2O.	Water.
HBSS.	Hanks balanced salt solution.
HCL.	Hydrochloric acid.
HFBA.	Heptafluorobutyric acid.

Abbreviations and acronyms.

HIF.	Hypoxia-Inducible factor.
HIV.	Human immunodeficiency virus.
HO-1.	Haemoxygenase-1.
H_2O_2.	Hydrogen peroxide.
HPLC.	High-performance liquid chromatography.
IFN-γ.	Interferon–gamma.
IGF.	Insulin-like growth factor.
IgG.	Immunoglobulin.
IL-1β.	Interleukin 1 beta.
IL-2.	Interleukin-2.
IL-6.	Interleukin-6.
IMS.	Industrial methylated spirits.
iNOS.	Inducible nitric oxide synthase.
iNOS-HRE.	Inducible nitric oxide synthase-hypoxic response element.
kD.	Kilodalton.
KPO_4.	Potassium phosphate.
LIF.	Leukaemia inhibitory factor.
LMMA.	L-N^G-monomethyl arginine.
M.	Molarity.
Mab.	Monoclonal antibody.
M-CSF.	Macrophage-colony stimulating factor.
Mg^{2+}.	Magnesium.
mg/dL.	Milligram per decilitre.
MMP.	Metalloproteinase.
MNGC.	Multi-nucleated giant cells.
Mn^{2+}.	Manganese.
Mo.	Molybdenum.
MT1-MMP.	Membrane-type-1 matrix metalloproteinase.
NAC.	N-acetyl cysteine.
NAD^+.	Nicotinamide adenine dinucleotide.
NADH.	Nicotinamide adenine dinucleotide, reduced form.
NADPH.	Nicotinamide adenine dinucleotide phosphate, reduced form.
$(NH_4)_2\ SO_4$.	Ammonium sulphate.

Abbreviations and acronyms.

NFkB.	Nuclear factor kappa B.
NIBSC.	National Institute for Biological Standards and Control.
NMR.	Nuclear magnetic resonance.
NO.	Nitric oxide.
NO_3^-.	Nitrate.
NO_2^-.	Nitrite.
$O_2^{\bullet-}$.	Superoxide.
OA.	Osteoarthritis.
OB.	Osteoblast.
OC.	Osteoclast.
$OH^{\bullet}$.	Hydroxyl radical.
$ONOO^-$.	Peroxynitrite.
ORSA.	Osteoclast resorption stimulating activity.
PAGE.	Polyacrylamide gel electrophoresis.
PBS.	Phosphate buffered saline.
PDGF.	Platelet derived growth factor.
PGE_2.	Prostaglandin (E_2).
PMN.	Polymorophonuclear leukocytes.
pmoles.	Pico ($x10^{-12}$) moles.
PMSF.	Phenylmethylsulphonyl fluoride.
PTH.	Parathyroid hormone.
RA.	Rheumatoid arthritis.
RCOBs.	Rat calvarial osteoblasts.
RGD.	(Arg-Glys-Asp).
ROS.	Reactive oxygen species.
RNS.	Reactive nitrogen species.
SCF.	Stem cell factor.
SCID.	Severe combined immune deficient.
SDS.	Sodium dodecyl sulphate (Lauryl Sulphate).
SOD.	Superoxide dismutase.
std.	Standard deviation.
S.E.M.	Standard error of mean.
TCM.	Tissue culture medium.

Abbreviations and acronyms.

TEMED.	N, N, N', N'-Tetramethylethylene diamine.
TGFβ.	Transforming growth factor beta.
TNFα.	Tumour necrosis factor-alpha.
TRAP.	Tartrate resitant acid phosphatase.
Tris-HCL.	Tris[hydroxymethyl] aminomethane hydrochloride.
Tween 20.	Poloxyethylene sorbitan monolaurate.
VCAM-1.	Vascular cell adhesion molecule-1.
VEGF.	Vascular endothelial growth factor.
XDH.	Xanthine dehydrogenase.
XO.	Xanthine oxidase.
XOR.	Xanthine oxidoreductase

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Chapter 1.

General Introduction.

Chapter 1

1.1. Functional evolution of the skeleton.

Movement and function in single cellular organisms is provided by an internal system, which is capable of adjusting between a solution and a gel. With the evolutionary onset of multicellular organisms, support for vital activities began to be provided by specialised tissues. Hence, epithelial cells began to be protected in their activities by connective tissues (Marks & Popoff, 1988). Ectoskeletal elements and dermal bones were the earliest forms of support for tissues. These went onto be replaced by the primitive form of the notochord, which was of an endoskeletal structure (Marks & Popoff, 1988). As organisms became larger and more complex the notochord was superceded by the vertebral column, where it gradually transformed from a cartilaginous material into a rigid bone structure (Romer, 1962). Thus, bone skeletal structures had evolved both in ontogeny and phylogeny, from the connective or mesenchymal tissues that proceeded them. This process is continually being repeated in our modern day life in the vertebrate embryo (Marks & Popoff, 1988).

The general consensus is that bone is an inert, solid piece of tissue which only serves two functions (support and protection), is due to the fact that we only see bone in an archaeological or anthropological setting. It seems quite remarkable that this rigid and very tensile structure is a dynamic piece of tissue that is capable of providing essential minerals for the body, and which undergoes highly regulated remodelling sequences throughout its entire life as a consequence of this.

1.2. The structure and dynamics of bone.

1.2.1. Introduction.

Bone is a living, highly specialised, complex dynamic form of connective tissue, required for an internal support system in higher vertebrates (Marks & Hermey, 1996). It has evolved to provide two main functions: firstly, the provision of rigidity and strength to the skeleton while still maintaining some elasticity, and secondly, to maintain mineral homeostasis, particularly calcium levels in the body (Marks & Hermey, 1996).

The major components of bone comprise of mineral and matrix phases. Bone is composed of approximately 70% mineral, 22% protein, and 8% water (Lane, 1979). Type I collagen makes up 95% of the organic matrix, the rest is composed of non-collagenous proteins mainly consisting of proteoglycans (Kresse *et al*, 1994), glycoproteins, such as alkaline phosphatase, osteonectin and the cell attachment sequence RGD (Arg-Gly-Asp)-containing proteins (osteopontin, bone sialoprotein, thrombospondin, fibronectin and vitronectin) (Robey, 1996). The crystalline salts found deposited in the organic matrix of bone are calcium and phosphate and are usually in the form of hydroxyapatite Ca_{10} $(PO_4)_6(OH)_2$ (Heaney, 1996). The hydroxyapatite deposited in the matrix provides the rigidity and strength of bone, whereas, the water component found in bone contributes mainly to the viscoelasticity of the bone composite (Einhorn, 1996).

Bone comprises of two structural forms, the compact (cortical) form and the spongy (cancellous/trabecular) form. In cortical bone, densely packed collagen fibrils form concentric lamellae and the fibrils in adjacent lamellae run in perpendicular planes. Consequently, the lamellae reinforce each other; producing beautiful concentric

structural units (osteons) that are designed to withstand twisting and high mechanical stresses that bone is subjected to. In contrast to cortical bone, spongy bone is composed of needle like pieces of bone called trabeculae (little beams). Although they appear to be haphazardly arranged, they're in fact carefully positioned to reveal where the bone is stressed and can provide a lattice like network that can help resist these stressful forces (Marieb, 1992).

The responsibility for the maintenance of skeletal integrity lies predominantly on four cell types, the osteoblast, the osteoclast, bone lining cells and the osteocyte (Marks & Hermey, 1996). Under a plethora of multifactorial influences these cells of the bone microenvironment work in conjunction with each other to maintain our skeleton providing support, locomotion, protection, growth and mineral homeostasis.

1.2.2. The osteoblast and bone formation.

Osteoblasts are mature differentiated skeletal cells derived from osteoprogenitors arising from mesenchymal stem cells, which are capable of producing a number of committed and restricted cell lineages, including the osteogenic cell line (Aubin *et al*, 1993). Osteoblasts are responsible for bone formation, where they synthesise and regulate the deposition and mineralisation of the extracellular matrix of bone (Marks & Hermey, 1996). Not all of the bones in the body are derived in the same way or even from the same embryonic tissue. Two types of bone formation have been described. Firstly, there is the direct intramembraneous form, where mesenchymal progenitors condense and then differentiate directly into osteoblasts, and secondly, the endochondral form, where mesenchymal progenitors condense to first form a cartilage model that is replaced by bone (Aubin & Liu, 1996). Both forms require a solid base and a well-developed vascular supply for growth and mineralisation of the extracellular

matrix. Interestingly, mobility or low oxygen tensions at these sites appear to favour the differentiation of chondrocytes or fibroblasts (Marks & Hermey, 1996).

During embryonic development, intra-membraneous ossification occurs by the direct transformation of mesenchymal cells into osteoblasts. The cranium, some facial bones and parts of the mandible and clavicle are representative of this type of ossification (Marks & Hermey, 1996). Endochondral ossification is responsible for most of the bones in the body, including the bones that are load bearing, and bones that participate in the joints. The unique properties of cartilage and bone are exploited to provide a mechanism for formation and growth of the skeleton. Here the condensed embryonic mesenchyme transforms into pre-chondroblasts and then into chondroblasts and finally into cartilage, resulting in a site where bone will be eventually formed. In the central part of such a bone, chondrocytes proliferate, hypertrophy and mineralise, forming a rigid scaffold that acts as a solid base upon which osteoblasts can deposit and mineralise bone matrix. This scaffold is partially resorbed by osteoclasts allowing for new growth of bone. Peripheral osteoblasts (periosteum) arrive with a blood supply where vessels penetrate the mineralised cartilage carrying cell progenitors for formation and turnover of bone (Marks & Hermey, 1996).

Fully differentiated osteoblasts express alkaline phosphatase on their cell surface and synthesize macromolecules such as osteocalcin, osteonectin, osteopontin and Type I collagen (Rodan & Noda, 1991). They are predominantly found along bone surfaces where they synthesise osteoid at a bone-forming site. The type I collagen produced by these osteoblasts are assembled extracellularly into fibrils which are organised into defined bands as one moves away from the cell surface (Marks & Popoff, 1988). These cells appear to be lined up at bone forming sites on the surface of unmineralised osteoid (Recker, 1996). This uncalcified bone tissue predominantly consists of Type I collagen

fibres which undergoes calcification by the formation of crystals of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (Heaney, 1996). As mineralisation proceeds osteoblastic cells either get incorporated into the new bone matrix becoming osteocytes, or, remain on the bone surface as very thin lining cells (Recker, 1996).

The osteoblast is the pivotal cell for the maintenance of bone metabolism. Osteoblasts are stimulated by paracrine factors such as growth factors to induce new bone formation. These include the Transforming Growth Factor beta (TGFβ) superfamily (including most of the Bone Morphogenic Proteins (BMP's)), Insulin Growth Factor's (IGF's), Fibroblast Growth factor's (FGF's), and Platelet Derived Growth Factor's (PDGF's) (Meikle, 1997). In addition, osteoblasts have been shown to be pivotal in regulating the resorptive process, and can no longer be thought of as purely osteogenic (Meikle, 1997).

1.2.3. Bone collagen and crosslinking.

Bone is predominantly made up of Type I collagen, which is composed of two α 1 chains and one α 2 chain coiled around each other in a triple helix format (Rossert & de Crombrugghe, 1996). Types I, II, III fibril-forming collagens are synthesized as procollagen molecules that have their entire N- and C-propeptides removed outside the cell before fibril formation (Eyre, 1996). These fibrils are stabilized by intermolecular cross-links derived from aldehyde forms of hydroxylysine and lysine, ensuring that the collagen laid down is very enduring under high amounts of stresses and strains (Dequeker, 1996).

The 3-hydroxypyridinium cross-links of mature collagen are pyridinoline (pyr) and deoxypyridinoline (D-pyr). They are only present in a mature form and are distinct to bone and cartilage (Dequeker, 1996). Osteoclasts that are actively degrading bone

collagen are unable to break down these peptides so they are released into the circulation. As pyr and D-pyr naturally fluoresce, they can be quantitatively measured in the urine by reverse-phased high performance liquid chromatography (HPLC) (Black *et al* 1988). This provides a sensitive and specific marker for bone resorption. Indeed it has been shown that in conditions with increased collagen breakdown such as RA, there is a correlation of increased secretion of these crosslinks into the urine (Black, 1989).

1.2.4. The osteocyte and canuliculi.

The osteocyte is the most abundant cell type of bone. According to Parfitt, 1977, adult bone has approximately 10 times the number of osteocytes in comparison to osteoblasts. Mature osteocytes are stellate-shaped cells enclosed within the lacuno-canilicular network of bone, probably osteoblastic in origin (Nijweide *et al*, 1996). The osteocytes in bone project elongated, narrow cytoplasmic structures which connect to neighbouring osteocytes by passing through the bone matrix via these small, elaborate canal networks (Nijweide *et al,* 1996). These canal networks in bone appear to possess similar properties to a nerve network.

This system, along with bone lining cells provides a cellular detection system allowing bone organs to determine increases and reductions in bone mass. Fluid flow rather than simple diffusion appears to be a popular hypothesis whereby osteocytes act and respond to microenvironmental signals (Cowin *et al*, 1991; Weinbaum *et al,* 1994). Interestingly, it has been found that osteocytes show a higher sensitivity to fluid flow (Klein-Nulend *et al*, 1995) than endothelial cells (Frangos *et al*, 1985).

1.2.5. The osteoclast and bone resorption/remodelling.

Osteoclasts are multinucleated giant cells with resorbing activities for mineralised bone, dentine and calcified tissue (Vaananen, 1996). Osteoclast progenitors

from haematopoietic tissue such as bone marrow (probably the macrophage-monocyte lineage) (Ash *et al,* 1980) proliferate and differentiate into mature osteoclasts through cell to cell interaction with osteoblastic stromal cells (Suda *et al,* 1996).

Osteoclasts are readily defined as large multinucleated cells that are found in large numbers at sites of active bone resorption (Athanasou, 1996). They are particularly rich in the acid phosphatase enzyme, tartrate-resistant acid phosphatase (Minkin, 1982; van de Wijngaert & Burger, 1986), and express calcitonin receptors, which when stimulated directly inhibit the osteoclasts activity (Chambers and Magnus, 1982). Mature osteoclasts possess a unique structure known as a ruffled border. It is a complex system of villous folds of the plasma membrane beneath which bone resorption occurs. Surrounding this is a clear zone, which assists in the anchorage of the osteoclast to the bone surface (Gothlin and Ericsson, 1976).

Once activated, osteoclasts in a normal bone remodelling sequence start to resorb bone. They attach to the bone surface and start to secrete hydrochloric acid, creating an acidic environment whereby hydroxyapatite can be solubilised. This is then followed by the degradation of the matrix by the secretion of several metalloproteinases (MMP's) and lysosomal cysteine proteinases (cathepsins, B, D, L, K, O and S), which have been localised to the osteoclast (Vaananen, 1996). Impairment of cathepsin-K which is highly expressed in the human osteoclast (Inoka *et al,* 1995) has recently been shown to cause an osteopetrosis-like disease in cathepsin-K-deficient mice (Saftig *et al,* 1998). A role for oxygen radicals has also been suggested in matrix degradation as Key *et al,* 1994 and Silverton, 1994 demonstrated that superoxide generation occurred in the resorption lacuna.

Osteoclasts are highly motile cells. *In vitro* they can undergo more than one resorption cycle by detaching themselves and moving onto a fresh site, repeating the

whole resorptive process again until external signals activate apoptosis and cell death (Kanehisa & Heersche, 1988; Mancini *et al*, 1998).

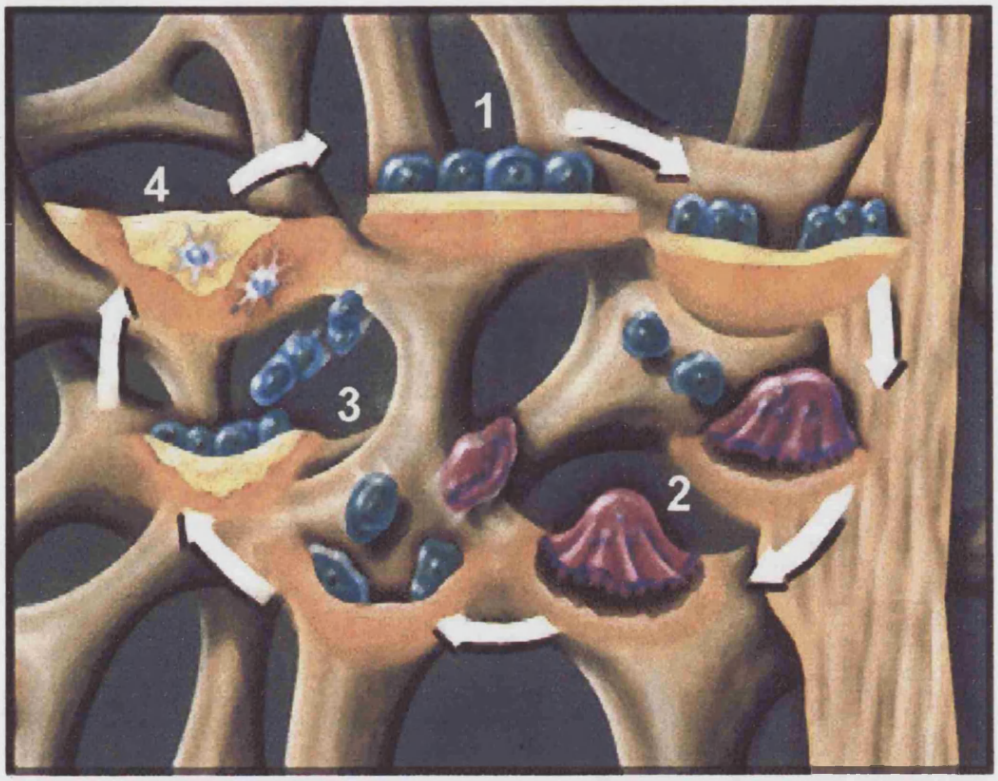


Figure 1.1. The remodelling sequence of bone: (1) Resting phase. (2) Osteoclast resorbing bone. (3) Osteoblasts recruited and start to lay down collagen to fill the excavated pit. (4) Mineralisation of the matrix.

Resorption is the first step of this highly regulated process as shown in the remodelling sequence of Fig. 1. Once resorption has occurred, osteoblasts are recruited to the excavated bone site laying down matrix that is subsequently mineralised, resulting in the formation of bone (section 1.2.2).

This sequence in bone is highly regulated, to the point where it seems that the osteoclast resorptive process is closely linked to the osteoblastic formation process, a term known as "coupling." Indeed many agents that cause or have an effect on bone resorption do not act on the osteoclast directly but produce their effects through osteoblasts or related cell types. Parathyroid hormone (PTH), 1,25 dihydroxyvitamin

D_3 (Vit D_3), prostaglandins such as PGE_2, Tumour Necrosis Factor-alpha (TNFα) and Interleukin-1Beta (IL-1β) all appear to exert their effects this way (reviewed by Russell, *et al,* 1998). The problem facing bone biologists today is the nature and the mechanism of the signals between the osteoblast and the oteoclast, which to date remain unclear. These signals could well be vitally important in the maintenance of skeletal integrity and especially in the communication of osteoclast-osteoblast coupling.

With bone remodelling having to be a precise mechanism, it is quite astounding that a vast number of agents can exert an influence on the bone remodelling process (Table 1) (reviewed in Bilezikian, Raisz & Rodan, 1996). The main influences appear to be the systemic hormones, cytokines, growth factors and, identified more recently, reactive oxygen and nitrogen species (Garrett *et al,* 1990; Bax *et al,* 1992 Fraser *et al,* 1996; Ralston *et al* 1995). With so many factors influencing bone metabolism, it is clear that an imbalance to either the resorptive side or to the formation side can have severe consequences on skeletal shape and structure.

		Stimulators of bone resorption	*Osteoclast formation*
Hormones			
Parathyroid hormone	PTH	+	+
1,25- Dihydroxy vitamin D_3	1,25Vit D	+	+
Glucocorticoids		+/-	+/-
Calcitonin	CT	-	-
Cytokines			
Interleukin's	IL-1	+	+
	IL-2	+/-	+/-
	IL-3	?	+
	IL-4	-	-
	IL-6	+/-	+
	IL-8	-	-
	IL-10	-	-
	IL-11	+	+
	IL-13	-	?
Tumour necrosis factor	TNFα	+	+
Colony stimulating factors	GM-CSF	+	+
	M-CSF	?	+
	LIF	+/-	+
	SCF	-	+
Interferon gamma	IFNγ	-	-
Growth factors			
Insulin like growth factors	IGF-1	+/-	+
	IGF-2	+/-	+
transforming growth factor	TGFb	+/-	+/-
Fibroblast growth factor	aFGF	+	+
	bFGF	+	+
Platelet derived growth factor	PDGF-AA	+	+
	PDGF-AB	+	+
	PDGF-BB	+	+
Epidermal growth factor	EGF	+	+
Bone morphogenic proteins	BMP's 1-8	+/-	+/-
Others			
Oestrogen		-	-
Heparin		+	?
Thyroid hormones		+	+
Bradykinin		+	+
Thrombin		+	?
Vitamin A.		+	+
Prostaglandin E_2		+	+
Reactive O_2 and N_2 species.			
Nitric oxide	$^{\bullet}NO$	+/-	?
Superoxide	$O_2^{\bullet -}$	+/-	?
Hydrogen peroxide	H_2O_2	+	?
Peroxynitrite	ONOO-	?	?

Table 1.1 The pathological and physiological regulators of bone resorption.

(+), positive effects of bone resorption, differentiation and activation; (-), negative effects;
(+/-), factors can stimulate or inhibit the bone resorptive process; (?), effects have yet to be elucidated.

1.2.6. Metabolic diseases of bone.

Abnormal bone remodelling can lead to skeletal disorders such as Paget's, disease, osteomalacia, osteopetrosis, and osteoporosis. Osteomalacia is where mineralisation is reduced because there is a deficiency of Vitamin D. This results in bowing deformities of long bones and the pelvis (Stamp, 1996). Osteopetrosis results from a reduction of resorption relative to bone formation due to inadequate osteoclastic activity. This disorder leads to the thickening of the cortical region reducing the size of the medullary space in long bones, resulting in reduced haematopoiesis (Key *et al*, 1996). Paget's disease is an increase in the remodelling cycle resulting in a disorganised structure of woven and lamellar bone at localised skeletal sites. This increase in activity is characterised by large osteoclasts and an enlarged vascular supply. Pain, brittleness and deformity (de Deuxchaisnes, 1996) are associated with this pathology.

The most common form of bone disorder is osteoporosis. Osteoporosis is a disease clinically characterised by loss of bone mass (osteopenia), microarchitectural deterioration of bone tissue, and a susceptibility to increase fracture risk (Dequeker *et al*, 1996). It is a multifactorial disease, which is not a consequence of aging and appears to be more common in women than men. Osteoporotic fractures are generally localized in the spine, the hip and the wrist, and is associated with high morbidity and, in the case of hip fractures, increased mortality (Dequeker, *et al*, 1996).

Finally there is rheumatoid arthritis, one of the most common inflammatory joint disorders in the western world. It is a symmetric polyarticular disease of the appendicular skeleton, sparing the axial skeleton except for the cervical spine. It is characterised by an inflammatory infiltrate in the lining of the synovial joints, periarticular soft-tissue swelling, osteoporosis, marginal erosions progressing to severe

erosions of subchondral bone, synovial cyst formation and a lack of bone repair. The distribution of the disease involves the hands, feet, knees, hips, cervical spine, shoulders and elbows, in decreasing order of frequency (Brower, 1988; Resnick, 1989).

1.3. Inflammation.

The inflammatory response is a pathological process associated with complex reactions that transpires in the affected blood vessels and surrounding tissues in response to an injury or abnormal physical, chemical, biological stimulant. It plays an important role in the repair of tissue by destroying, diluting and isolating the damaging agent (Gallin *et al*, 1992). Unfortunately in some settings this system can do more harm than good.

The classic signs of inflammation were first described in the first century A.D by Cornelius Celsus as *rubor, tumor, calore,* and *dolore* (redness, swelling, heat, and pain) (Weissman, 1992). Later on, a fifth sign *functio laesa* (loss of function) was added to the classification of inflammation (Gallin *et al*, 1992).

Firstly, in an inflammatory response, blood flow is increased due to vasodilation of blood vessels (causing swelling and redness). Secondly the vessels become highly permeable allowing for increased movement of fluid with a high-protein concentration out of the vessels, and at the same time, fluid return to the blood is impaired. This results in an exudate accumulation in the tissue creating the oedema. Finally, white blood cells infiltrate the area of injury, removing and degrading the injurious agent and damaged tissue. The polymorphonuclear leukocytes (PMN's), lymphocytes, monocytes or histiocytes, and plasma cells participate in the inflammatory process. PMN's that engulf microbes, crystals or any other particulate matter rapidly consume high amounts of oxygen, (respiratory burst). As a consequence of this, oxygen is reduced to superoxide $O_2^{\bullet-}$(reaction 1 Fig. 1.2), which in turn is converted to hydrogen peroxide (H_2O_2) by the enzyme, superoxide dismutase (SOD) (reaction 2 Fig. 1.2). $O_2^{\bullet-}$ and H_2O_2 can combine via the Haber Weiss mechanism and generate the hydroxyl radical ($OH^{\bullet}$) via two reactions which are catalysed by ferric ions (reactions 3a & 3b Fig. 1.2).

This reaction product is then able to induce lipid peroxidation as well as polypeptide chain cleavage (Merry *et al*, 1990; Robinson, 1996). Once this is complete the process of tissue reparation can now occur.

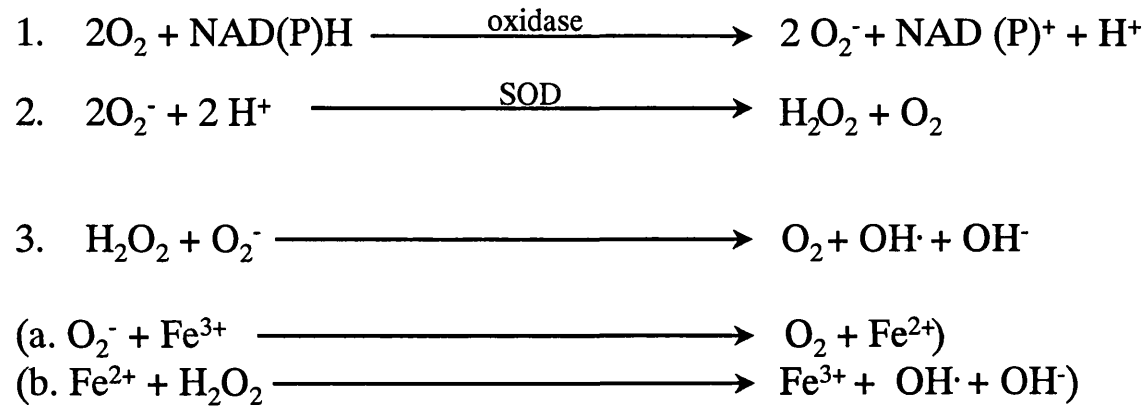


Figure 1.2. Chemical reactions that involve the generation of reactive oxygen species.

Inflammation can be either acute or chronic, but both elements express themselves some time during the process (Cotran *et al*, 1989). Acute inflammation usually has a sudden onset but normally only lasts for a few hours or at most a few days. Chronic inflammation on the other hand can last for weeks or even years. This prolonged condition is the result of either the perpetuation of an acute reaction, or may begin as a small latent response, which rarely has the classic features of redness, heat, swelling, pain, loss of function as seen in acute inflammation. However, a persistent episode of dull pain, hard swelling, and granulation in the tissue characterise chronic inflammation. Along with the infiltrate of proliferating fibroblasts and mononuclear leukocytes, such as macrophages, lymphocytes, and plasma cells (Robinson, 1996), autoimmune reactions, which persist over a long period of time as seen in rheumatoid

arthritis and Sjogren's Syndrome, can initiate these chronic inflammatory reactions resulting in highly destructive tissue damage (Robinson, 1996).

1.3.1. Inflammatory bone destruction in rheumatoid arthritis.

1.3.1.1. Introduction.

Rheumatoid arthritis may be defined, in part, as an example of an often persistent symmetrical synovitis that is associated with erosive bone and cartilage damage, both of which contribute to a progressive loss of function (reviewed by Kanczler *et al*, 1998). It affects approximately 1% of all adults involving all ethnic and racial groups (Firestein & Zvaifler, 1992). There are a number of persistent inflammatory forms of synovitis that are not associated with erosive bone damage. With other conditions such as psoriatic arthritis and crystal induced arthritis, erosive damage is a feature, but the location and nature of the erosive damage are distinctive and different from those in rheumatoid arthritis. Conversely, in inflammatory forms of oesteoarthritis erosions are not a feature. Indeed an osteophytic, perhaps reparative stabilising response, is a hallmark of the pathology.

1.3.1.2. Where do erosions develop?

In infective arthritis, where the relationship between synovial inflammation and erosive bone damage appear to be the most direct, erosions are found in the bare areas where the synovium directly abuts the bone. In rheumatoid arthritis, a periarticular osteopenia is an early feature of disease. This is thought to occur early in the disease in relation to changes in synovial blood flow. In addition, three different forms of erosive damage are found as well as sub-chondral cyst formation (Resnick, *et al*, 1993). The first is the marginal erosion that occurs at bare sites. Such sites are also in close physical proximity to the insertion of the capsule ligaments and this area is believed to be considerably innervated. Secondly, compressive erosions develop when osteoporotic

bones collapse or one bone invaginates another. In the third type of erosion, namely surface erosions, bone underlying inflamed tendons is resorbed. This process most characteristically occurs around the ulnar styloid process in the wrist. These broad clinical descriptions favour the conclusion that inflammation in the overlying tissue (either synovium or tendon sheath) is the major driving force for the initial erosive pathology. The possibility must also be considered that micro-erosions breaking the periosteal surface release bone debris, which may initiate synovial inflammation and augment the damage.

1.3.1.3. A question of balance. Multiple mediators of bone resorption: A recipe for chaos.

As already alluded to in section 1.2.5, bone remodelling is uniquely balanced, and has to stay balanced in order to maintain skeletal integrity. The bone microenvironment involves complex sequences of cellular events, which are modulated by many mediators. When this is imbalanced, especially towards the resorptive side, bone loss is inevitable. Table 1 shows the vast array of agents that can influence bone resorption under physiological and pathological conditions. The main influences appear to be the systemic hormones, cytokines, growth factors and more recently, reactive oxygen and nitrogen species.

1.3.1.4. Hormonal modulation.

The systemic hormones, parathyroid hormone (PTH) and 1,25-dihdroxyvitamin D_3 have a dramatic influence on osteoclastic bone resorption. Parathyroid hormone is a central component of calcium homeostasis by its actions to reclaim filtered calcium in the kidney, to facilitate absorption of calcium from the gastrointestinal tract and to remodel bone (Fitzpatrick and Bilezikian, 1996). PTH is involved in the differentiation and activation of osteoclasts to resorb bone (Miller, 1978; Tatevossian, 1973), which is

mediated indirectly by signals from the osteoblast (Chambers *et al*, 1985). Similar bone resorptive effects are seen with 1,25 dihydroxyvitamin D_3 (Suda *et al*, 1997).

1.3.1.5. Inflammatory cytokines.

The most significant conceptual advance in bone pathology during the last 15 years was the realisation that many cytokines could act as autocrine and paracrine regulators of pathophysiological bone resorption. The problem now facing bone biologists today is the sheer numbers of cytokines and their complex mechanisms and interactions. An inflammatory induction of many of these cytokines could easily be described as chaotic, especially in pathological situations where cytokine responses are non-linear and interacting. This premise of non-linearity and interactive response drives the mathematics of chaos. Additionally, it has been demonstrated that some of these pro-inflammatory cytokines are elevated in diseases such as RA (Fontana *et al,* 1982; Hopkins & Meager, 1988).

The initial discovery that the pro-inflammatory cytokines IL-1β and TNFα could stimulate bone resorption (Gowen *et al,* 1983; Bertolini *et al,* 1986) led to several other cytokines being implicated. As the bone microenvironment contains cells that can produce many of these cytokines, it is possible that the action of one cytokine may influence the action of another. This appears to be the case for IL-6, which dramatically enhances bone resorption stimulated by IL-1β and TNFα. However, IL-6 does not stimulate bone resorption mediated by PTH and 1,25 $VitD_3$, and subsequent inhibition of IL-6 generation prevents IL-1β or TNFα from resorbing bone (Mundy, 1991). These data highlight the complex interactions of these cyokines that exist in a bone-resorbing environment.

Currently, several cytokines and colony stimulating factors have been implicated in the differentiation and development of osteoclasts and osteoblasts. These include

TNFα, the interleukins (IL-1, IL-3, IL-6, IL-11), granulocyte/macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (M-CSF). The pro-inflammatory cytokines TNFα and IL-1β do not mediate their osteoclastogenic actions directly but via factors derived from stromal/osteoblastic cells (Thomson, *et al,* 1987; Chambers, 1988). Inhibitors of bone resorption appear to be IL-4 (Riancho, *et al,* 1993), IL-8 (Fuller, *et al,* 1995) and IFNγ (Gowen & Mundy 1986). Indeed IFNγ has been shown to synergistically augment TNFα and IL-1β stimulated NO by cultured osteoblasts (Ralston *et al,* 1994), which at the levels being produced causes an inhibitory effect on mouse calvarial bone resorption in comparison to low levels of NO which stimulate bone resorption (Ralston *et al,* 1995).

It is well documented that the cytokines IL-1β, TNFα, IL-6 and M-CSF are elevated in the rheumatoid joint whereas levels of IL-2 have been found to be low (Westacott *et al,* 1990). This unusual pattern of cytokine response may be linked to the hypoxic nature of the mobile inflamed joint.

The extracellular matrix of bone is a rich source of growth factors containing the FGFs, IGFs, PDGFs and the TGFβ super-family which contain the bone morphogenic proteins (BMPs). The latter are implicated in the stimulation of new bone formation via the osteoblastic lineage (Mundy, *et al,* 1995). TGFβ, a growth regulatory factor that is synthesised by many tissues, including bone, inhibits osteoclast formation and differentiation (Mundy, *et al,* 1995). Since TGFβ is within the extracellular matrix, it may be an important factor in inhibiting osteoclastic activity and promoting bone formation when released during resorption of the bone matrix (Mundy, *et al,* 1995).

With increased levels of cytokines in inflammatory arthritis, the levels of other factors such as growth factors released from the bone matrix may not be adequate to balance the formation of bone in comparison to its destruction.

1.3.1.6. The unique environment within the inflamed joint.

The inflamed rheumatoid joint is a chronically hypoxic environment. Synovial tissue analysed by nuclear magnetic resonance spectroscopy has shown a profile of low molecular weight metabolites, which are consistent with hypoxic metabolism (Naughton, *et al*, 1993). The main characteristics are an increased lactate and a lowered glucose content. More recent studies utilising a polarographic needle electrode have measured O_2 levels in synovium directly and shown generally lower levels in rheumatoid knees with pockets of tissue that are profoundly hypoxic. This is supported by the morphometric analysis of the inflamed synovial tissue, indicating inadequate perfusion due to failure of angiogenesis to vascularise the innermost layer of synovium (Stevens *et al,* 1991). In addition to this, movement of the inflamed joint results in pressure changes that lead to repeated cycles of ischaemia/reperfusion. The inflamed joint tissue is therefore exposed to the influences of hypoxia-induced events, which can be modulated by the intermittent reperfusion, particularly in the sub-synovium where the vasculature is more adequately developed. These characteristics create a unique redox-controlled environment within the inflamed synovium where both reducing (due to chronic hypoxia) and oxidising (due to intermittent reperfusion) events prevail. Fig.1.3 summarises the events that are believed to occur in the inflamed synovium.

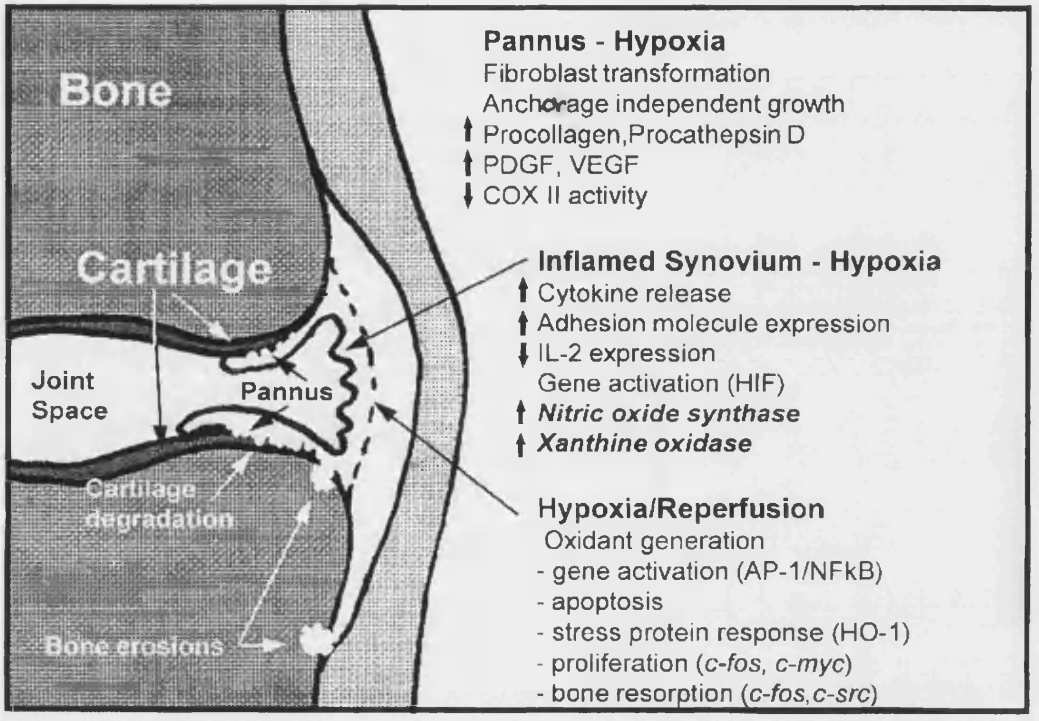


Figure 1.3. Summary of hypoxia-and hypoxia/reperfusion-induced events in inflamed synovium, which modulates bone resorption.

1.3.1.7. Free radicals, and the environment of resorbing bone in RA.

As already mentioned in section 1.3.1.6 the inflamed joint is relatively hypoxic, creating a unique redox-controlled environment where both reducing and oxidising events prevail. With the induction of pro-inflammatory cytokines the role of local mediators produced by the osteoblast to activate the osteoclast has been the subject of much debate.

Prostaglandin E_2 (PGE_2) is one of the most potent prostanoids that induce bone resorption. It was thought that TNFα and IL-1β-induced resorption might be mediated in part, by prostaglandins, by inducing cyclooxygenase (COX-2) synthesis in the osteoblast. (Tashjian *et al,* 1987); Sato *et al,* 1986). However, there have been a variety of studies demonstrating that cyclooxygenase inhibitors, though able to block PGE_2 biosynthesis by TNFα, are incapable or only partially able to inhibit bone resorption

(Lerner & Ohlin, 1993). This indicates that there must be a prostaglandin independent mechanism involved in cytokine-induced bone resorption. Cytokines have been shown to stimulate cells from the synovium, cartilage and bone to produce both reactive oxygen (ROS) (Schmitz *et al,* 1987; Tiku *et al,* 1998; Garrett *et a*l, 1990) and nitrogen species (RNS) (Stefanovic-Racic *et al,* 1994; Grabowski, *et al,* 1996). Free radicals are small labile molecules that could act as intermediates in the signalling mechanism between osteoblasts and osteoclasts in the remodelling of bone.

What are free radicals? Any molecular species containing one or more unpaired electrons in an orbital surrounding an atom capable of existing independently can be described as free radicals (Halliwell, 1991). In fact, most biological molecules are non-radicals containing only paired electrons. Radicals can react with other molecules in a number of ways. Firstly, when two radicals meet they can combine their unpaired electrons to form a covalent bond. Secondly, they can react with non-radicals by either donating the unpaired electron (reduction) or they may take an electron from another molecule (oxidation). Thirdly, a radical may attach itself to a non-radical. In general, when any of these reactions take place the non-radical species becomes a radical. This usually has a knock on effect creating a chain reaction of these events (Halliwell, 1991).

Cellular responses to oxidants are diverse. The nature of these responses is not only dependent on the extent of the oxidative stress, but also to the intrinsic antioxidant status of different cell types. Thus, differential 'oxidant sensitivity' is observed within different cell populations. The involvement of oxidants such as H_2O_2 and NO in processes as diverse as apoptosis, stress protein response, and proliferation for example, clearly reflects the complexity of their interactions. Although complex, and with largely unknown mechanisms of action, these oxidants are known to act as signaling molecules

(Brenneisen *et al*, 1997; Clementi, 1998). Oxidant-mediated induction of the growth competence genes, c-myc, c-fos, and c-jun is well documented (Li & Spector, 1997). The significant role of the c-fos oncogene in the regulation of bone remodelling (Grigoriadis *et al*, 1994) infers potential modulation of related processes by oxidants. Furthermore, the c-src proto-oncogene, known to play a critical role in the development of the ruffled border in osteoclasts for resorptive activity (Boyce *et al*, 1992), is also activated by oxidant mediated phosphorylation of its associated protein product $pp60^{src}$ (Gonzalez-Rubio *et al*, 1996).

Two transcription factors, AP-1 and NFκB are also oxidant sensitive (Sen & Packer, 1996). Collagenase and stromelysin genes both have AP-1 binding elements in their promoter regions, which thus confer oxidant sensitivity to these genes integral to the bone matrix dissolution process (Crawford & Matrisian, 1996). Oxidant-sensitive AP-1 also mediates the transcriptional activation of resorption modulating cytokine genes such as TGFβ and IL-2. (Birchenall-Roberts *et al*, 1990; Jain *et al*,1992).

Oxidant sensitive transcription factor AP-1 mediated modulation of the osteopontin gene (Hwang *et al* 1994), integral in several processes involving bone remodelling (mineralisation, cell attachment, and migration), also infers modulation of such responses by oxidants. Hypoxia-response element (HRE), another redox regulated promoter on the iNOS gene (iNOS-HRE) has also been reported (Melillo *et al*, 1995).

Oxidants also mediate the activation of the transcription factor, nuclear factor κ B (NFκB). NFκB regulates the transcription of a multitude of pro-resorptive cytokine genes including TNFα, IL-1, IL-6, and GM-CSF (Kus *et al*, 1995; Lieb *et al*, 1996; Thomas *et al*, 1997). It has also been shown that several antioxidants such as vitamin E and pyrrolidinedithiocarbamate can inhibit NFκB activation (Meyer *et al*, 1992; Kus *et*

al, 1995). In addition, the genes for adhesion molecules E-selectin (Rahman *et al,* 1998) and vascular cell adhesion molecule-1 (VCAM-1) (Iademarco *et al,* 1992), the acute phase proteins angiotensinogen and serum amyloid A precursor (Kus *et al,* 1995), as well as the proliferation linked proto-oncogene c-myc (Kessler et al, 1992), are target genes for NFκB, reflecting its role in the maintenance of the inflammatory reaction as well as the bone resorptive processes.

How do reactive oxygen species influence the resorptive processes? The answer is predictably complex but we can begin to unravel the problem based on recently published work. There are several levels at which reactive oxygen species may be involved. Superoxide is known to be generated via NADPH oxidase in the ruffled border (osteoclastic margin of the resorption lacuna) and released into the site of resorption (Key *et al,* 1990; Darden, *et al,* 1996). Superoxide dismutase can inhibit resorption at this level, implying those oxidative processes due to superoxide or oxidising derivatives may enhance the degradation of the matrix itself.

There are several steps in the process leading up to the initiation of resorption activity by the osteoclast that could potentially involve reactive oxygen species. Osteoclasts, which are blood borne multinucleated giant cells from the same haematopoietic mononuclear stem cell lineage as macrophages, have to be recruited and differentiated by the process of osteoclastogenesis. It has been shown that hydrogen peroxide, a product of superoxide dismutation but also generated directly by many cell types, can induce osteoclastogenesis and osteoclast motility *in vitro* (Bax *et al,* 1992; Fraser *et al,* 1996; Steinbeck *et al,* 1998). Fully differentiated osteoclasts have the potential to resorb bone, but only after appropriate stimulation. We have reviewed earlier the factors that are known to induce bone resorption, and it is clear that it is a

diverse group. However, they have in common, the requirement for their effect to be partially mediated via signals from the osteoblast.

It is widely held that in response to stimulation, osteoblasts induce osteoclastic bone resorption by secreting into the medium a small, soluble, labile factor (McSheehy & Chambers, 1987). These are the characteristics of reactive oxygen species and other free radicals such as nitric oxide which has been implicated already in the cytokine-induced bone resorptive processes (Ralston *et al*, 1995). Hydrogen peroxide, in fact, as well as increasing osteoclast number, may be unique in its ability to directly activate osteoclastic bone resorption in the absence of PTH-responsive osteoblasts or other pro-resorptive stimuli (Bax *et al*, 1992; Steinbeck, *et al*, 1998). Osteoblasts directly generate hydrogen peroxide in response to cytokines (Stevens, *et al*, 1993) but, it is also formed by the action of SOD on superoxide, which is also generated in the resorptive environment. Interestingly, a novel, developmentally regulated 150kD plasma membrane glycoprotein related to cytosolic Mn^{++} superoxide dismutase has been reported on osteoclasts and identified as the ligand for the osteoclast-specific monoclonal antibody (MAb) 121F (Oursler, *et al*, 1991). This superoxide dismutase-related membrane component may serve as a signal-transducing molecule at the osteoclast surface, converting osteoblast or osteoclast-derived superoxide to hydrogen peroxide, since its functional blockade using Fab fragments of MAb 121F, dose-dependently inhibit osteoclastic bone particle and pit resorption activity (Collin-Osdoby *et al*, 1998).

It is evident that reactive oxygen (Garrett *et al*, 1990; Bax *et al*, 1992; Steinbeck *et al*, 1998) and nitrogen species (Ralston *et al*, 1995) are involved in the resorptive mechanism. It is therefore important to identify the sources of such reactive oxygen species in bone. There are potentially more than one source, but the ideal candidate

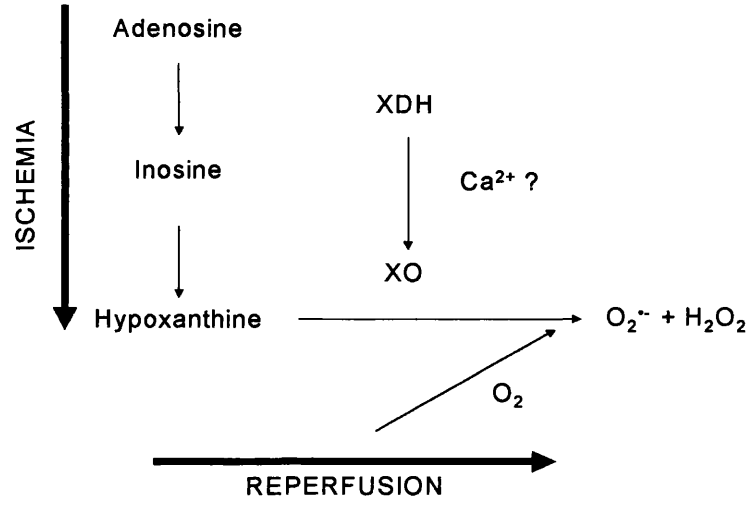
should be controllable and sensitive to the stimuli that have been identified as pro-resorptive. One of the strongest candidates that fulfill these criteria is the enzyme xanthine oxidoreductase.

1.4. Xanthine Oxidase.

1.4.1. Introduction.

Schardinger initially discovered in 1902, that samples of fresh milk decolourised methylene blue upon addition of formaldehyde. It was not until 1922 that Morgan *et al* found a factor in bovine milk, which was able to convert hypoxanthine and xanthine to urate under both aerobic and anaerobic conditions. That factor was identified to be xanthine oxidoreductase. Due to its abundance in bovine milk much work has been performed on this enzyme. It plays a crucial role in purine metabolism to urate, which can result in Gout crystals of urate being deposited in the joints. It is a homodimer of 150kDa subunits existing as two interconvertible forms, xanthine dehydrogenase (XDH; EC 1.1.1.204) and xanthine oxidase (XO; EC 1.1.3.22) (Harrison, 1997). It is believed to play a role in ischaemia-reperfusion injury, via its ability to generate damaging reactive oxygen species, either by the conversion of XDH to XO (Fig. 1.4A) (McCord, 1985) or by XDH (Fig. 1.4B) (Harrison, 1997).

A



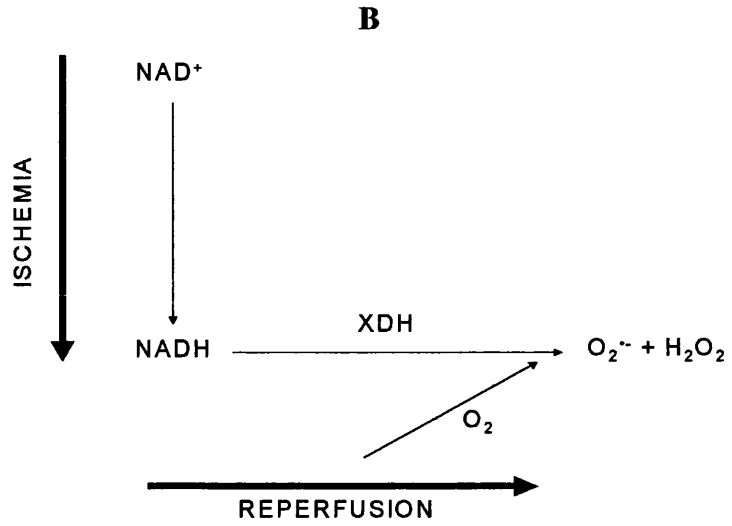


Figure 1.4. Mechanism of ROS production by XOR during ischaemic/ reperfusion by McCord, 1985 (A), and the mechanism for production of ROS from XOR proposed by Harrison, 1997.

1.4.2. Structure and function of xanthine oxidoreductase (XOR).

XOR is a cytosolic and membrane-bound complex molybdoflavoprotein with two Fe-S clusters and is a constitutive enzyme involved in the purine metabolism of most cells (Bray, 1988). During the catalysis of xanthine to uric acid, XDH preferentially uses NAD^+ (dehydrogenase form) whereas; XO uses molecular oxygen (oxidase form) as an electron acceptor. The oxidase form thus generates the superoxide anion radical ($O_2^{\bullet-}$) and H_2O_2 and is therefore a candidate for the source of communication signals in bone resorption. The gene for human XOR has been cloned and investigated. It is located on chromosome 2 at band p22 (Xu *et al*, 1994) and mouse chromosome 17 (Cazzaniga *et al*, 1994) and contains two transcriptional initiation sites consisting of sequences coding for a number of promoter elements associated with the acute phase response genes. These included four CAAT/Enhancer Binding Protein sites, three IL-6 regulatory elements, an NF-κB site and five interferon-γ responsive elements. Other than these inflammation related promoters the gene also

contains an AP-1 motif, three potential AP-2 sites, a potential responsive element for glucocorticoid hormone regulation and a putative hypoxia-inducible factor-1 (HIF-1) binding site (Xu *et al*, 1996). The latter factor may explain the observed upregulation of XOR by hypoxia in a variety of cell types. It can also be seen that the control of the XOR gene itself may be influenced by the oxidants generated by the enzyme, since its promoter elements include oxidant regulated transcription factor binding sites.

As depicted in Fig. 1.5, there are some compounds that can inhibit the function of this enzyme. Most commonly known are allopurinol and its corresponding analogue oxypurinol which exert their effects via the molybdenum (Mo) site of the enzyme. BOF 4272 (Sodium-8-(3-methoxy-4-phenylsulfynilphenyl)pyrzolo[1,5,-a]-1,3,5-tria-zine-4-olate monohydrate) is another compound that also exerts its effects via the Mo site of the enzyme. Another inhibitor of the enzyme is DPI Diphenyliodonium chloride although not as specific as the others it inhibits the enzyme at the FAD site.

Interestingly, work by Sergeev *et al*, 1985 showed that under anaerobic conditions nitrates could be reduced by XO. Recent work by Millar *et al*, 1998 has taken this a step further and have elucidated a novel function for the enzyme. Under hypoxic conditions XOR can reduce inorganic nitrates to nitrite, which can be subsequently reduced to nitric oxide (Fig. 1.5). With hypoxia and reperfusion events taking place in the cell environment, XOR can generate both reactive oxygen and nitrogen species, which could lead to the production of the powerful oxidant, peroxynitrite ($ONOO^-$). The analysis of the XO gene also supplies potential mechanisms for its upregulation in various cell types by the inflammatory cytokines such as TNFα and IL-1β. These cytokines are established inducers of bone resorption both *in vitro* and *in vivo* and it can be construed that their abundance in rheumatoid joints may facilitate the destruction of joints by bone erosion.

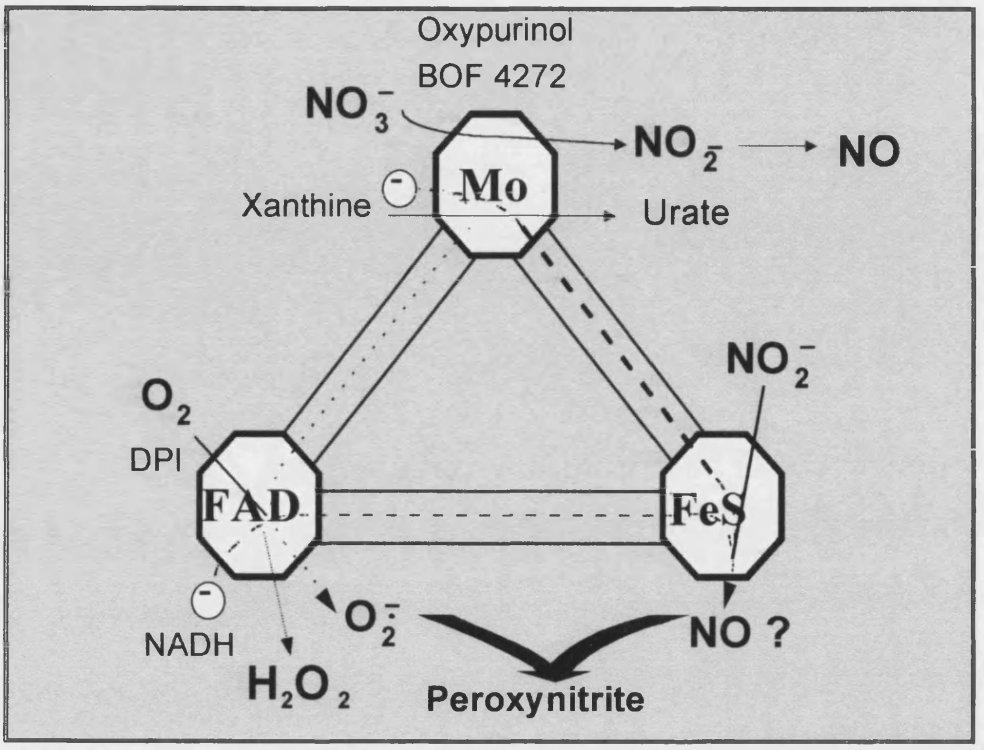


Figure 1.5. A schematic diagram of xanthine oxidoreductase and its abilty to generate reactive oxygen and nitrogen species.

1.5. Summary and formulation of hypothesis.

Bone remodelling involves many stimuli, which act predominately on the two main cell types of bone, namely the osteoblast and the osteoclast. In pathological conditions such as rheumatoid arthritis, this regulated process is altered by the infusion of pro resorptive stimuli resulting in erosion of bone and cartilage. The recognition of the requirement for osteoclast/osteoblast coupling for bone resorption has ascribed further significance to the identification of the signaling processes.

Our ability to halt ersoive damage in diseases such as RA appears at present to be minimal. In the short term steroids have been shown to inhibit erosive development but the effects are not very significant. Non-steroidal cyclo-oxygenase inhibitors have a clear effect on synovitis, but have no effect on preventing bone erosions. This begs the question as to what other mechanism are involved in the erosive process of these diseases.

It is now felt that the fluctuations of the redox environment within the joint especially the synovium may well be a key driving force in the pathology of bone erosion. As XOR has been localised to the endothelium of the rheumatoid synovium (Stevens *et al,* 1991), it was an obvious step forward to investigate possible roles for this multifunctional enzyme in the complex and subtle mechanisms of erosive bone damage in RA. In this respect, this thesis asks the following questions to substantiate the hypothesis that XOR plays an important role in bone resorption. Firstly, is XOR present in the bone microenvironment? Secondly, can pro- inflammatory factors such as TNFα and IL-1β modulate its ability, to generate reactive oxygen species and thus modulate bone resorption. Finally, can inhibitors of XOR and ROS alter the redox state in bone to limit or control cytokine or hormonally-induced bone resorption.

1.6. Hypothesis.

XOR plays a central role in cytokine-induced bone resorption by the generation of reactive oxygen intermediates, which can differentiate and activate osteoclasts to resorb bone. Therefore, the suppression of XOR activity in the pathological bone microenvironment (Fig. 1.6) may have an abating effect on inflammatory or calcitropic hormone-driven bone destructive diseases.

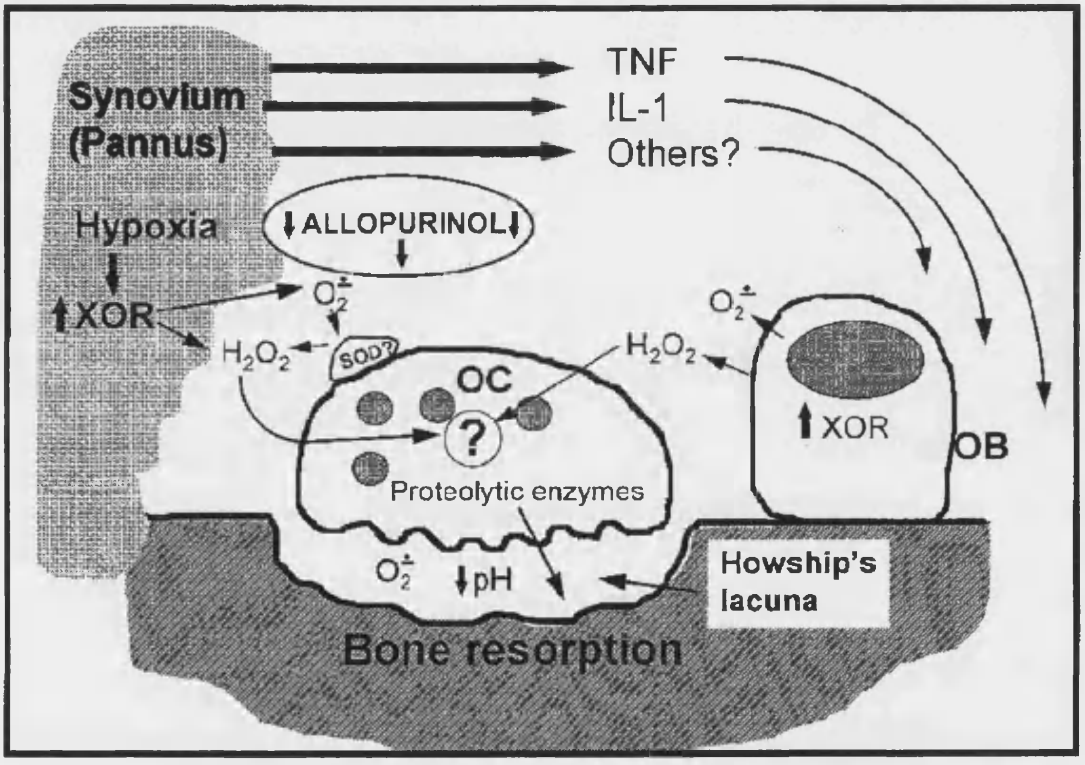


Figure 1.6. Oxidative influences on rheumatoid bone erosions. XOR is a source of oxidants from cells in the synovium and from the osteoblasts (OB). We propose that the influence of these oxidants on the osteoclast (OC) and its precursors can be inhibited by scavengers of these reactive oxygen species and by inhibitors of XOR such as allopurinol.

Chapter 2.

The culture and characterisation of human and rat osteoblasts and the localisation of xanthine oxidase.

Chapter 2

2.1. Introduction.

2.1.1. Osteoblast culture-historical aspects.

Cell culture has been in use by scientists for almost 100 years but its application to bone cells has only become routine since the 1960's (Peck *et al,* 1964). Most of our information on the biology of bone tissue has been derived from morphological studies. Studying the molecular and cellular aspects of osteoblasts is made difficult because of the rigid mineralised composite, the extensive cross-linked extracellular matrix and the heterogeneous cell population in bone. However, recent literature contains numerous and varied reports of cell culture systems for the study of osteoblasts. Bone cultures containing "osteoblastic" cells have been developed from several species, using tissues of different ages, anatomical location and pathophysiological states. Moreover, some systems have been developed by some groups to focus on different cell populations in the lineage of osteogenic cells (Majeska, 1996).

Generally in cell culture, osteoblastic cells are either studied relatively soon after removal from the tissue (human osteoblasts or neonatal animal osteoblasts i.e., rat calvarial osteoblasts) (Wong *et al,* 1986), or after they have been immortalised as permanent cell lines (Majeska, 1996).

2.1.2. Techniques used for characterisation of osteoblasts.

In serial subcultures there is plenty of evidence demonstrating that there is a loss of osteoblastic phenotype and osteogenic potential (Rao *et al,* 1977; Bellows *et al,* 1990; Nakahara *et al,* 1990). Even in long-term cultures of clonal osteoblast cells, phenotypic traits have been shown to be lost (Rao *et al,* 1977). However, not all is lost with osteoblast cells in culture. In 1993, Schmidt & Kulbe documented that some osteoblastic features are retained over numerous passages in mixed bone cell

populations. In contrast, permanent cell lines that are immortalised may offer advantageous stability of osteoblastic phenotype. However, there are some drawbacks to this process. When cells undergo immortalisation (neoplastic transformation) only a part of the phenotype is exhibited. It has been shown that these cells sometimes spontaneously undergo this process in vitro, with loss of the initial phenotype of the cell that was immortalised (Freshney, 1987).

Biochemical markers have been used to identify and characterise osteoblasts in cell culture. One of the most widely used biochemical markers for osteoblasts is alkaline phosphatase activity, which has been implicated in the process of mineralisation. Its expression pattern in osteoblasts can be easily identified histochemically (Doty & Schofield, 1976), or its biochemical activity can be determined by a simple assay (Sodek & Berkman, 1987). Although some cell types such as adipocytes (Beresford *et al*, 1993), fibroblastic cells (the bane of bone biology cell culture work) express levels of alkaline phosphatase, for the most part, they do not express high levels of this enzyme. So if cartilage and marrow can be eliminated during initial dissection of bone specimens then the expression of alkaline phosphatase can be a reasonably specific indicator of the osteoblastic cell phenotype.

The production of bone matrix molecules such as Type I collagen has been found to be a good indicator of osteoblast activity. The ability to respond to calcitropic hormones has also helped characterise the osteoblast, where it has been demonstrated that PTH can modulate alkaline phosphatase activity (Majeska & Rodan, 1982), while Vitamin D_3 can stimulate the production of osteocalcin (bone-Gla protein) in osteoblasts (Owen *et al*, 1991).

Probably the ultimate test of whether you have phenotypically correct osteoblastic cells is the ability to produce an organised, mineralised tissue that

resembles bone. Assays have been developed to assess osteogenesis. This involves osteoblastic cells being cultured as monolayers, supplemented with ascorbic and β-glycerol phosphate acids, which act as co-factors for collagen cross-linking to form stable matrices, and to facilitate mineralisation respectively (Beresford, *et al*, 1993).

2.1.3. Evidence for radical production from osteoblasts.

As previously discussed in chapter 1, the signalling factors released by osteoblasts upon stimulation by pro-resorptive factors has been the subject of much debate in the bone field. The literature has clearly shown that superoxide ($O_2^{\bullet-}$) (Garrett *et al*, 1990; Datta *et al*, 1996) and hydrogen peroxide (H_2O_2) (Bax *et al*, 1992; Fraser *et al*, 1996) potentiate the resorptive mechanism, which begs the question as to what the source of reactive oxygen species are. Ralston *et al*, 1994 have shown that the free radical nitric oxide (NO) influences the resorptive process upon stimulation of the osteoblast by the pro-inflammatory cytokines, TNFα, Il-1β and IFN-γ. Inducible nitric oxide synthase (iNOS) in the osteoblast was found to be the source of this NO. Indeed in an ischaemic, reperfusing system, perfused osteoblasts have been shown to generate measurable amounts of $O_2^{\bullet-}$ and H_2O_2 (Gasbarrini *et al*, 1997), although the source of these radicals were not alluded to.

The gene for the enzyme xanthine oxidase (XO) has a putative hypoxia-inducible factor-1 (HIF-1) in its promoter (Hoidal *et al*, 1997), and the enzyme activity is also sensitive to changes in oxygen tensions (Hoidal, *et al* 1996; Hassoun *et al*, 1998). This makes XO an ideal candidate for the generation of $O_2^{\bullet-}$ and H_2O_2 from the osteoblast. This molybdo-flavoprotein has been implicated in ischaemia/reperfusion mediated damage of vessels (Beetsch *et al*, 1998) and has been shown to be released into the circulation from organs rich in XO activity. It has been demonstrated that XO

can bind to glycosaminoglycans (GAGs) especially to heparin, compared to heparan sulphate, chondroitin sulphate, and dermatan sulphate. However, the binding of XO to endothelial cells has indicated the enzyme to have a higher affinity to chondroitin sulphate in comparison to heparin (Houston *et al*, 1999). Proteoglycans are an integral component of the bone matrix. The latter consists of a core protein to which one or more GAG side chains are covalently attached. Four types of GAGs have been found in bone, chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid (Nijweide, *et al*, 1996). It is possible then that GAGs produced by osteoblasts especially within the developing bone could actually bind XO to the matrix.

2.2. Aims and objectives.

- To isolate, characterise, and culture osteoblasts from animal and human sources.
- To determine whether XO is expressed constituitively in these osteoblasts.
- To determine whether XO is localised to the extracellular matrix of osteoblastic cells.

2.3. Materials and methods.

All reagents used in the following methodology were obtained from Sigma, UK unless otherwise stated.

2.3.1. Rat calvarial osteoblast isolation and culture.

The calvariae is the bone that makes up the cranial portion of the skull – that which lies above the level of the plane which passes through the supra-orbital margins and the superior nuchal lines of the occiput.

Rat calvarial osteoblasts (RCOBs) were isolated using the method of Bellows *et al*, 1986. Calvariae from 5-day-old Wistar rats (Harlan Olac, UK) were dissected out, minced and incubated with 5mls of an enzymatic cell dissociation solution (1mg/ml collagenase, Type II (Sigma) in Hanks balanced salt solution see appendix 1). The calvariae were incubated for 10mins at 37°C/ 95% air and 5% CO_2 in a petri dish and then washed up and down using a sterile plastic pipette. The cells were dissaggregated from the calvariae and the solution was discarded and replaced with 5mls of fresh enzymatic solution and the process was repeated. On the third occasion, the calvariae were incubated for a period of 20 mins. The resulting supernatant with dissaggregated cells were placed in 15ml Falcon™ (Farenheit) tube containing 5mls of serum containing tissue culture medium (10% FCS/DMEM, L-glutamine (2mM), penicillin (100 IU/ml) streptomycin (100ug/ml) with ascorbate (50μg/ml) see appendix I) to deactivate the collagenase. This solution was then pelleted by centrifugation, 1100 rpm (Bench top centrifuge, Denley) for 10mins. The supernatant was aspirated, leaving just the pellet of cells. This pellet was re-suspended in 5mls of tissue culture medium and transferred to a T-25 ($25cm^2$) filter capped cell culture flask (Falcon™). This was placed in a 37°C humidified incubator (5%CO_2/95% air) overnight to allow cells to adhere to the plastic. This represented population III of osteoblastic cells isolated from

the rat calvariae. This procedure was further repeated twice on the same samples providing osteoblast cell populations, IV and V. The next day fresh TCM replaced the existing one in the T-25 flask and from there on, the culture medium was replaced every 3-4 days. When the cells reached confluence the cell populations III-V were disaggregated by a 1x solution of Trypsin/ EDTA (Sigma, see appendix III) pelleted down as before and re-suspended in fresh TCM. The resuspended cells were then transferred to 4, T-75 ($75cm^2$) flasks (Falcon^{TM}) which were then incubated. Cells from passages 2-4 that were greater than 85% alkaline phosphatase positive (section 2.3.4) were used in the experiments.

2.3.2. Isolation and culture of human osteoblast-like cells.

Bone samples from patients undergoing elective surgery for either hip or knee replacements were collected in sterile pots. Sterile Hank's Balanced Salt Solution (HBSS) was added to the pots and then transferred to a Class II sterile cabinet. The samples were placed in a 100mm dish, and washed 2-3 times with HBSS to remove any blood. The method of Gallagher *et al*, 1996 was used to isolate human osteoblast like cells. Firstly, extraneous synovium, fibrotic and connective tissue was removed from the outer surfaces of the bone samples. If the fragments were large they were diced into pieces 3-5mm in diameter. The bone fragments were then transferred to a 50ml polypropylene tube (Falcon^{TM}) containing HBSS. The tubes were then vortexed three times for 10 seconds and left to stand for 30 seconds to allow bone fragments to settle. The supernatant containing the haematopoietic tissue and dislodged cells were carefully decanted off and the whole process was repeated at least three times, or until no remaining haematopoietic marrow was visible and the bone fragments had assumed a white, ivory-like appearance.

The bone fragments were cultured as explants (approximately 10 explants per 100mm dish) in 7mls of medium at 37°C in an humidified incubator of 95% air, 5% CO_2. The cultures were left for 7 days, where the TCM was carefully replaced (appendix II). This was repeated again and cultures were then subjected to TCM changes twice weekly thereafter. Cell cultures generally reached confluence at about 4-6 weeks postplating.

2.3.3. Human cell lines (MG63 and Saos2).

The human osteoblast-like cell lines, Saos-2 (ECACC number 89050205) and MG-63 (ECACC number 86051601) derived from osteosarcomas, were kindly provided by Dr. Jon Beresford at the University of Bath. They were grown in osteoblast culture medium (see appendix I) in a 37°C humidified incubator (5%CO_2/95% air).

2.3.4. Characterisation of osteoblasts *in vitro*.

An azo-dye coupling method (Bancroft & Stevens, 1982) with slight modification was used to determine the activity of alkaline phosphatase (ALP), [orthophosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] in osteoblasts in culture (Ali *et al*, 1993). Osteoblast cells and fibroblasts (controls) were grown on six-well plates (Falcon™) and washed in cold 1x PBS w/o Ca^{2+} & Mg^{2+}(Gibco) and fixed in cold 4% formal saline (BDH) for 10 mins. Cells were washed again 1x PBS (3 times) and incubated for 30mins in a freshly prepared, filtered solution of napthol AS-MX phosphate dissolved in dimethylformamide (10mg/ml) and Fast Blue BB salt (10mg/ml) in 50mM Tris buffer at pH 9 (see appendix IV). Alkaline phosphatase activity of osteoblasts was confirmed by positive blue staining of the cells. Cells were observed microscopically using a Nikon TMS® inverted microscope and photographed using a Nikon Diaphot TMD® inverted microscope with an attached Nikon F-601 automatic focus camera.

2.3.5. Immunolocalisation of xanthine oxidase staining in osteoblasts in culture.

Confluent osteoblasts were harvested by non-enzymatic cell dissociation solution (appendix III) and pelleted by centrifugation, 1100 rpm (Denley). The cells were suspended in a known volume of 1x PBS and 0.4% Trypan Blue (1:1 ratio, see appendix III) and a 100μL aliquot was used to determine the total number of viable cells by counting on a haemocytometer. The cells were again pelleted by centrifugation, and re-suspended in a volume of culture medium (10% FCS/ DMEM/penicillin/streptomycin; see appendix1) and plated onto an 8-well chamber culture slide (Nunc, Gibco, see appendix VI) at a seeding density of 15,000 cells per chamber. The culture slides were placed in a humidified 37°C; 5% CO_2; 95% air incubator (Techgen, UK) and incubated overnight. The cells were removed from the incubator and washed three times in cold 1x PBS (w/o Ca^{2+} and Mg^{2+}) (Gibco, UK), fixed with ice-cold methanol:acetone (1:1) for 3 minutes and washed again three times with 1X PBS.

The cells were incubated in blocking buffer (5% non-fat dried milk, Marvel® in 1x PBS/0.5% Tween 20, see appendix IV) for 1hr and further incubated with diluted (1:100) rabbit polyclonal anti-bovine XO Ab (Chemicon, Harrow, UK) in blocking buffer overnight at 4°C. Control slides were incubated with the blocking buffer alone (-ve control) in parallel. The cells were washed with 1x PBS/0.5% Tween 20 (x 6, 5 mins each) and then incubated with biotinylated goat anti-rabbit IgG (1:100) (Vector Labs, Peterborough, UK.) in blocking buffer for 1hr. After rinsing the cells again (x 6, 5 mins) they were incubated with a 1:100 dilution of an alkaline phosphatase-conjugated avidin biotin complex (ABC) (Vectastain, Vector Labs, Peterborough, UK.) for 30mins. The cells were washed again with 1x PBS/0.5% Tween 20, and finally developed with Sigma Fast Red™ (Fast Red TR/Napthol AS-MX Phosphate (4-chloro-2-methyl-

benzenediazonium/3-hydroxy-naphthoic acid, 2,4-di-methylanilide phosphate (a napthol AS-MX) in Tris Buffer (0.1M) at room temperature. Levamisol (0.15mg/ml) was also added to the solution to block endogenous alkaline phosphatase activity. The cells were rinsed in cold tap water to stop the reaction, counterstained with Mayer's haematoxylin rinsed with H_2O and mounted in Aquamount (Merck, Lutterworth, UK). Slides were photographed using a Zeiss photomicroscope mark III.

2.3.6. Isolation of the extracellular matrix network from cultured rat calvarial osteoblasts for XO immunolocalisation.

Extracellular matrix (ECM) of RCOB's was prepared according to methods of Rinehart *et al*, 1993 and Ho, *et al*, 1996 with slight modifications. RCOB's were plated into 4-well plates (Nunc) with 0.5mls of TCM and grown to confluence. The cells were washed three times with HBSS at room temperature. The HBSS was removed and the confluent RCOB's were treated with 25mM ammonium hydroxide for a period of 5 mins. The cells were then washed again with HBSS a further five times ensuring careful agitation of the solution. The ECM was then fixed with ice-cold methanol: acetone 1:1 for three mins and then washed three times with 1x PBS (w/o Ca^{2+}, Mg^{2+}). XO immunolocalisation was carried out as previously described in section 2.3.5.

2.4. Results.

2.4.1. Morphological characterisation of primary human osteoblastic cells, rat and human osteoblastic cell lines.

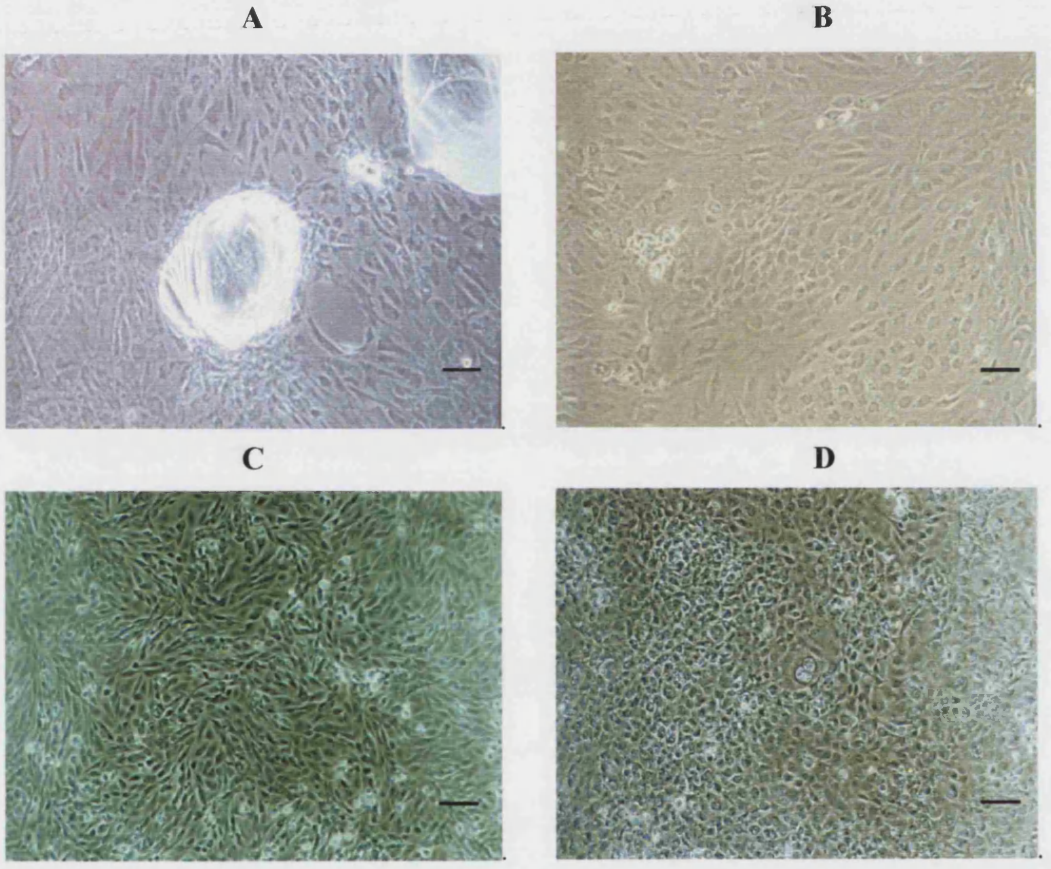


Figure 2.1. Photomicrographs of osteoblasts in cell culture. (A) Human osteoblasts, (B) rat calvarial osteoblasts, (C) Saos-2 and (D) MG-63 human osteoblastic cell lines. Scale bars represent 50μm.

Confluent monolayers of osteoblasts are shown in Fig. 2.1 (A) human osteoblasts from an explant of trabecular bone, (B) RCOB's (passage 2) and human osteoblast cell lines (C) Saos-2 (passage 41) and (D) MG-63 (passage 109). All the osteoblasts from varying sources presented the classical "cuboidal" shape. RCOB's and the cell lines had comparable growth rates (approx. 5 days to obtain a confluent monolayer of a T-75 cell culture flask) unlike the human osteoblast, which took between 3-4 weeks to acquire a confluent monolayer in a T-75 cell culture flask.

2.4.2. Cytochemical characterisation of osteoblasts using alkaline phosphatase activity as an index.

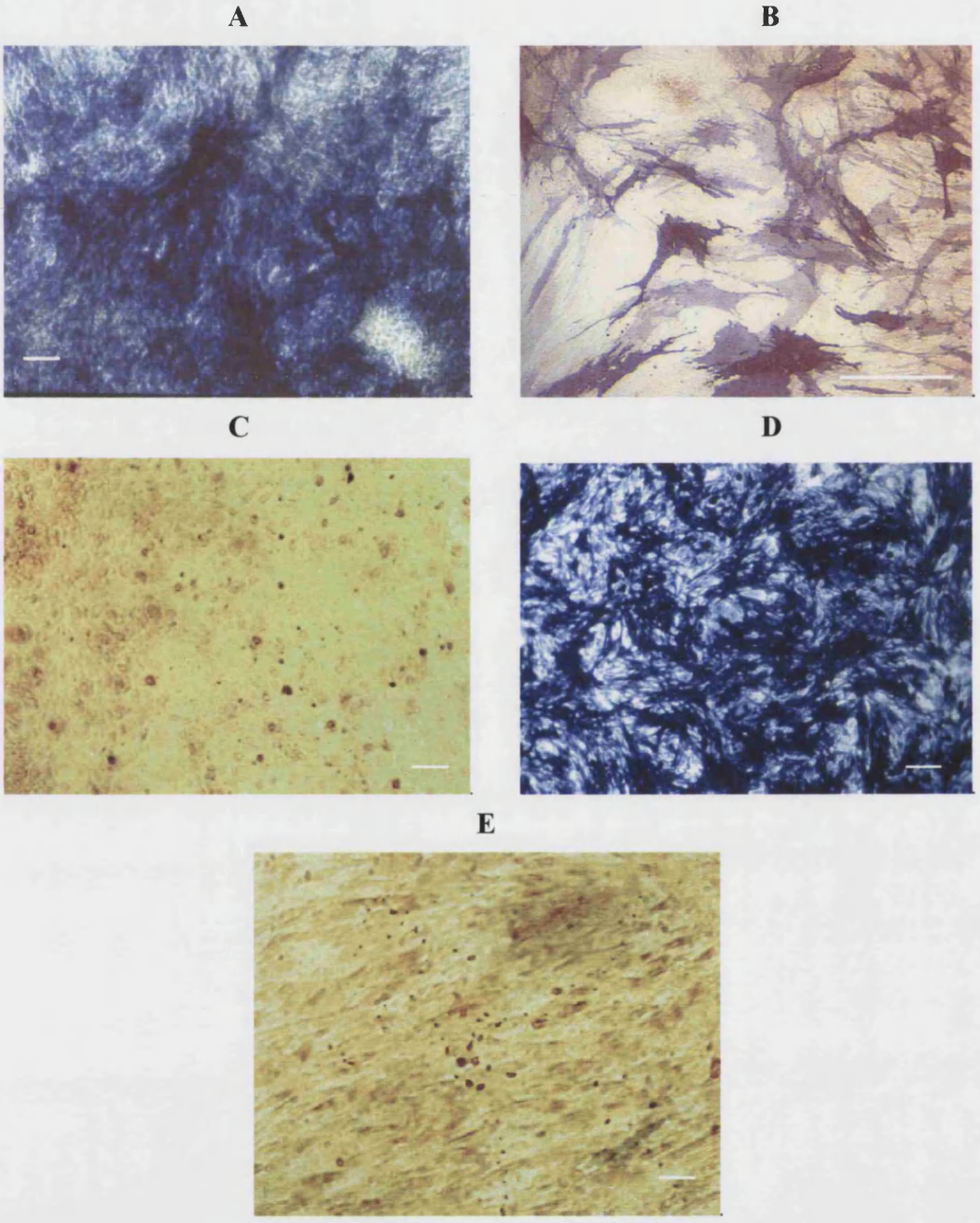


Figure 2.2. Photomicrographs of osteoblasts stained for alkaline phophatase activity. (A) RCOB's, (B) Human osteoblasts, (C) Human osteoblast cell lines MG-63 and (D) Saos-2. (E) Fibroblasts. Scale bars represent 50μm.

As depicted in Fig. 2.2 RCOB's (P2) (A) and Saos-2 (P41) (D) showed very strong positivity for alkaline phosphatase activity whereas the MG-63 (P109) (C) cell

line was negative. Human osteoblasts (P1) (B) derived from trabecular bone explants at a sub-confluent density showed positive alkaline phosphatase activity. Fibroblasts cultured from the adventitial layer of an artery were used as a negative control for the staining of alkaline phoshatase activity. Fig. 2.2 (E) shows negligible amounts of alkaline phosphatase in fibroblasts *in vitro* at P2.

2.4.3. Localisation of xanthine oxidase in rat calvarial osteoblasts.

As depicted in Fig. 2.3. rat calvarial osteoblasts showed positive immunoreactivity for xanthine oxidase. It appears from both slides that the majority of immunoreactive protein appears to be localised to the peri-nuclear regions of the cells. A blue nucleus represents the counterstain Mayer's haematoxylin.

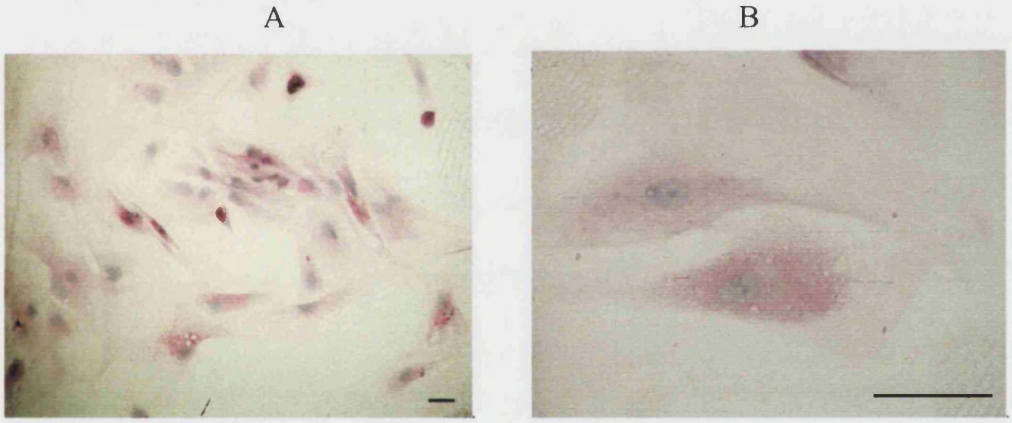


Figure 2.3. Photomicrographs depicting (A & B) immunolocalisation of XO (red) in RCOB's. Cells were counterstained with Mayer's Haematoxylin. Scale bars represent 50μm.

2.4.4. XO immunolocalisation in human osteoblast-like cells.

Figures 2.4 A-C indicate human osteoblast-like cells (alkaline phosphatase positive) isolated from bone specimens obtained from patients undergoing elective surgery showing positive immunoreactivity for XO in sub confluent cultures. Osteoblastic cells from the first three passages were used in these experiments. To

visualise all cells, including non-immunostained cells, a haematoxylin counterstain was applied which depicts a blue nucleus.

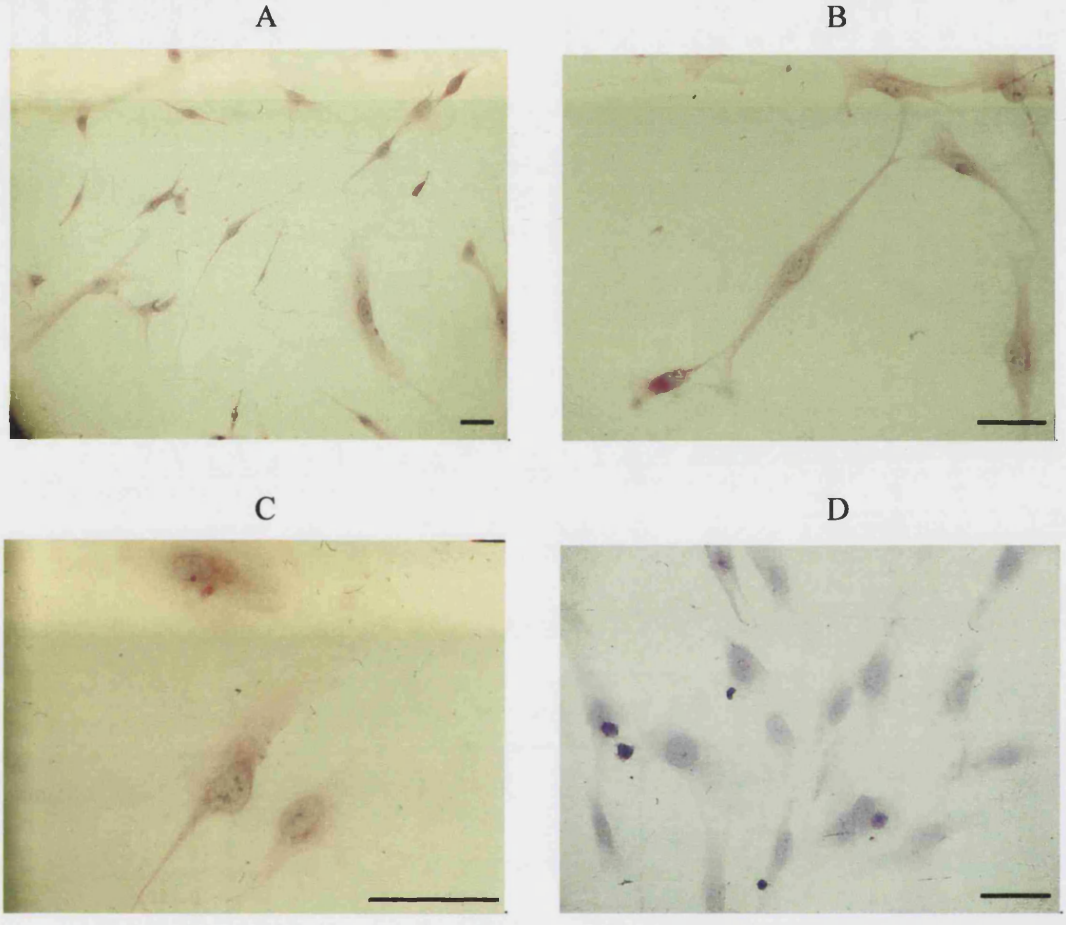
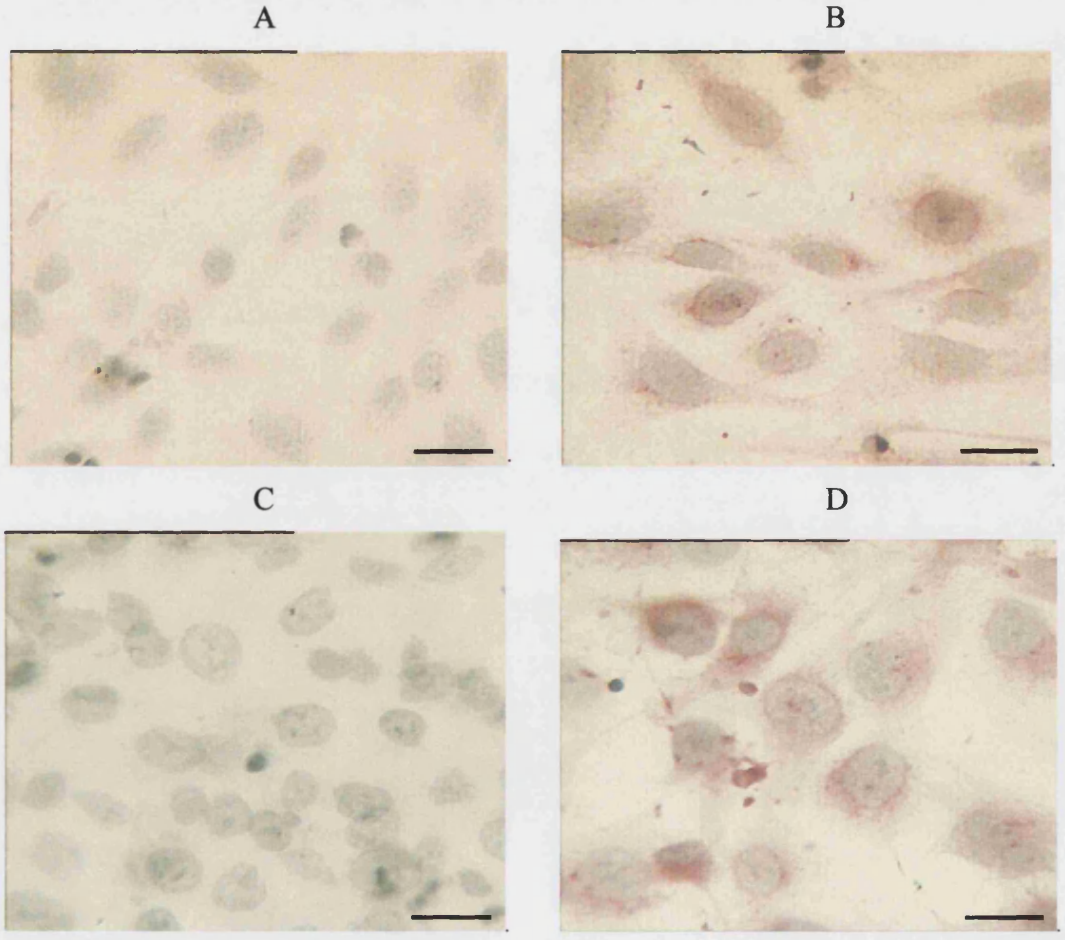


Figure 2.4. Photomicrographs showing the immunolocalisation of XO (red) in human osteoblast like cells (A-C). Control (D) stained for Mayers haematoxylin. Scale bars represent 50μm.

2.4.5. Immunolocalisation of XO in human osteoblastic cell lines Saos2 and MG-63.

The human osteoblast-like cell lines SaOS2 & MG-63 derived from human osteosarcomas also showed immunoreactivity for XO. Both cell lines showed a similar pattern of staining for XO which is predominately localised to the perinuclear region of the cells (Fig. 2.5 B, D & E). Control cells (primary antibody omitted) were negative for any XO (Fig. 2.5 A & C). All cells were counterstained with Mayer's haematoxylin to depict the nuclei.



E

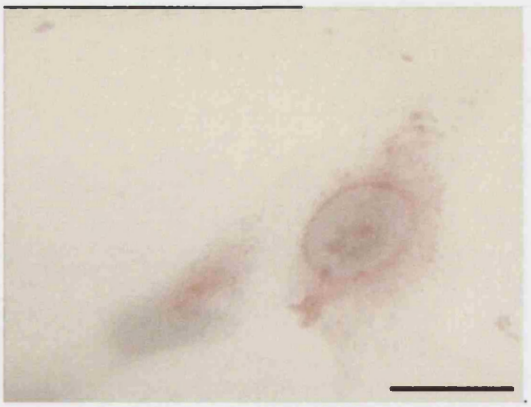
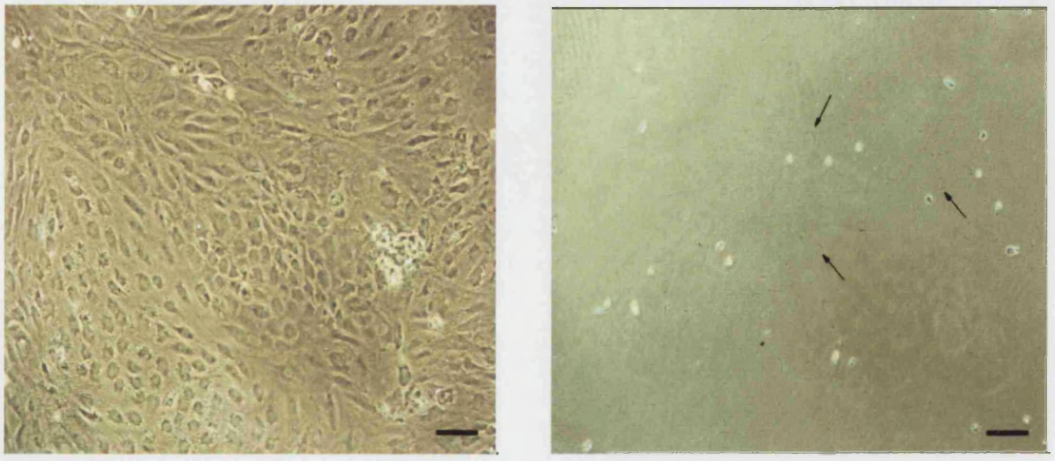


Figure 2.5. Photomicrographs showing immunolocalisation of XO (red) in the human osteoblast cell lines Saos2 (B), MG-63 (D & E). Control; (plates A & C). XO; (plates B, D & E. All cells were counterstained with Mayer's haematoxylin. Scale bars represent 30μm.

2.4.6. Localisation of XO to the extracellular matrix (ECM) of osteoblasts.

After the addition of 25mM ammonium hydroxide to the confluent RCOB monolayer (Fig. 2.6 A), although very difficult to see, the extra cellular matrix of some of the cells ("ghost-like" images) (arrows) were isolated (Fig. 2.6 B). ECM isolated from these RCOB's stained positively for XO (arrows). (Fig. 2.6 C & D). This investigation was carried out twice using two preparations as controls and two for XO immunolocalisation in each study.

A B



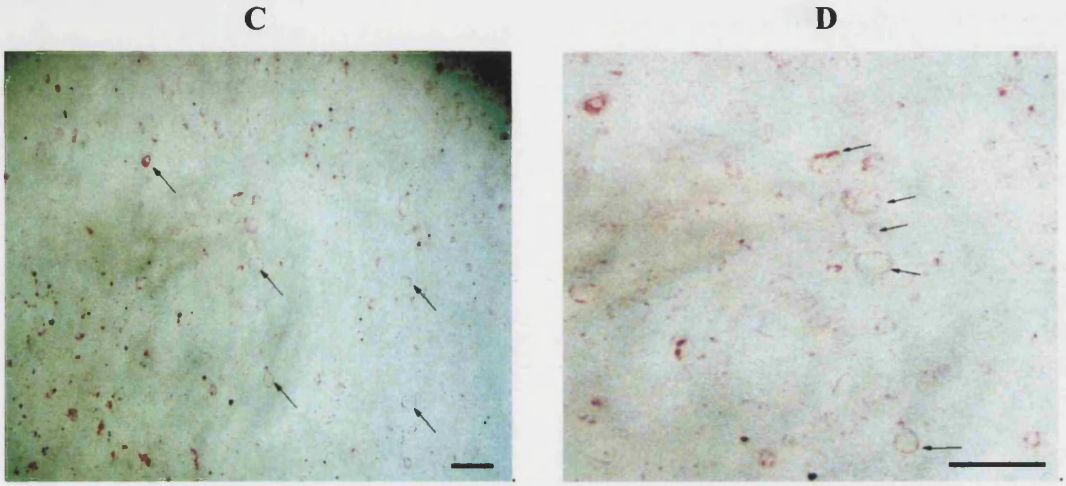


Figure 2.6. Phase contrast micrographs showing the localisation of XO to the ECM (arrows) of rat calvarial osteoblasts. (A) Confluent monolayer, (B) control ECM, (C) XO staining of ECM, (D) XO staining of ECM (high magnification). Scale bars represent 50μm.

2.5. Discussion.

Analysis of bone tissue-related research reveals a general consensus that many of the resorptive stimulating agents do not act directly on the osteoclasts but produce their effects through osteoblasts or other cells in the bone micro-environment. Factors that have been shown to induce resorption via signals from the osteoblast through co-culture experiments are 1,25-dihydroxyvitamin D_3, PTH, prostaglandins and the inflammatory cytokines TNFα and IL-1β. The nature of these, small, labile intercellular signals that are involved in the communicative mechanism of osteoblast/osteoclast coupling still remain to be identified. Originally the factor involved in cytokine-induced bone resorption was thought to be prostaglandins, but work by Lerner *et al,* 1993 showed that cytokines could still initiate bone resorption even if the prostaglandin activity was blocked. Relatively recent reports have identified reactive oxygen and nitrogen species as stimulating agents for osteoclastic bone resorption (Garrett *et al,* 1990; Bax *et al,* 1992; Ralston *et al,* 1995; Fraser *et al,* 1996; Steinbeck *et al,* 1998). In these studies, inhibition of NO by L-N^G-monomethyl arginine (LMMA) reduced TNFα and IL-1β -induced bone resorption in vitro (Ralston *et al,* 1995) whereas, catalase was shown to inhibit H_2O_2-induced bone resorption (Fraser *et al,* 1996).

H_2O_2 appears to be a pivotal metabolite not only for the differentiation of osteoclast progenitors to mature osteoclasts but in the activation of mature octeoclasts to resorb the bone matrix. It has been shown that H_2O_2 can be generated from osteoblast cells that have been subjected to cycles of hypoxic/reperfusion (Gasbarrini *et al,* 1997) and that increased levels of H_2O_2 have also been detected in osteoblast culture media after TNFα stimulation by ^{1}H-NMR spectroscopy (Stevens *et al,* 1993). It is clear from previous reports that ROS are involved in the process of bone resorption. However, the

likely source(s) of these species has not been thoroughly questioned previously. Xanthine oxidase has the potential to generate the superoxide anion and H_2O_2. Results presented in this chapter show that XO is present in rat calvarial osteoblasts as well as human osteoblast-like cells. The two osteosarcoma osteoblast cell lines studied SaOS2 and MG-63, also stained positively for XO although they showed varying patterns of alkaline phosphatase activity staining. The predominant perinuclear immunolocalisation of XO may be an indication of its involvement in signal transduction, since reactive oxygen intermediates such as H_2O_2 are implicated in the activation of ubiquitous transcription factors such as the nuclear factor Kappa B (Schmidt *et al*, 1995).

In 1991 Fuller *et al* showed that an osteoclast resorption-stimulating activity (ORSA) factor could bind to glycosaminoglycans (GAGs) on the surface or the ECM of osteoblast cells. These findings imply that GAGs may facilitate localisation of ORSA, which would be a critical factor in activating the coupling of osteoblasts and osteoclast progenitors. The identity of this metabolite still remains elusive but on the results presented in this thesis it is postulated that XO could be correlated with ORSA.

Although XO is generally thought to be a cytoplasmic enzyme, its precise localisation is not clear since it has also been localised to peroxisomes (Angermuller *et al,* 1987; Dikov et al, 1988) as well as the cytosol (Ichikawa *et al*, 1992). However, recent studies have demonstrated that XO is localised on the outer surface of human endothelial and epithelial cells (Rouqette *et al*, 1998), and has the ability to bind to GAGs (Adachi *et al*, 1993) such as heparan sulphate, chondroitin sulphate and dermatan sulphate (Radi *et al*, 1997). Houston *et al,* 1999 demonstrated chondroitin sulphate to be one of the major GAGs on the endothelial cell surface that circulating XO could bind to. The bone matrix is known to contain such GAG macromolecule proteoglycans,

which consist of a core protein to which one or more GAG side chains are covalently attached. Chondroitin sulphate was shown to be synthesised by the osteoblast-like cell line UMR 106-01 (McQuillan, *et al*, 1991) and involved in bone mineralisation (Slater *et al*, 1994). This particular GAG was previously localised to the thin layer of un-mineralised matrix of the bone lacuna walls surrounding the osteocyte and in the canaliculus (Takagi *et al*, 1991). Results presented in this chapter showing localisation of XO to the ECM of RCOB's may support the notion that XO, like some growth factors, may be incorporated into the ECM of osteoblasts and subsequently into the bone matrix. This provides a putative source of a low level ROS generation which would play a significant role in regulating the bone resorptive process as suggested by Fuller *et al*, 1991.

Chapter 3.

The effect of hydrogen peroxide, catalase and XO inhibitors on mouse calvarial bone resorption.

Chapter 3

3.1. Introduction.

Free radicals have been reported to play a role in regulating the activities of osteoclastic bone resorption (Collin-Osdoby *et al*, 1995; Ralston *et al*, 1995). Though it was originally shown by Garrett *et al* (1990), that superoxide ($O_2^{\bullet-}$) generated from xanthine/xanthine oxidase was responsible for resorption, it has now been demonstrated that another reactive oxygen species, namely hydrogen peroxide (H_2O_2) can stimulate osteoclastogenesis and increase the resorbing activity of the osteoclast (Bax *et al,* 1992; Suda *et al*, 1993; Fraser *et al,* 1996; Steinbeck *et al*, 1998). $O_2^{\bullet-}$ and H_2O_2 may have differing roles in bone resorption due to their biological characteristics in cells. H_2O_2 can easily penetrate the membranes of surrounding cells whereas $O_2^{\bullet-}$ cannot (Okabe *et al,* 1990). H_2O_2 has been demonstrated to be involved in the signalling activation of nuclear transcription factors such as NF kappa B (Schmidt *et al*, 1995) and as previously mentioned involved in osteoclastogenesis. $O_2^{\bullet-}$ on the other hand, may have a role in osteoclastogenesis indirectly by being dismutased to H_2O_2 by cell-surface superoxide dismutase enzymes, but its main role, as evidenced by Ries *et al* (1992) and Steinbeck *et al* (1994), is to facilitate bone resorption by degrading collagen in the sealed zone of the lacuna underneath the osteoclast.

We have already demonstrated XO to be localised in "osteoblast-like" cells (Chapter 2). It therefore seems possible that this enzyme could play an integral part in the production of $O_2^{\bullet-}$ and H_2O_2 leading to the activation of the resorptive processes of bone. Before trying to establish whether XO is involved in the process of cytokine or hormonal induction of bone resorption, it has to be identified whether it is directly involved in low level signalling of osteoblast/ osteoclast coupling, or is induced by external stimulating factors.

One of the best-characterised XO inhibitors is allopurinol (1,5-dihydro-4H-pyrazolo [3,4-d] pyrimidin-4-one) (Watts *et al,* 1965). Its main function is to act on purine catabolism without disrupting the biosynthesis of purines. It reduces levels of uric acid by inhibiting its production. It is a widely accepted inhibitor of XO, which is responsible for the conversion of xanthine to hypoxanthine to uric acid. Allopurinol exerts its effect at the molybdenum site of the enzyme where it is metabolised to the corresponding analog oxypurinol (alloxanthine), the tight-binding competitive inhibitor of XO.

Gout is a metabolic disorder, which is characterised by hyperuricaemia with resultant deposition of monosodium urate in the tissues, particularly in the joints and kidneys. The aetiology of this hyperuricaemia is the overproduction of uric acid in relation to the patient's ability to excrete it. Administration of allopurinol generally results in a fall in both serum and urinary uric acid within two to three days. The degree of this decrease can be tightly controlled since the effect is dose-dependent.

Another inhibitor of XO is BOF-4272, which binds tightly to both the oxidised and reduced forms of the enzyme (Okamoto & Nishino, 1995). It has been shown *in vivo* that it inhibits rat liver xanthine dehydrogenase for longer periods compared to allopurinol (Sato *et al,* 1991). Furthermore, BOF-4272 is not a strong scavenger of superoxide radicals (Okamoto & Nishino, 1995). In addition to this, the compound has a (+)- isomer which is much weaker inhibitor than the (-)- isomer. This may be a valuable asset in studies of the role of xanthine oxidase in *in vivo* models of pathology (Okamoto & Nishino, 1995). The addition of these XO inhibitors to the calvarial cultures should elucidate whether XO is involved in basal bone resorption.

As already demonstrated in the literature, H_2O_2 is involved in bone resorption. A scavenger of this reactive oxygen species is catalase. It is a haem-containing redox

enzyme that is found in high concentrations in cell compartments known as peroxisomes (del Rio *et al*, 1992). It has the ability to disproportionate H_2O_2 to H_2O and molecular oxygen (Deisseroth & Dounce, 1970). Again, before applying catalase to cytokine- or hormone-induced bone resorption in culture, it is important to determine whether catalase has any effect on basal levels of mouse calvarial bone resorption.

One of the standard indicators of bone resorptive activity in the mouse calvarial assay is the release of Ca^{2+} into the culture medium. This can either be detected by initially radio-labelling neonatal mouse calvariae with ^{45}calcium chloride and measuring the amount of ^{45}Ca release by liquid scintillation counting (Lerner & Ohlin, 1991), or by using a colourimetric assay such as cresolphthalein complexone (Toffaletti & Kirvan, 1980). Another indicator of bone resorptive activity is the detection of [^{3}Hydroxy]-proline released from pre-labelled bones into the culture medium (Lerner, 1987).

Although release of calcium is an established marker of bone resorption, it does have its drawbacks. As the calvariae in culture are capable of the active processes of both resorbing and forming bone, Ca^{2+} may be incorporated by the osteoblast in the formative process of new bone, therefore altering the net release of calcium by the active osteoclasts. Also, the isolation of calvariae from neonatal mice is not 100% clean. Although calvariae are rigorously washed and incubated for 24 hrs before starting any experiments, other cell types such as blood cells may still be attached and might have a slight effect on the levels of Ca^{2+} release. Another good marker of active bone resorption is the release of the 3-hydroxypyridinium cross-links of mature collagen, pyridinoline (pyr) and deoxypyridinoline (D-pyr). They are only present in a mature form and are specific to bone and cartilage (Dequeker, 1996). As these peptides are naturally fluorescent and are unable to be broken down by osteoclasts they are released into the circulation. Levels of these peptides have been shown to be

quantitatively measured in the urine by reverse-phase high performance liquid chromatography (HPLC) (Black *et al,* 1988), providing a sensitive and specific marker for bone resorption. If calcium release is a true reflection of bone resorption activity then the amount of calcium release should correlate with the level of pyridinoline crosslinks that are released upon osteoclastic breakdown of the collagen matrix.

3.2. Aims and Objectives.

- To test the sensitivity limits of our calvarial assay using the cresolphthalein complexone detection system of calcium.
- To determine whether the release of Ca^{2+} from calvariae correlates with other indices of resorptive activity.
- To establish if hydrogen peroxide can stimulate bone resorption, using this mouse calvarial assay system.
- To determine whether allopurinol, BOF-4272 (xanthine oxidase inhibitors) and catalase (hydrogen peroxide scavenger) have any effect on the basal levels of bone resorption.

3.3. Materials and methods.

3.3.1. Mouse calvarial bone resorption.

Calvariae were isolated from MF-1 strains of mice (Harlan-Olac, UK). Five-day-old mice were collected and terminated by cervical dislocation and then placed in Hank's balanced salt solution (HBSS) (appendix I). This took place under strict guidelines set out by the Home Office, and was undertaken in a designated area of the animal house at the University of Bath.

The mice were then transferred to a class II cabinet where they were sterilised by immersion in industrial methylated spirits (IMS). They were then individually laid out on a sterile board and pinned down at the nose and the tail of the body. The skin was removed by pinching the back of the neck with a pair of forceps and cutting it away from the calvariae, limiting any damage to the calvariae.

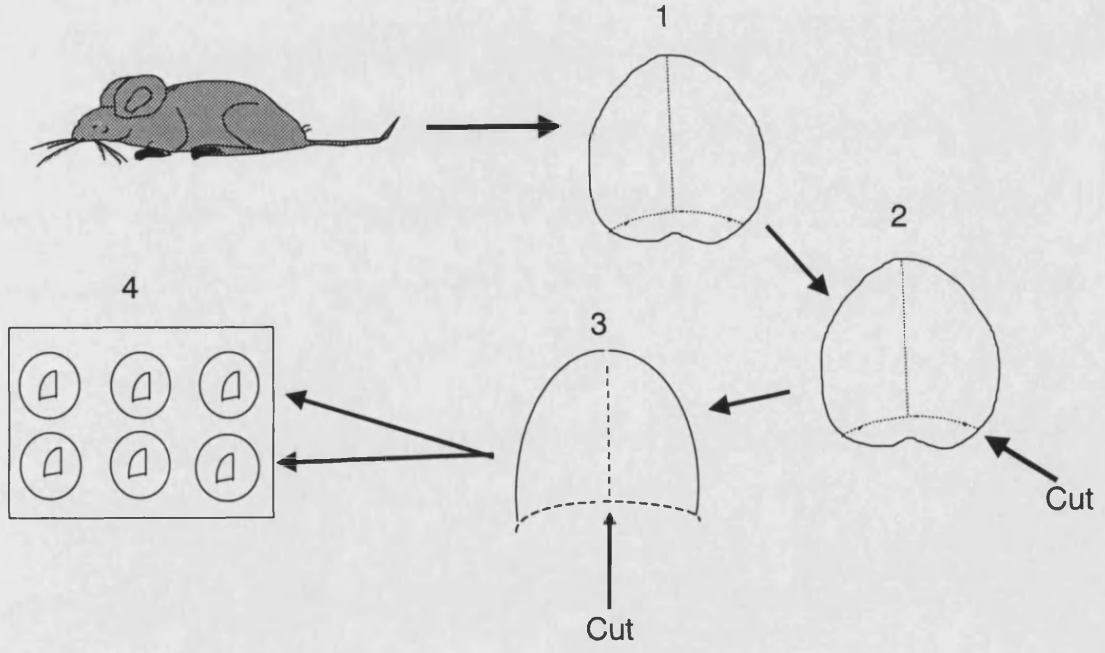


Figure 3.1. Isolation of mouse calvariae.

Fig. 3.1 is a schematic representation of the procedure used to isolate mouse calvariae. Briefly, using sterile forceps and curved scissors, the calvariae was cut away

from the body by first cutting through one ear across the back of the neck to the other ear, then cutting down the sides of the calvariae to the apex (1). Holding the bottom of the calvariae with the forceps the extraneous soft tissue was cut away and the calvariae was trimmed to obtain a uniform size, which was then placed in a dish containing HBSS (2). The calvariae were then washed three times with HBSS, and then transferred to a new petri dish where they were divided into two equal pieces by cutting down the saggital suture using a No. 22 surgical scalpel blade (3). Using the scalpel blade, the hemi-calvariae were carefully transferred to a new petri dish containing HBSS and again were washed three times. Individual calvariae were placed in six well plates (Falcon^{TM}) containing 1.5mls of bone resorption TCM (appendix II) and transferred to a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air. They were left to equilibriate overnight (4).

After 18-24hrs, the calvariae were washed three times with HBSS and incubated in the test media. Each treatment contained no less than 4 hemi-calvariae. After 4 days of incubation, the medium from each well containing the calvariae were collected individually and placed in a screw cap plastic tube (Alpha labs, see appendix II) and stored at 4°C. The calvariae from each experiment were stored in either formal saline (appendix 1.) for immunohistochemical analysis, or frozen in OCT/liquid N_2 and stored at –70°C.

Analysis of the tissue culture medium was performed using the calcium kit (Sigma N°. 587, see appendix II). Briefly, in disposable plastic 1.5ml cuvette (Kartell), 10μL of tissue culture medium was added to 990μL of cresolpthalein complexone producing a red complex at pH 10-12 with an absorbance maximum at 575nm. This was measured using a Cecil, series 2 spectrophotometer (model CE 373).

Figure 3.2. The reaction of Ca^{2+} with o-cresolphthalein produces a red complex at pH 10-12 with an absorbance maximum at 595 nm.

The original experiments at The Royal London Hospital, Whitechapel, involving cytokine- and hormone-induced bone resorption were performed on calvariae isolated from Swiss albino CD-1 mice (Harlan Olac, UK). The protocol was followed as before with the MF-1 mice, but the analysis of calcium released into the tissue culture medium was performed, using an automated spectrophotometer (Monarch 2000, Instrumentation laboratory, Warrington.) (see appendix VI).

A serial dilution of 2.5mmols/L of calcium standard solutions (Sigma, see appendix II) were prepared and incubated with cresolphthalein and measured at 575nm on a spectrophotometer. Similarly, 0.5mmols/L-10mmols/L doses of calcium standards solution (Instrumentation Labs, see appendix II) were incubated with cresolphthalein and measured on the Monarch 2000 to determine standard curves for calcium.

3.3.2. The effect of hydrogen peroxide on mouse calvarial bone resorption.

Increasing doses of hydrogen peroxide (1nM-1mM) (Sigma, UK) was added to the culture medium. 1.5mls of the TCM including the H_2O_2 were placed in 6-well plates (Falcon,UK see appendix VI) to which the hemi-calvariae were added. The hemi-calvariae were then incubated for a period 96hrs in a humidified atmosphere 5% CO_2/ 95% air. The media from the 6-well plates were collected and 10μL aliquots were placed in a 1.5ml cuvette to which 990μL of the cresolpthalein complexone mixture was added. The absorbance of the solution was then measured using a spectrophotometer at 575nm.

3.3.3. The effect of catalase on basal levels of calvarial resorption.

To determine whether catalase, a haem-containing redox enzyme that converts H_2O_2 to water and to molecular oxygen, had any effect on the basal levels of bone resorption, increasing doses were added to the culture medium and Ca^{2+} release from the hemi-calvariae were determined. Briefly, 5-day-old mouse calvariae were isolated as previously described (section 3.3.1) and incubated for 24hours in resorption TCM. The calvariae were washed three times with HBSS in 6-well plates, where they were incubated for a further 96 hours with 1.5mls of TCM that included varying doses of murine catalase (0-100 IU/ml) (Sigma, UK), (see appendix II). Following incubation, the TCM was collected and assayed for the amount of calcium.

3.3.4. The effect of allopurinol & BOF-4272 on basal levels of calvarial resorption.

Allopurinol (0.01μM-10μM) (Sigma, UK) a xanthine oxidase inhibitor, was added to the TCM containing the calvariae and incubated for 96hrs. Aliquots of the TCM were assayed for Ca^{2+} as described before.

The same procedure for allopurinol was applied to another XO inhibitor, BOF-4272 (sodium-8-(3-methoxy-4-phenylsulfynilphenyl) pyrzolo[1,5,-a]-1,3,5-tria-zine-4-olate monohydrate) (Ozuka Pharmaceuticals, Japan) (0.01μM-10μM).

3.3.5. Correlation of matrix degradation with mineral dissolution.

In the resorption experiments discussed in Chapter 4, the assay of calcium release into the TCM from isolated mouse calvariae as a measure of bone resorption in response to various stimuli was routinely used. However, it is unclear whether the free calcium measured in this system is a true reflection of osteoclastic activity or in part due to mineral dissolution. To determine whether the resorption assay of Ca^{2+} release into the TCM is a true reflection of matrix degradation, random samples from resorption

experiments were analysed for the more specific pyridinoline crosslinks, which reflect resorption of the mature extracellular matrix. These crosslinks are widely accepted as sensitive markers of bone turnover in clinical situations.

The method initially developed by Black *et al* (1988) and modified by James *et al* (1996) to analyse pyridinium crosslinks of collagen in urine using ion-paired reversed-phase HPLC was used to determine pyridinoline and deoxypyridinoline release from hemi-calvariae in culture. Briefly, samples of TCM from mouse calvarial resorption experiments were collected and stored at +4°C. Each sample was hydrolyzed with an equal volume of concentrated HCl (final concentration 6M) for 20hrs at 116°C. The hydrolysates were centrifuged in 2-ml microcentrifuge tubes at 13,000g for 10mins to remove any precipitant prior to extraction. Hydrolysates were diluted 10-fold with 25mM disodium tetraborate, pH (Merck Ltd) giving a final pH of 0.6. Extraction steps were performed using a Gilson Aspec XlLi solid-phase extraction system (Anachem Ltd). Samples were extracted on 1-ml Isolute Confirm HCX SPE cartridges which were conditioned with 2ml of 5mM sodium formate, pH 2.75, before adding 2.5mls of the dilute hydrolysate at 0.5ml/min. After washing with 6mls of 5mM sodium formate, pH 2.75 containing methanol (40% v/v) followed by 2mls of 5mM sodium formate pH 2.75 and drying with nitrogen for 30secs. Crosslinks were eluted with 400μL of 100mM sodium formate (pH5) and collected in tubes containing 40μL of heptafluorobutyric acid (HBFA). After repeated aspiration and dispensing, 50μL of sample was injected onto the HPLC column. The chromatographic system composed of a Shimadzu LC6A pump (Dyson Instruments, UK) and a Jasco FP 920 fluorescense detector (Jasco Ltd, UK). Crosslinks were separated on a 150 X 4.6-mm (i.d.) Techsphere5μm ODS column (HPLC Technologies Ltd) eluted with 30mM HFBA in DDW plus acetonitrile (83:17) at 1ml/min. The pyridinium crosslinks were measured using fluorescense spectroscopy

with an excitation at λ 295nm and an emission at λ 400nm. The results were expressed as nanomoles of pyridinoline or deoxypyridinoline.

3.4. Results.

3.4.1. Quantitative determination of Ca^{2+} using o-cresolphthalein complexone as a detection system.

Calcium reacts with cresolphthalein complexone forming a coloured complex. The increase in absorbance due to complex formation is measured at 575nm. The "IL Test™ Calcium" reagent from Instrumentation labs (Warrington, UK) contained two calcium reagents: Ca 1 and Ca 2 (see appendix II) that were combined in equal volumes and added to the sample that was being tested for levels of calcium. Using the Monarch 2000 the loading parameters were as follows: sample-4µL; sample diluent-16µL; Ca 1 reagent-85µL; Ca 2 reagent-85µL, at 37°C. Linearity was achieved between 0.5mmol/L-5mmol/L (2mg/dL-20mg/dL) (Fig. 3.3B). Concentrations above this range did not show linearity. (Fig. 3.3A).

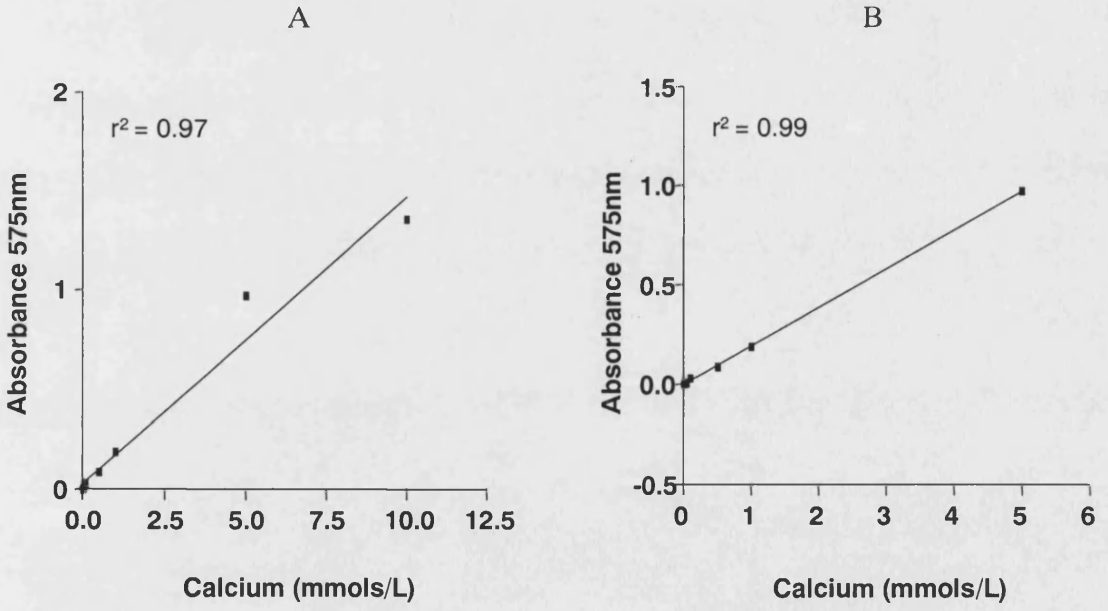


Figure 3.3. Calcium standard curves, (A) 0.5mmols/L-10mmols/L of Ca^{2+} and (B) 0.5mmols/L-5mmols/L of Ca^{2+} using the cresolphthalein complexone reaction to quantify levels of Ca^{2+}.

During this study access to the Monarch 2000 automated analyser became no longer available. Therefore, an alternative method was adopted to measure calcium levels in bone resorption culture medium. This was based upon the same principle of Ca^{2+}-cresolphthalein complexone formation as before, but the assay used in this procedure was supplied in kit form by Sigma, UK (see appendix II). 10μL of the sample was added to 990μL of the cresolpthalein solution and placed in a cuvette. This was read manually on a Cecil, series 2 spectrophotometer (model CE 373) at 575nm. Although samples had to be read manually, consistently accurate results were obtained from this method. Fig. 3.4A and 3.4B shows that the linearity of the Ca^{2+}/cresolpthalein complexone reaction of this kit to be between 0.15-2.5mmols/L (Fig3.4B) anything above was non-linear (Fig. 3.4A).

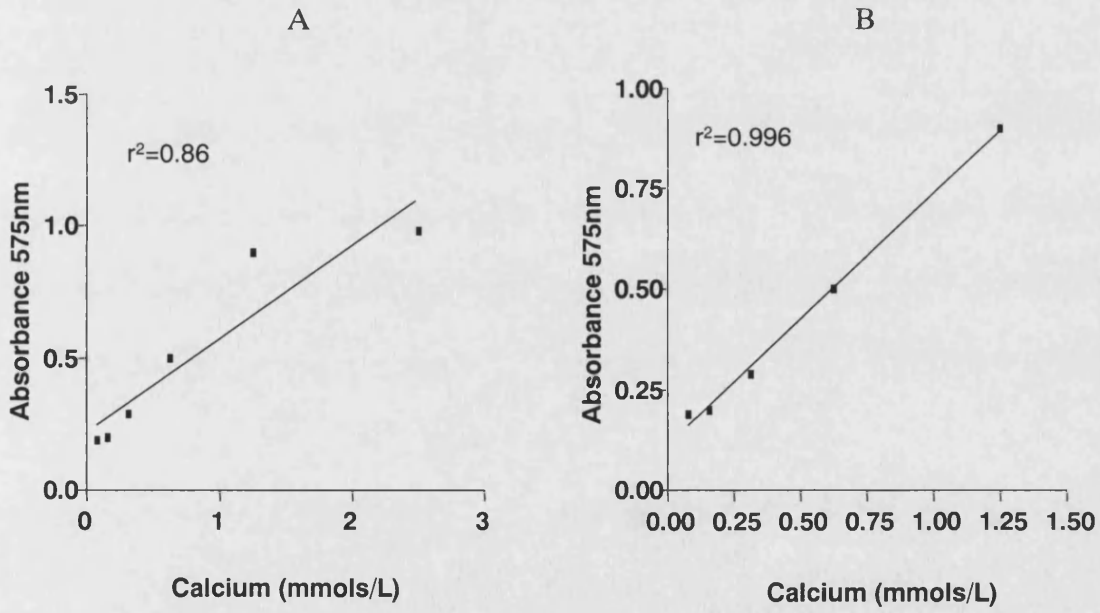


Figure 3.4. Calcium standard curve, serial dilution of Ca^{2+}(A) 2.5mmols/L (10mg/dL) and (B) 1.25mmols/L (5mg/dL) of Ca^{2+} using the cresolphthalein complexone assay kit (Sigma, UK) to quantify levels of Ca^{2+}.

3.4.2. Dose response of H_2O_2 on mouse calvarial bone resorption.

H_2O_2 that was added to the TCM at the start of the resorption experiment and incubated for 96hrs caused a dose-related increase in mouse calvarial resorption from 0.1nM-1000μM $^{**}p < 0.01$, $^{***}p < 0.001$ above control respectively (Fig. 3.5). Data are representative of 2 separate experiments (mean ± SEM) where n= 4 calvariae per individual treatment.

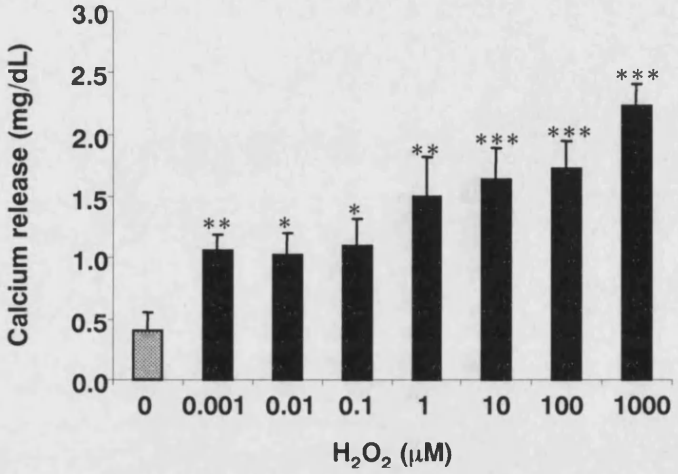


Figure 3.5. The dose effects of H_2O_2 on mouse calvarial bone resorption in culture (▒ Control).

3.4.3. The effect of catalase on basal resorption.

Catalase, a scavenger of H_2O_2 had no inhibitory effect on basal levels of mouse calvarial bone resorption (Fig. 3.6A). On the contrary, it demonstrated the ability to stimulate resorption (increase in Ca^{2+} levels) at 1 IU/ml ($^{**}p < 0.01$) 100U/ml ($^{***}p < 0.001$) respectively. In TCM that contained 100 IU/ml of catalase a change in pH towards acidic levels was observed. The other concentrations of catalase showed no change of pH whatsoever. The data are representative of 3 separate experiments (mean ± SEM) where n =4 calvariae per individual treatment.

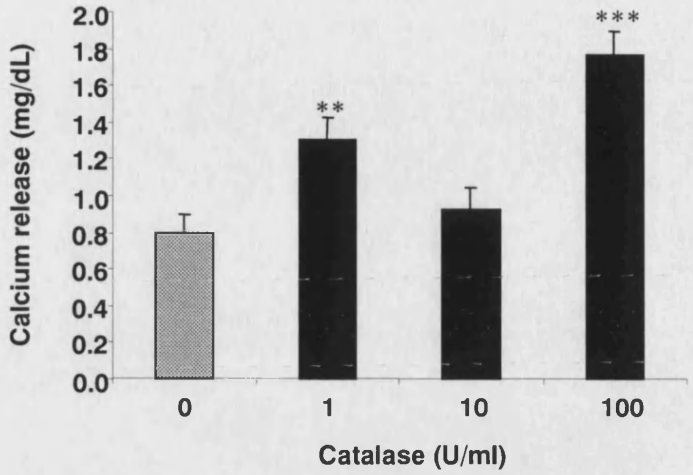


Figure 3.6A. The effect of catalase on basal levels of mouse calvarial bone resorption in culture (▒ Control; ■ Catalase).

The catalase used in these resorption experiments was made up in a stabilising solution of ammonium sulphate $(NH_4)_2\ SO_4$ at a concentration of 2.1M. To determine whether or not $(NH_4)_2\ SO_4$ was having an inhibitory effect on bone resorption or changing the pH levels to an acidic environment in the TCM, calvariae were incubated with the equivalent concentration of $NH_4\ SO_4$ that was being added in the catalase preparations. Fig. 3.6B shows that $NH_4\ SO_4$ had no inhibitory effect on basal levels of bone resorption but had a stimulatory effect at 15mM (***$p < 0.001$). Again, we observed a fall in pH levels at the concentration that was equivalent to 100 IU/ml of catalase (15mM $(NH_4)_2\ SO_4$). The data are representative of three separate experiments (mean ± SEM) where n=4 calvariae per individual treatment.

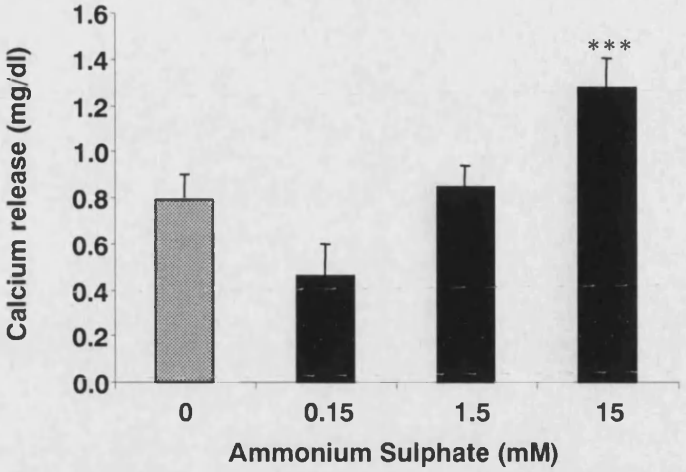


Figure 3.6B. The effect of ammonium sulphate on basal levels of mouse calvarial bone resorption in culture (▒ Control; ■ ammonium sulphate).

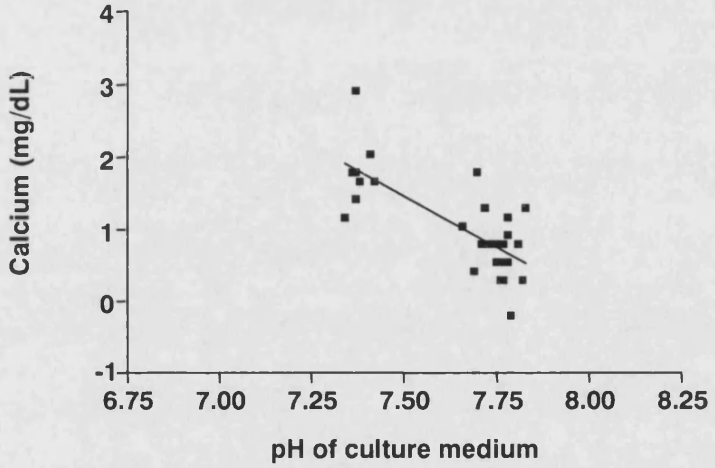


Figure 3.6C. The effect of pH changes by catalase and NH_4 SO_4 on basal levels of mouse calvarial bone resorption in culture.

On analysis of the pH of these samples, it was found that there was a strong correlation between increase in calvarial bone resorption and a decrease in the pH concentration. The control samples were approximately around pH 7.6-7.8 whereas, the high concentration of catalase (100U/ml) and $(NH_4)_2$ SO_4 (15mM) had reduced the pH to around 7.2 and subsequently caused an increase in the resorptive process $r^2 = 0.55$; $***p < 0.0001$ (Fig. 3.6C). Data represents 28 samples.

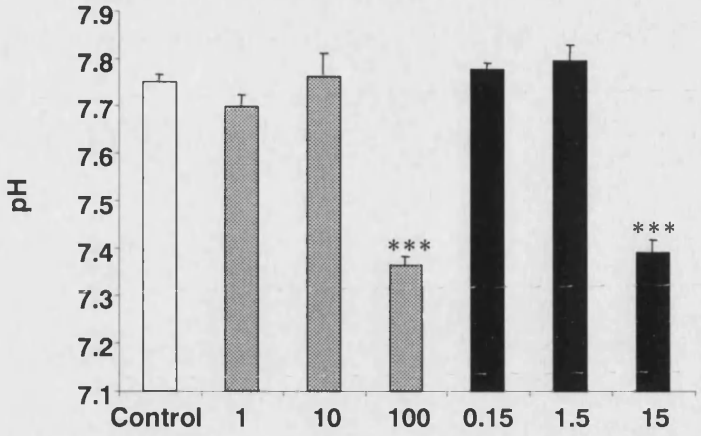


Figure 3.6D. The effect of catalase (IU/ml) ▒ and $NH_4 SO_4$ (mM) ■ on the pH levels in TCM.

Fig. 3.6D shows that catalase (100 IU/ml) and $NH_4 SO_4$ (15mM) caused a significant decrease in pH levels (***$p < 0.001$) of the TCM of mouse calvarial cultures. At the lower concentrations of catalase and $NH_4 SO_4$, there was no pH effect on the resorptive process in comparison to controls.

3.4.4. The effect of allopurinol on basal resorption.

Incubation with increasing concentrations of allopurinol (0.01μM-10μM) had no effect on basal levels of calvarial bone resorption (Fig. 3.7). Data are representative of 2 separate experiments (mean ± SEM) where n=6 calvariae per treatment).

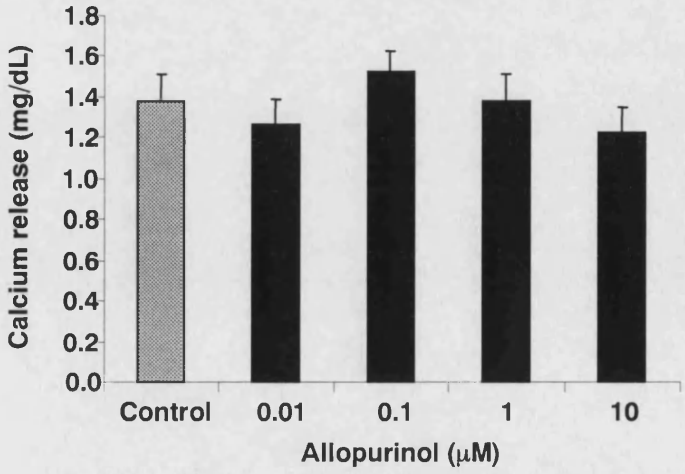


Figure 3.7. The effect of allopurinol on basal levels of mouse calvarial bone resorption in culture.

3.4.5. The effect of (-)- BOF-4272 on basal calvarial bone resorption.

The tightly binding xanthine oxidase inhibitor BOF-4272 had no effect on the basal levels of mouse calvarial resorption (Fig. 3.8). There was a slight decrease in the Ca^{2+} values for the TCM that had 10μM BOF-4272, but this was not statistically significant. The data were representative of one experiment only (mean ± std), where n=6 calvariae per treatment.

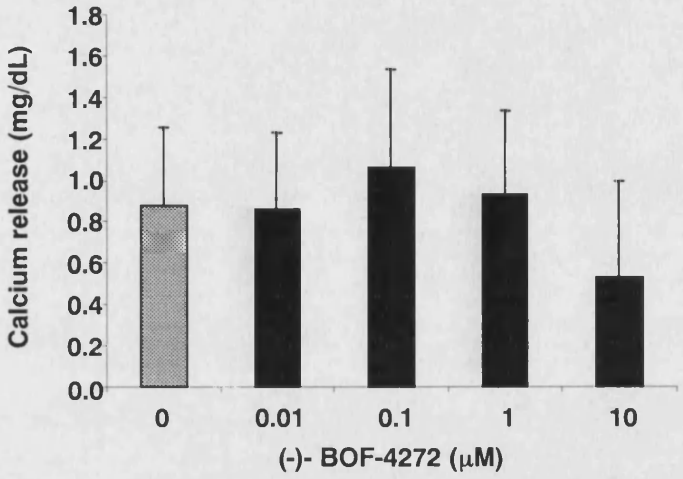


Figure 3.8. The effect of (-)- BOF-4272 on the basal levels of mouse calvarial bone resorption *in vitro*.

3.4.6. The correlation of matrix degradation and mineral dissolution.

Random samples of tissue culture medium from three separate mouse calvariae resorption experiments (n=43) were analysed for pyridinoline crosslinks. This analysis showed a strong correlation with release of calcium into the culture medium from the calvariae. $r^2 = 0.4$; $***p < 0.0001$ (Fig. 3.9). Data are representative of 43 samples assayed.

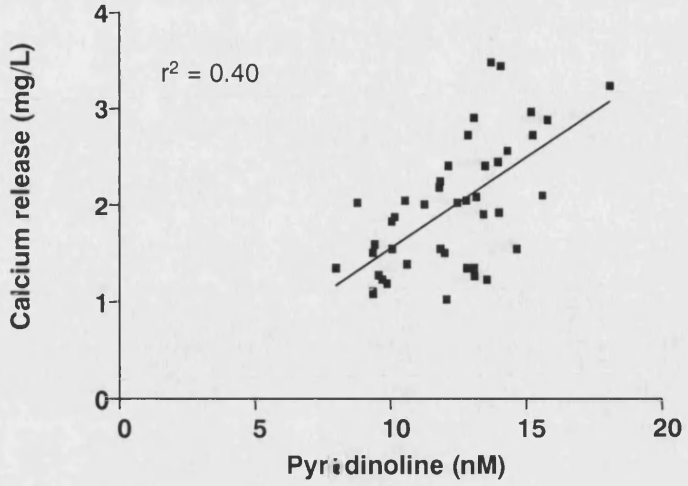


Figure 3.9. The correlation between calcium and pyridinoline crosslink release from mouse calvariae in tissue culture.

3.5. Discussion.

Using the cresolpthalein complexone assay we were able to detect low levels of Ca^{2+} both by automation, using the Monarch 2000 auto analyser and manually, using the Sigma kit. Although the automated detection of cresolpthalein was more accurate than the manual kit at higher concentrations, detection of Ca^{2+} was linear for the concentrations that were being released by mouse hemi-calvariae in the resorption assays.

As previously reported in the literature (Fraser *et al,* 1996), H_2O_2 is a potent inducer of mouse calvarial bone resorption *in vitro*; stimulating new osteoclast formation and enhancing the activity of existing mature osteoclasts. My findings showed that significant increased resorption could be attained as low as 1nM. My results differ slightly from Fraser *et al* (1996), in respect of their finding that H_2O_2 concentrations of 10μM displayed an inhibitory effect on bone resorption. The data presented here, in contrast, show that a stimulatory effect on bone resorption is achieved by H_2O_2 across a concentration range of 1μM-1mM. This discrepancy may be due to the differing culture systems that were employed. Fraser *et al,* 1996 used BGJ medium that did not contain phenol red whereas, the CMRL-1066 medium employed in the present study contained phenol red (20mg/L). As described in the literature, phenols or phenolics are natural chemicals found in most plants. They are abundantly present in fruit, wine, tea, and chocolate, and their antioxidant properties have been documented (Waterhouse *et al,* 1996). Phenol red, which is used in most culture medium as an indicator of pH changes, has been shown to be oxidised by H_2O_2 in the presence of horseradish peroxidase (Pick & Keisari, 1980; Maslen *et al,* 1987). This might explain why high concentrations of H_2O_2 continue to have a stimulatory, rather than an inhibitory, effect on bone resorption. Therefore, the effective H_2O_2 concentration in this

system may be considerably lower due to the scavenging effect of phenol. One question does arise from the work of Fraser and colleagues. Does H_2O_2 at high doses inhibit bone resorption via a mechanistic inhibitory effect or via a cytotoxic effect? They showed that high concentrations of H_2O_2 inhibited resorption only back to the basal levels. It would seem that high doses would be deemed cytotoxic and if so the whole of the resorptive process including basal resorption should have been inhibited.

Allopurinol and BOF-4272 (XO inhibitors) had no effect on basal levels of bone resorption, implying that XO is not a critical enzyme for normal metabolic resorption in mouse calvariae.

Catalase had no inhibitory effect on basal mouse calvarial resorption but did stimulate bone resorption at 1 IU/ml and 100 IU/ml. The TCM (100 IU/ml) n these cultures became slightly acidic, which was probably due to the $(NH_4)_2SO_4$, a stabiliser for the catalase solution. This confirms what has already been published in the literature that a lowering of pH in the TCM results in an increase in osteoclastic activity (Arnett & Dempster, 1986; Walsh *et al*, 1990). This implies that the resorption assay has to be carried out very carefully as to avoid slight changes in pH, which if allowed to occur could cause serious problems in interpreting results from these resorption experiments. It is not clear why 1 IU/ml of catalase increased resorption, but the effect appears not to be linked to changes in pH. It may, however, be due to low levels of H_2O_2 being converted to H_2O and O_2 and providing an increased source of O_2 that may be converted to superoxide, which is generated by the osteoclast during its resorptive cycle. However, 10 IU/ml should have had a similar effect.

Finally, the detection of Ca^{2+} release into the TCM from calvariae has been shown to be a good indicator of matrix degradation. This is strengthened by the correlation shown between the levels of pyridinoline crosslinks released during matrix

degradation of bone and the release of Ca^{2+} into the resorptive medium of some randomised samples from cytokine experiments described in Chapter 4.

In conclusion; data in this chapter showed that the cresolphthalein complexone assay is sensitive enough for the detection of Ca^{2+} release from mouse calvariae in culture, and this correlated with pyridinoline crosslinkage release, another marker of bone resorbing activity. We also determined in this chapter that hydrogen peroxide induces mouse calvarial bone resorption, and that the XO inhibitors, allopurinol and BOF-4272 and the H_2O_2 scavenger, catalase had no inhibitory effect on basal calvarial bone resorption.

Chapter 4.

The role of XO in mouse calvarial bone resorption influenced by pro-inflammatory cytokines; TNFα, IL-1β and Interferonγ.

Chapter 4

4.1. Introduction.

The pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), Interleukin-1 beta (IL-1β) and interferon gamma (IFNγ) are important mediators in bone turnover (Mundy, 1991; Heath, *et al,* 1985; Gowen & Mundy, 1986; Bertolini *et al,* 1986). TNFα and IL-1β, both 17 kD cytokines are generated by immune cells such as monocytes in the marrow cavity (Mundy, 1991) and by other bone cells (Gowen *et al,* 1990; Keeting *et al,* 1991). They have similar effects on osteoclasts and their precursors. IL-1β stimulates bone resorption by its action on an 80 kD receptor on cells of the osteoblastic lineage, whereas TNFα acts on its own receptor. Conversely, IFNγ (17kD) is a multifunctional cytokine, produced by immune cells, which is capable of inhibiting both proliferation and differentiation of osteoclast progenitors. It appears to exert its effect on osteoclast formation which has been stimulated by TNFα and IL-1β rather than stimulation by the systemic hormones, PTH and 1,25 Dihydroxyvitamin D_3 (Mundy, 1991).

The mechanism by which cytokines stimulate osteoclastic bone resorption remains unknown, but it is thought to be mediated by an initial action on osteoblasts, which results in the release of a small labile factor producing a paracrine stimulant of the osteoclast (Thomson *et al,* 1986). Tashjian *et al,* 1987 reported that abolition of TNFα-induced PGE_2 formation by nonsteroidal anti-inflammatory drugs completely inhibited TNFα-induced bone resorption. The amount of PGE_2 formation by TNFα stimulation could not really account for the amount of Ca^{2+} release in neonatal mouse calvariae. However, Lerner & Ohlin, 1993 found that complete inhibition of TNFα-induced PGE_2 formation by different cyclooxygenase inhibitors resulted in only partial inhibition of TNFα-induced bone resorption.

Chapter 4

Reactive oxygen (ROS) and nitrogen species (RNS) such as superoxide, hydrogen peroxide and NO have been alluded to as playing an integral part in bone remodelling. Ralston *et al* (1995), have already shown that NO plays a pivotal role in cytokine-induced bone resorption. Conversely, other ROS have been ascribed roles in the coupling mechanism of bone resorption, although the actual source of these ROS has yet to be identified. As already described in Chapters 2 and 3 hydrogen peroxide is a potent stimulator of bone resorption, which may be produced by XO localised in osteoblasts. This chapter investigates the possibility of XO induction in the bone cell environment by pro-inflammatory cytokines, resulting in ROS production and activation of bone resorption.

4.2. Aims and Objectives.

- To determine whether the cytokines, TNFα, IL-1β and IFNγ can increase XO protein and activity in osteoblasts.
- To determine whether these particular cytokines exert their bone resorptive effects via ROS
- To determine whether mouse calvarial bone resorption induced by these cytokines can be inhibited by xanthine oxidase inhibitors (allopurinol, BOF 4242).

4.3. The effect of XO in TNFα-induced bone resorption.

4.3.1. Materials and methods.

4.3.1.1. The effect of TNFα on bone resorption.

Mouse calvarial bone resorption was carried out as previously described (section 3.3.1). TNFα (1-100ng/ml) (Boehringer Mannheim) was added to the culture medium (5% FCS in CMRL-1066, pen/strep and L-glutamine see appendix II) and incubated with mouse hemi-calvariae for a period of 96hrs. Effects of TNFα on bone resorptive activity were determined by measurement of Ca^{2+} release into the culture medium as previously described in section 3.3.1.

4.3.1.2. Inhibition of TNFα-induced bone resorption.

The optimal dose, 20ng/ml, of TNFα (determined from experiments described in 4.3.1.1) was added to the culture medium and hemi-calvariae. A dose range of inhibitors including allopurinol (XO inhibitor, 0.05μM-50μM), catalase (H_2O_2 scavenger, 0.01 IU/ml-10 IU/ml), N-acetyl cysteine (H_2O_2 scavenger, 0.1μM-10μM) and superoxide dismutase ($O_2^{\bullet-}$ scavenger, 0.1μg/ml-100μg/ml) (see appendix II) were added to these cultures.

4.3.1.3. Immunohistochemical XO localisation in mouse calvarial sections.

Calvariae from TNFα-induced resorption experiments were fixed in 4% buffered formalin (appendix IV) for at least 24-hrs. Calvariae were then processed using a Shandon hypercenter wax-embedding station (appendix VI). Sections (5μM) were cut using a microtome blade cutter (Leitz), and placed on a microscope slide and left to dry overnight at 42°C. The sections were then dewaxed in xylene and rehydrated using a graded series of alcohol solutions through to distilled water (appendix IV). The microscope slides were previously coated with poly-L-lysine to aid adhesion, (appendix

IV) and to avoid loss of tissue sections during staining procedures.

The sections were washed and incubated with blocking buffer (5% non-fat, dried milk, Marvel® in 1X PBS) for 1hr, and then with diluted (1:100) anti-bovine XO (Chemicon, Harrow, UK) overnight at 4°C. The sections were again washed and incubated with biotinylated goat anti-rabbit IgG (1:100) (Vector Labs, Peterborough, UK.) for 1hr, ABC-Alkaline Phosphatase conjugate (AP) complex (1:100) (Vectastain, Vector Labs, Peterborough, UK.) for 30mins, and finally with Fast Red TR/Napthol AS-MX in Tris Buffer (Sigma, Poole, UK) at room temperature. The sections were mounted in Aquamount (Merck, Lutterworth, UK) (The specific details of antibodies and kits used are listed in appendix IV).

Some sections were counterstained with Haematoxylin for 5mins and washed in H_2O until the blue nuclear colouration was distinct before the addition of Aquamount and a coverslip.

Tartrate resistant acid phosphatase (TRAP) was used as a marker to identify osteoclasts in these calvarial sections. After staining calvarial sections for XO, they were subjected to the TRAP assay (Suda *et al*, 1997). The assay components included 5mgs of AS-MX phosphate in 0.5mls of dimethyl formamide + 30mg Fast blue BB salt in 50ml of 0.1M sodium acetate (pH 5) + 50mM sodium tartrate. The sections were incubated for no more than 10mins and then counterstained with haematoxylin and mounted using aquamount.

4.3.1.4. XO activity induced by TNFα in rat osteoblasts.

Osteoblasts, isolated from 5-day-old rat calvariae (section 2.3.2), were stimulated with a dose range of TNFα (0.01-10ng/ml) for 24hrs. After this time, xanthine oxidase enzyme activity in RCOB homogenates were measured using a

fluorometric assay monitoring the conversion of pterin (a xanthine substitute) to the fluorescent reaction product isoxanthopterin (Beckman *et al*, 1989). The cell monolayers were washed three times in cold PBS (w/o Ca^{2+}, Mg^{2+}) (see appendix I) and homogenised in an ice-cold buffered solution (pH 7.4), containing 0.05 M potassium phosphate, 1mM EDTA, 0.1mM PMSF, 1μg/ml pepstatin A, 1μg/ml antipain and 1μg/ml leupeptin (Sigma, see appendix III).

The homogenates were then centrifuged at 10,000 rpm, for 10mins at +4°C and the supernatant was retained. An aliquot of this lysate (200μL) was diluted in 780μL 1x PBS and placed in a cuvette and introduced to a fluorimeter (excitation wavelength of 345nm, and an emission wavelength of 390nm), where an initial control reading was taken. Stock pterin (Sigma) solution (20μL of 1mM) was then added to the cuvette (final concentration 20μM) at time 0, and emission readings were taken at regular intervals until a constant rate of change was achieved. Lysate pterin oxidation activity was completely abolished by the addition of the XO inhibitor allopurinol (10μM). Values for XO activity were normalised to total protein in the sample, as estimated by the Bio-Rad Protein Assay (see appendix III). This is a dye-binding assay based on the differential colour change of a dye (Comassie Brilliant Blue G-250) in response to various concentrations of protein (Bradford, 1976). Briefly, several dilutions of bovine serum albumin (protein standard II) containing 1-25μg/ml were prepared. 0.8mls of diluted sample (usually 10μL of cell lysate) and 0.8ml sample buffer as experimental blank was assayed. 0.2ml of dye reagent was then added and the 1.0ml solutions were transferred to a 1.5ml plastic cuvette (Kartell, Merck). After 5 mins the optical density at λ_2 595nm was measured on a spectrophotometer (Cecil series 2 CE-373). A standard plot was constructed using the corresponding OD values and the protein concentrations

in the test samples were estimated from their corresponding OD values using the standard plot.

4.3.1.5. Quantification XO protein levels after TNFα treatment of rat and human osteoblasts.

4.3.1.5.1. Preparation of cell lysates.

Monolayers of RCOBs were stimulated with a dose range of TNFα (0.1-10 ng/ml) for 24hrs. After stimulation, the cells were washed twice with 1x phosphate buffered saline w/o Ca^{2+} and Mg^{2+} (see appendix I) and lysed with 500μL of 1x SDS loading buffer (50mM Tris-HCL pH 6.8, 100mM DTT, 2% SDS and 20% glycerol) that was heated to 85°C. Cell lysates were transferred to Eppendorf tubes and boiled for a further 10mins, and the released DNA was sheared by passing the samples through a 26-gauge hypodermic needle three times. Samples were centrifuged at 10,000g for 10 mins, and the supernatants were transferred to fresh tubes. A portion was retained for protein determination using the Bio-Rad protein assay kit (section 4.3.1.4).

4.3.1.5.2. Polyacrylamide gel electrophoresis SDS-PAGE.

Materials. (See appendix V)

Acrylamide gels (8%) were prepared according to Laemmli 1970 (see appendix V). An equal volume of 2x reducing loading buffer (see appendix V) was added to the lysate in an eppendorf and boiled for 10mins. 10μl of rainbow markers (molecular weights, 250-10kD) (Amersham) were mixed with an equal volume of 2x reducing loading buffer. The samples were then centrifuged at 10,000 rpm (Biofuge, Heraeus Instruments) for 5mins. The gel plates were placed in the ATTO electrophoresis set up and filled with running buffer containing 25mM Tris base, 190mM glycine and 0.1% SDS (w/v) pH 8.3 (see appendix V). An equal volume (10-30 μL) of each sample

containing equal amounts of total protein (10-30μg) was carefully loaded into the gel wells. The gel was then subjected to electrophoresis at a constant voltage of 120V for approximately 1½ hours, or until the dye reached the base of the gel.

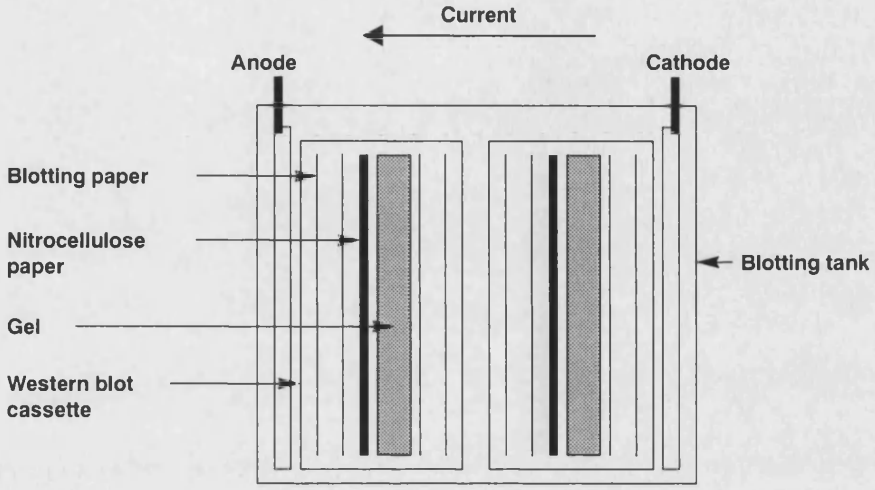


Figure 4.1. A schematic diagram of the blotting apparatus used to transfer proteins onto nitrocellulose paper.

4.3.1.5.3. Protein blotting and immunodetection of XO by ECL.

Following electrophoresis, the gel was carefully removed and incubated in blotting buffer, which contained 190mM glycine, 25mM Tris base, 0.05% SDS and 20% methanol for 10 mins. At the same time nitrocellulose paper and Whatman paper (see appendix V) were also cut to the size of the gel and incubated in the blotting buffer for 10mins. Using a western blot cassette holder the 4 filter papers were placed on top of the sponges on both sides of the holder. The nitrocellulose paper and the gel were placed on one set of filter papers carefully removing all air bubbles. The second set of filter papers were then placed on top of the gel and the cassette was then closed. This was then transferred to a western blotting tank (Hoeffer Scientific) making sure that the nitrocellulose paper was nearest to the positive electrode (anode) as shown in Fig. 4.1. Blotting buffer was added to the tank and the proteins were blotted at 100V for 1½ hours. After this the cassette was removed from the tank and the gel was stained with

Comassie Brilliant Blue (see appendix V) for analysis of complete transfer of protein to the nitrocellulose paper. The positions of different molecular weight markers were enhanced on nitrocellulose paper with a pencil. The nitrocellulose was then incubated in blocking buffer consisting of 5% non-fat dried milk in 0.5% Tween 20/1x PBS (see appendix V) for 1 hour at room temperature (can be left overnight at +4°C).

After a 5-minute wash with 0.5% Tween 20/1x PBS, the blot was then transferred to a 50ml falcon tube containing 5mls of the diluted primary antibody (rabbit anti bovine xanthine oxidase (Chemicon), 1:1000 dilution in 5% non-fat dried milk in 0.5% Tween 20/ 1x PBS). This was incubated on a roller plate for 1½ hours, and thoroughly washed in 0.5% Tween 20/1x PBS 6 times, 5mins each. The blot was then incubated with the diluted secondary antibody (conjugated horseradish peroxidase swine-anti rabbit (Dako, see appendix IV) 1:1000 dilution in 5% non-fat dried milk in 0.5% Tween 20/1x PBS) for 1½ hours. The blot was again washed in 0.5% Tween 20/1x PBS 6 times, 5mins each.

Equal volumes of solution A and solution B from the ECL Amersham detection kit (see appendix V) was mixed and added to the membrane making sure the whole of the membrane was covered. The membrane was incubated for 90 secs and the excess drained off and covered in SaranWrap™ ensuring that all bubbles were smoothed out and then placed in a film cassette, protein side up. In a dark room, a sheet of Kodak autoradiography film (see appendix V) was carefully placed on top of the membrane and exposed initially for 30secs. This film was then developed using an automated developer (Fuji RG-II X-ray film processor).

4.3.2. Results.

4.3.2.1. Dose effect of TNFα on mouse calvarial bone resorption.

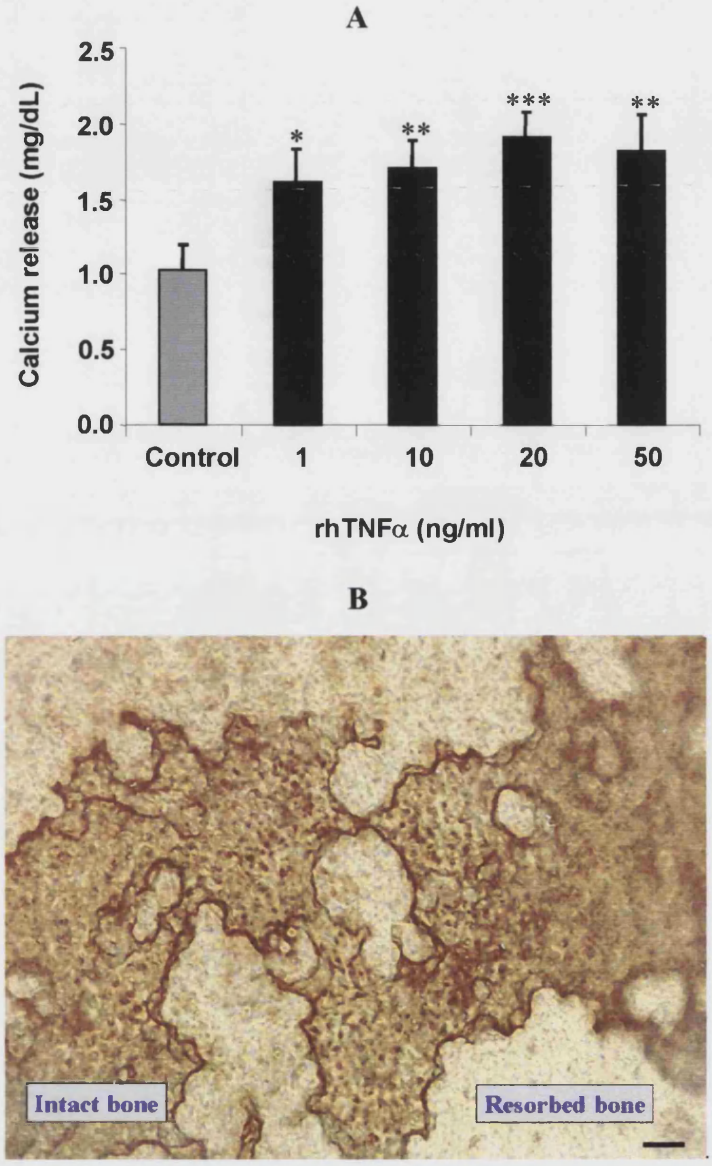


Figure 4.2. (A) The dose effect of TNFα on mouse calvarial bone resorption. (B) A photomicrograph depicting osteoclastic bone resorption in a mouse hemi-calvariae stimulated with TNFα (20ng/ml) for 96 hrs in culture. Bar represents 100μm

Stimulation of bone resorption was observed between 1ng/ml-50ng/ml with a maximal stimulation occurring at the 20ng/ml dose (Fig. 4.2A). Significant increases in the levels of bone resorption above control are represented by $*p < 0.05$; $**p < 0.01$;

***$p < 0.001$. Data presented are means ±S.E.M from 4 separate experiments where n=4-8 calvariae per treatment. Fig 4.2B is a photomicrograph depicting the osteoclastic resorption activity in these mouse calvariae in culture after 96-hrs incubation with TNFα (20ng/ml).

4.3.2.2. The effect of allopurinol on TNFα-induced resorption.

Allopurinol, dose dependently inhibited TNFα (20ng/ml)-induced mouse calvarial bone resorption, with maximal inhibition occurring between 5-50μM (Fig 4.3) Interestingly, the maximum inhibition of TNFα-induced resorption by allopurinol brought calcium levels down only to basal bone resorption levels. Data presented are means ±S.E.M from 3 separate experiments, where n=6-12 calvariae per treatment.

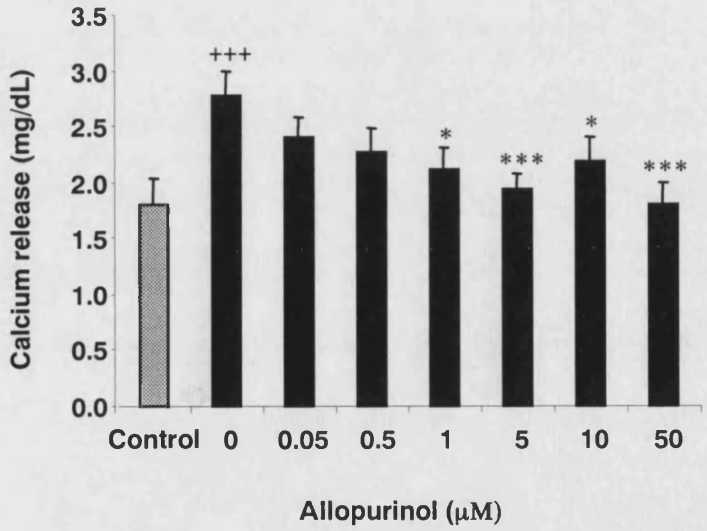


Figure 4.3. The effect of allopurinol on TNFα-induced mouse calvarial bone resorption. ▒ Control; ■ 20ng/ml TNFα.

The majority of the resorption experiments were carried out at the Royal London Hospital where Swiss albino CD-1 mice were used. However, a similar strain of mouse (MF-1) was used in experiments at the University of Bath. To establish similar responses in these different mice the studies carried out with TNFα and allopurinol were repeated. Fig. 4.4 indicates that allopurinol similarly inhibited TNFα induced resortption in MF-1 mice Data presented are means ±std. from 1 experiment, where n=

4 calvariae per treatment. A significant increase in resorption above control levels is represented by $^{++}p < 0.01$ and significant inhibition of TNFα-induced resorption by allopurinol is indicated as $^{*}p < 0.05$.

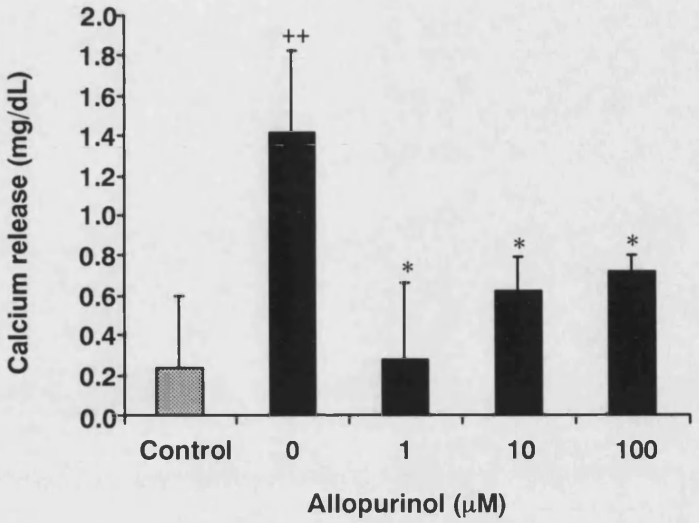


Figure 4.4. The effect of allopurinol on TNFα-induced mouse calvarial resorption. (MF-1 strains of mice). ▒ Control; ■ 20ng/ml TNFα.

4.3.2.3. The effect of catalase on TNFα induced resorption.

Catalase, a scavenger of hydrogen peroxide, inhibited (20ng/ml) TNFα-induced bone resorption at 10 IU/ml (***p, < 0.001), confirming that hydrogen peroxide as one of the mediators involved in the bone resorptive process in this system. Data presented are means ±S.E.M from 2 separate experiments where n=4 calvariae per treatment.

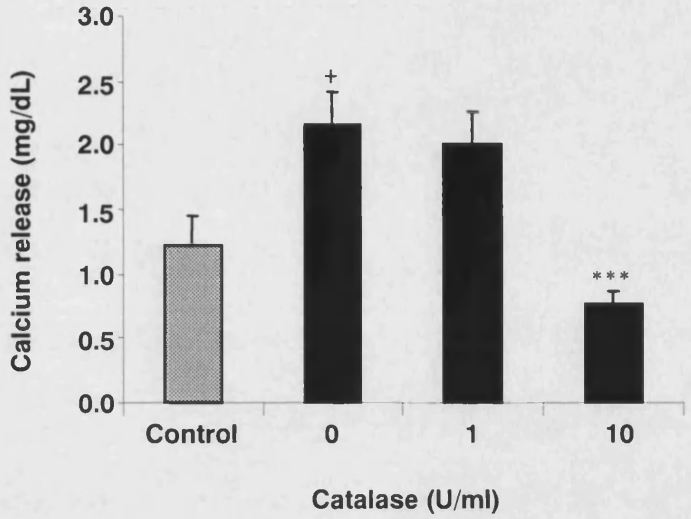


Figure 4.5. The effect of catalase on TNFα-induced mouse calvarial bone resorption. ▒ Control; ■ 20ng/ml TNFα.

4.3.2.4. The effect of N-Acetyl Cysteine on TNFα-induced resorption.

N-acetyl cysteine (NAC) is also a scavenger of hydrogen peroxide. In order to compound the inhibitory effects of catalase a single experiment was also performed. The result of this experiment revealed that 10μM NAC was also capable of inhibiting TNFα (20ng/ml) induced bone resorption (Fig 4.6). Data presented are means ±std. of 1 experiment, where n=8 calvariae per treatment. A significant increase in resorption above control is given by $^{+++}p < 0.001$. A significant inhibition of TNFα-induced calvarial resorption is indicated by $***p < 0.001$.

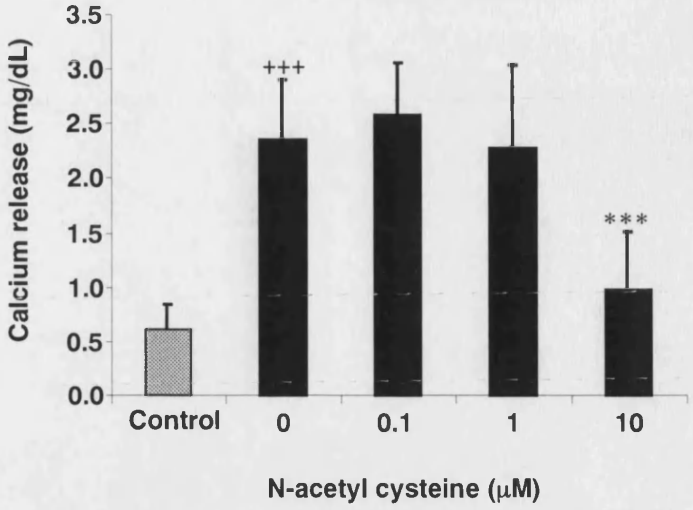


Figure 4.6. The effect of NAC on TNFα-induced resorption ▒ Control; ■ 20ng/ml TNFα.

4.3.2.5. The effect of superoxide dismutase (SOD) on TNFα induced resorption.

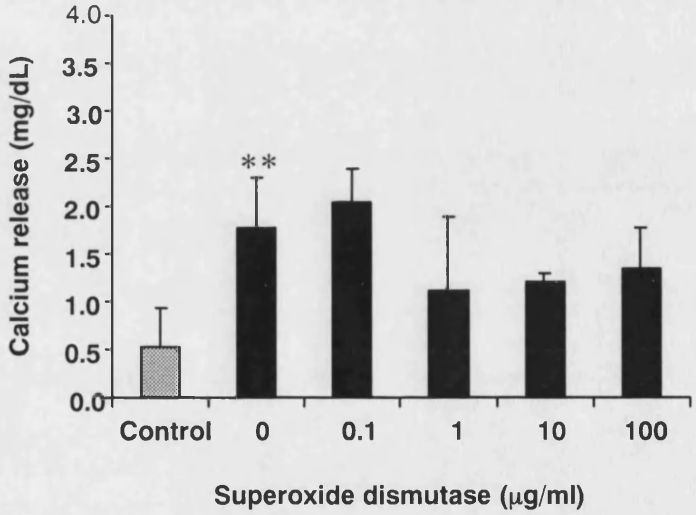


Figure 4.7. The effect of SOD on TNFα-induced mouse calvarial bone resorption.

In contrast to catalase and NAC, a dose range of 0.1-100μg/ml superoxide dismutase (which converts $O_2^{\bullet-}$ to H_2O_2 and molecular O_2) (Boehringer Mannheim) had little effect on TNFα-induced mouse calvarial bone resorption (Fig. 4.7). There seem to be a slight decrease in the resorptive activity (1-10μg/ml SOD), but this was found not to be statistically significant. A significant increase in resorptive activity above

control is given by $**p < 0.01$. Each treatment was representative of 4 hemi-calvariae. Results presented are expressed as means ±std.

4.3.2.6. Immunohistochemical XO localisation in mouse calvarial.

Sections (5μM) from both control and TNFα-treated calvariae were stained for XO to determine if there were any qualitative changes to XO expression in situ. The sections from both control and TNFα treated calvarial stained for XO (Fig. 4.8 B-D). The staining of XO was particularly stronger at the surface of the calvarial sections where osteoblasts line the bone matrix. Although, both specimens were positively staining for XO, it proved difficult to identify qualitative differences in the extent of staining between control and TNFα-treated sections. In the TNFα treated sections, the osteoclasts that had been stained for TRAP (blue), could clearly be distinguished resorbing the bone matrix. Interestingly, in some of the calvarial sections, XO staining in the regions of the lacuno-canalicular compartments that house the osteocytes were noted.

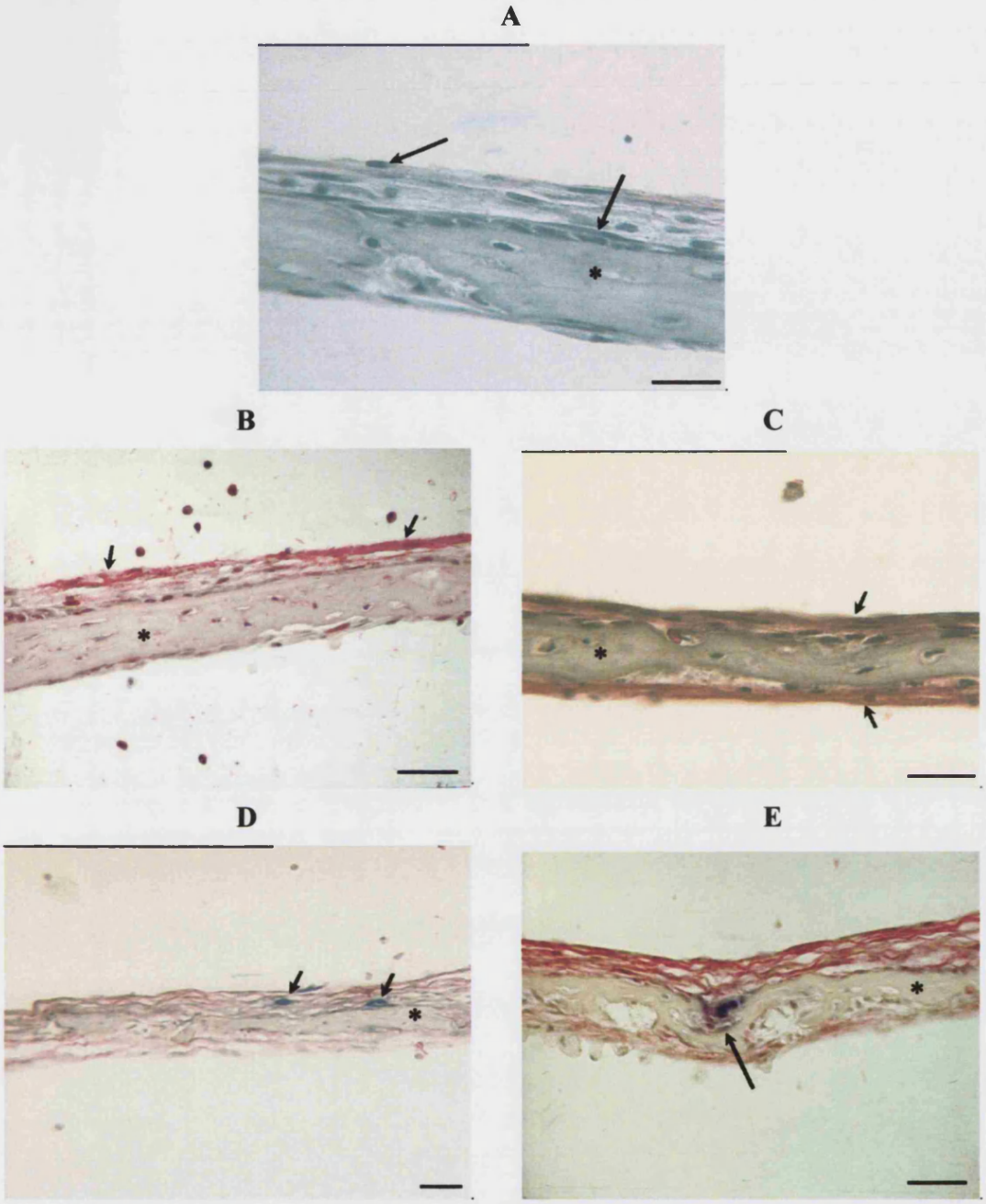


Figure 4.8. Photomicrographs of neonatal mouse calvariae after 96hrs in culture. Plate A represents control calvariae stained with Mayer's haematoxlin depicting mineralised bone (*) and osteoblasts (arrows). Plates B & C represents control calvariae staining the bone lining cells for XO (arrows). Plates D & E represents TNFα-treated calvariae showing osteoclasts (staining blue for TRAP (arrows)) resorbing the bone matrix. XO staining =red. Bar represents 25μm for plates A & C; and 50μm for plates B, D & E.

4.3.2.7. The dose effect of TNFα on XO activity in rat calvarial osteoblasts.

Due to difficulties in establishing clear differences in the XO immunoexpression in control and treated calvariae, a fluorometric assay was alternatively utilised to determine whether the osteoblast XO activity was being affected upon stimulation by TNFα. Initially, 10ng of bovine XO (Biozyme) was used as a positive control for XO activity using the fluorometric assay. The assay medium (1x PBS) contained 20μM of pterin with or without 50μM the XO inhibitor allopurinol. The experimental blank consisted of 1x PBS and 20μM pterin alone. Fig. 4.9 shows a typical reaction rate of pterin oxidation with bovine XO (Biozyme see appendix III). The addition of 50μM of allopurinol 35 mins after initiation of the reaction completely abolished the XO activity.

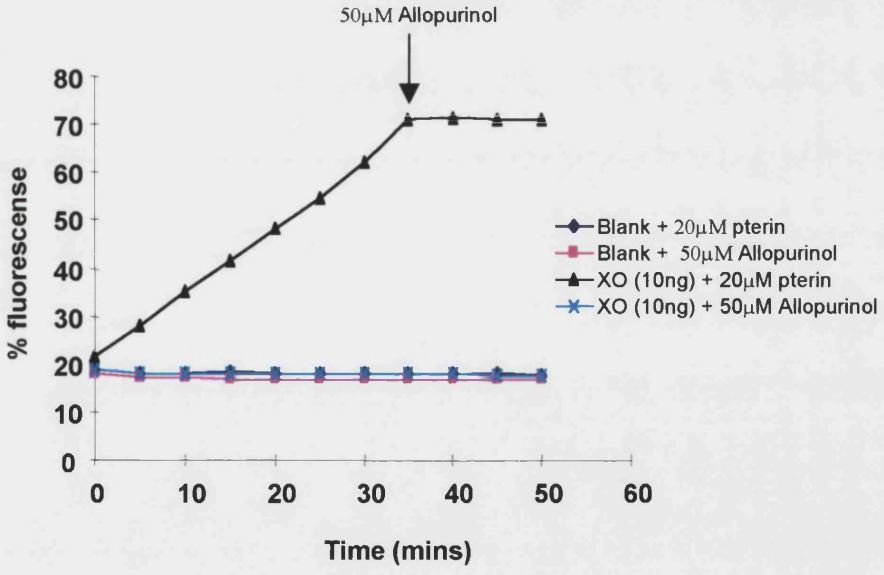


Figure 4.9. A typical graph, showing the rate of XO-catalysed conversion of pterin to its fluorometric reaction product isoxanthopterin.

Figure 4.10 shows the relationship between increased concentrations of TNFα and XO activity in rat calvarial osteoblasts as determined by the fluorometric assay described above. A significant increase in XO activity occurred with TNFα treatment with a dose range of 0.05ng/ml-1ng/ml. Maximal stimulation of XO activity was observed at 5ng/ml. Almost a 3-fold increase was observed with 5ng/ml of TNFα. $*p < 0.05$ $**p < 0.01$. Results were expressed as means ± S.E.M. for 3 separate experiments.

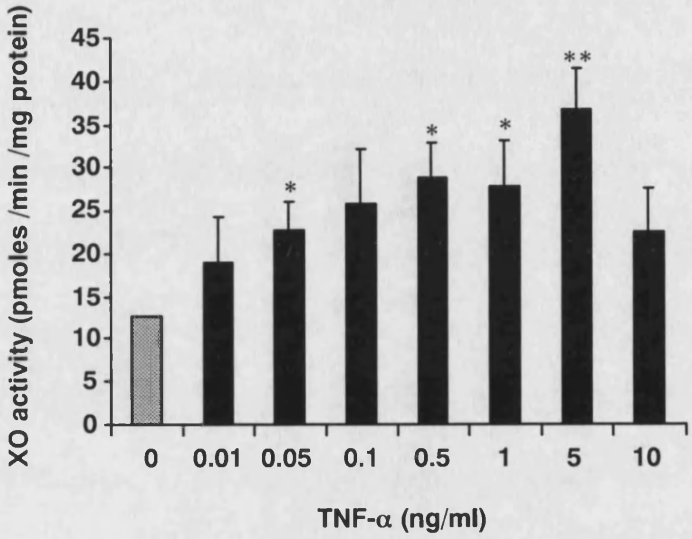


Figure 4.10. The effect of increasing doses of TNFα on RCOB XO activity.

4.3.2.8. The effect of TNFα on the expression of XO protein in osteoblasts.

On the basis of the observed stimulatory effects of TNFα on XO activity in osteoblasts, the effects of TNFα treatment on osteoblastic XO protein levels were investigated.

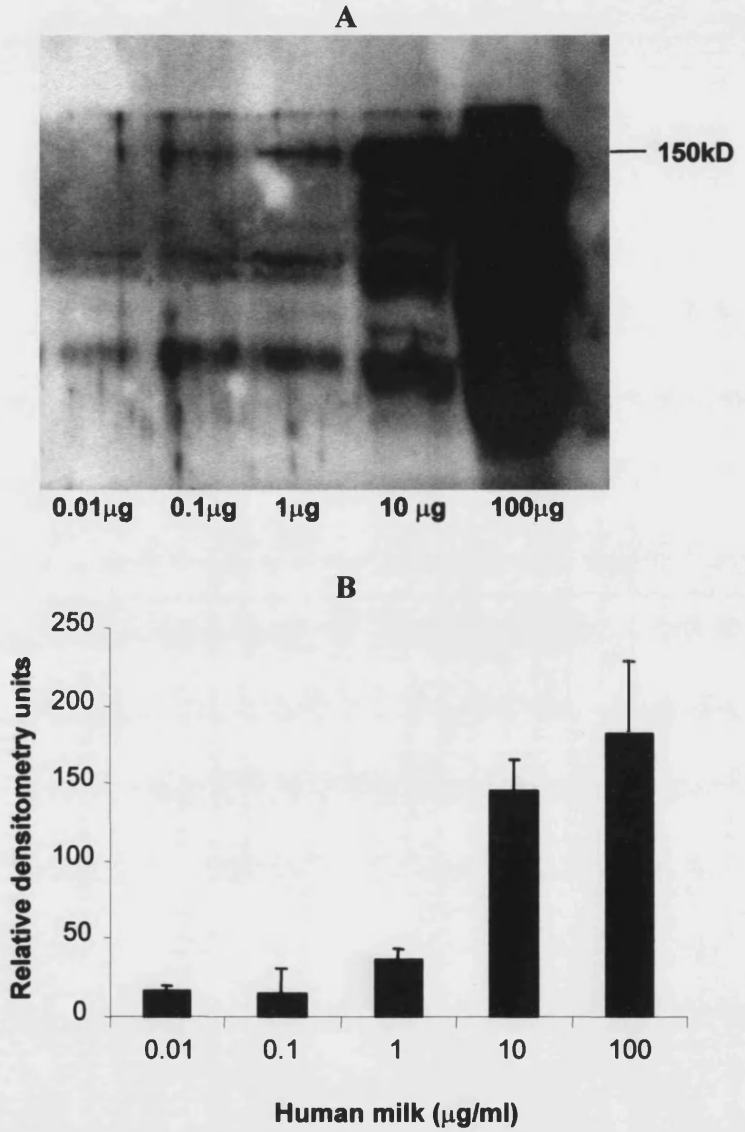


Figure 4.11. (A) Western blot of human breast milk XO after SDS-PAGE. (B) XO protein levels (relative densitometry units) in a sample of human milk with increasing amounts of total protein. (0.01μg/ml-100μg/ml) (n=2).

Initially however, western blotting using purified bovine milk XO (Biozyme) and human breast milk was used to test the affinity and the specificity of the available XO antibodies.

Human breast milk was used for the initial antibody tests, since it is an abundant source of XO, and allows for the purification of the human form of the enzyme (Abadeh *et al*, 1992; Sarnesto *et al*, 1996). Fig.4.11 (A) shows a representative blot of human breastmilk XO depicting a band at the estimated weight of 150kD. XO protein from human milk increased with increasing total protein in milk samples (0.01μg-100μg/lane) electrophoresed on an 8% SDS-polyacrylamide gel (Fig.4.11 (B)) Data are derived from two separate western blots.

Commercially-available bovine XO enzyme in varying quantities (0.1ng-100μg/lane) was subjected to SDS-PAGE, and a 150kD band was detected using a rabbit polyclonal XO antibody (Chemicon) (Fig. 4.12) n=1 western blot.

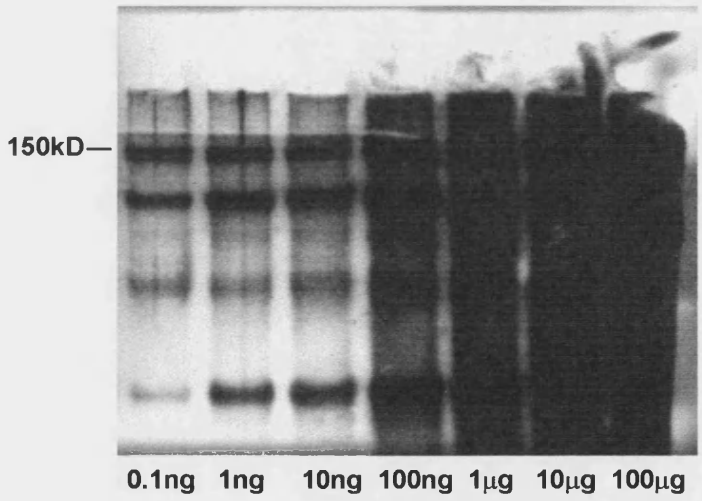


Figure 4.12. Western blot of increasing protein concentrations of the Biozyme bovine XO enzyme subjected to SDS-PAGE (8% polyacrylamide gel).

Fig. 4.13(A) is a representative blot showing XO protein levels in TNFα-treated RCOBs for 24hrs. The result of three separate experiments with TNFα treatment (0.01-20ng/ml) over 24hrs did not indicate any significant increase in the levels of XO protein (Fig. 4.13 (B)). Data presented are means ±S.E.M from 3 separate experiments.

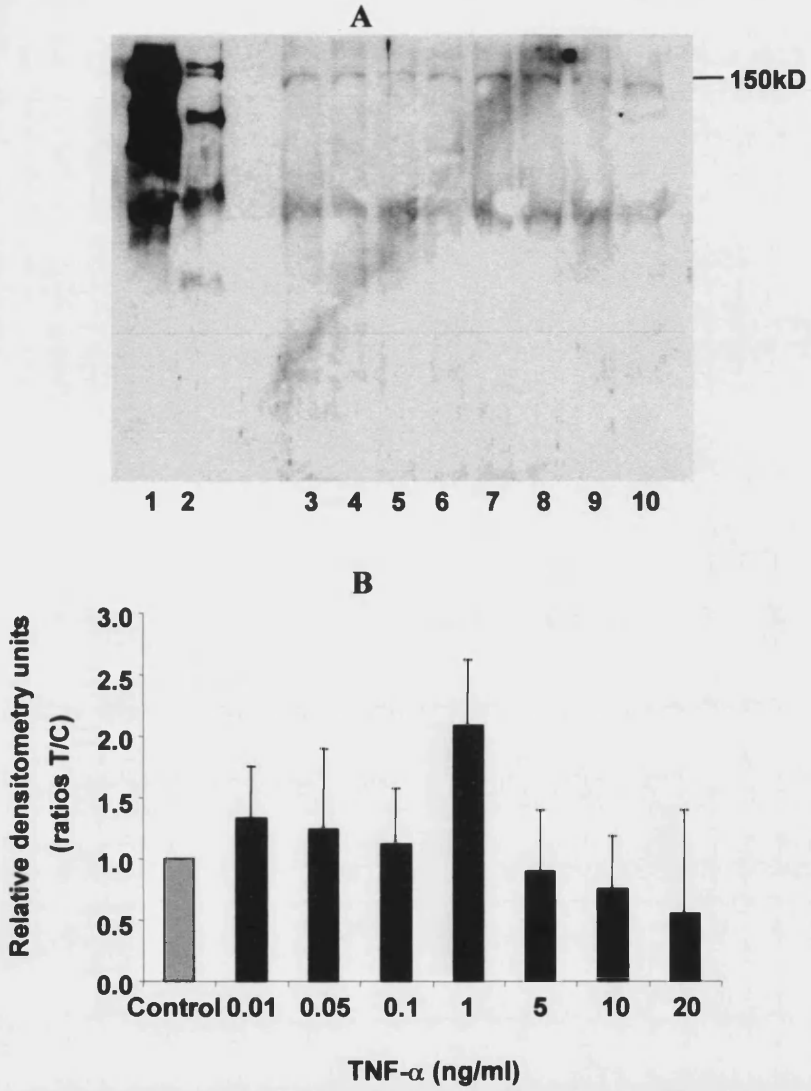


Figure 4.13. A representative blot of XO protein levels after incubation with TNFα for 24hrs. Bovine XO (lane 1) Human milk (lane 2). RCOB control (lane 3) 0.01, 0.05, 0.1, 1, 5, 10, 20ng/ml =lanes 4-10 respectively. (B) Ratio of XO protein levels in treated (TNFα) over control levels (n=3 experiments).

4.3.2.9. Summary.

We have demonstrated that TNFα-induced resorption can be inhibited by the XO inhibitor allopurinol and by scavenging H_2O_2 by catalase. Although we could not determine a difference immunohstochemically of XO in TNFα treated calvariae in comparison to control calvariae we did find a dose effect increase in the activity of the enzyme when rat osteoblasts were stimulated with TNFα. Contrary to these results we

could not determine any increase in protein levels of the enzyme when stimulated with TNFα.

4.4. The effect of XO in IL-1β-induced bone resorption.

4.4.1. Materials and methods.

4.4.1.1. The effect of IL-1β on bone resorption.

The effect of the cytokine Interleukin 1β (Boehringer Mannheim, UK) on mouse calvarial bone resorption was investigated. The same protocol was followed as previously described for the TNFα experiments (section 4.3.1.1) replacing TNFα with IL-1β. Mouse hemi-calvariae were incubated with the resorption culture medium (section 4.3.1.1) which included a dose range (1-100 IU/ml) of IL-1β for a period of 96-hrs in a humidified, 5% CO_2/95% air incubator. Calcium levels in the culture medium were assayed using the cresolphthalein complexone reaction as described before (section 3.3.1).

4.4.1.2. Inhibition of IL-1β-induced bone resorption.

To identify whether XO was involved in IL-1β-induced mouse calvarial bone resorption, the optimal dose of IL-1β (50 IU/ml) was added to the culture medium and calvariae, together with the addition of increasing doses of allopurinol (0.01μM-10μM), the XO inhibitor. Again, release of calcium into the culture medium was used as an indicator of the resorptive activity. To determine whether H_2O_2, $O_2^{\bullet-}$ or both these radicals are involved in IL-1β-induced bone resorption, catalase (Sigma) and superoxide dismutase (SOD, Boehringer Mannheim) were added to the culture medium respectively. As described before, resorption experiments were carried out over a 96-hr period in a humidified incubator, 5%CO_2/95% air.

4.4.1.3. XO activity induced by IL-1β in rat osteoblasts.

The same protocol was used as described in section 4.3.1.4 to ascertain whether IL-1β had any effect on XO activity in rat calvarial osteoblasts. RCOBs were incubated for 24hrs with increasing concentrations of IL-1β (0.1 IU/ml-100 IU/ml) and, as described in section 4.3.1.4, assayed for XO activity using the fluorometric pterin oxidation assay.

4.4.1.4. XO protein expression induced by IL-1β in rat and human osteoblasts.

To ascertain whether XO protein expression was affected by IL-1β we followed the method previously described in section 4.3.1.5 but replaced TNFα with a dose range of IL-1β (0.1 IU/ml-100 IU/ml).

4.4.2. Results.

4.4.2.1. Dose effect of IL-1β on mouse calvarial bone resorption.

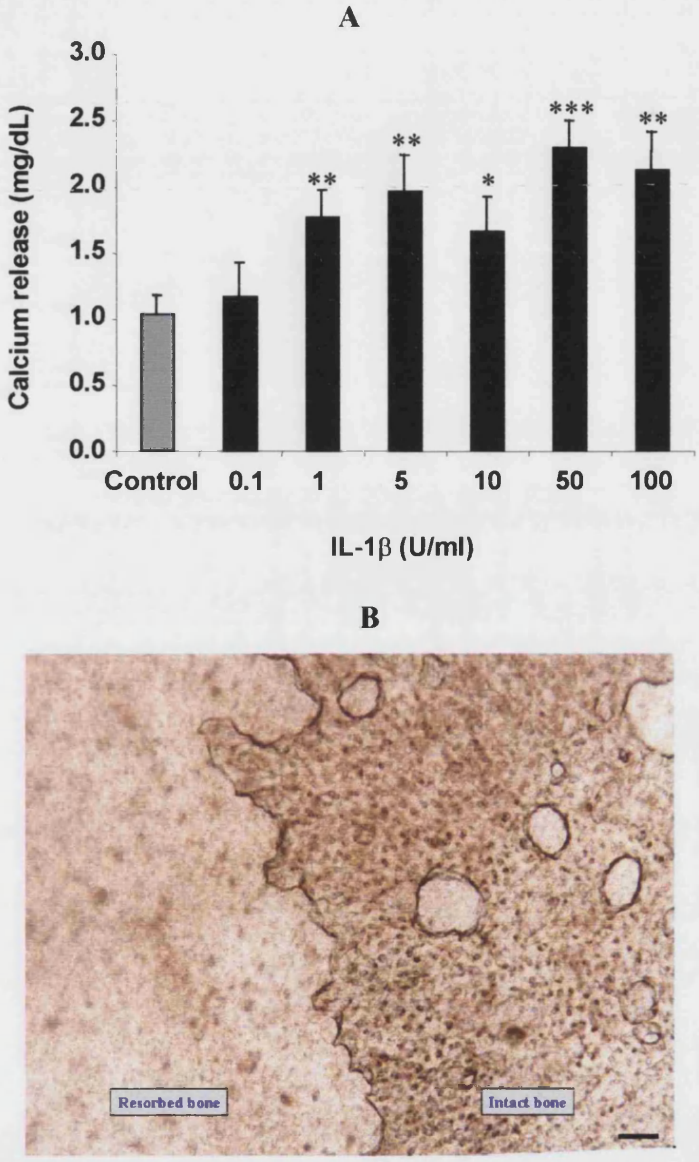


Figure 4.14. (A) The effect of IL-1β on mouse calvarial bone resorption. (B) Photomicrograph of a mouse calvariae in culture stimulated with 50 IU/ml IL-1β after 96 hrs in culture. Bar represents 100μm.

Fig 4.14 (A) shows that IL-1β caused a dose dependent increase in mouse calvarial resorption (1-100 IU/ml) after 96hrs in culture, with a maximal stimulation occurring at 50 IU/ml. A significant increase in resorption above control basal levels are represented by* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ respectively. Data presented are

means ±S.E.M from 2 separate experiments, where n=6 calvariae per treatment. As indicated in the photomicrograph (Fig. 4.14 (B)) Il-1β (50U/ml), like TNFα, is a potent inducer of bone resorption.

4.4.2.2. The effect of allopurinol on IL-1β-induced bone resorption.

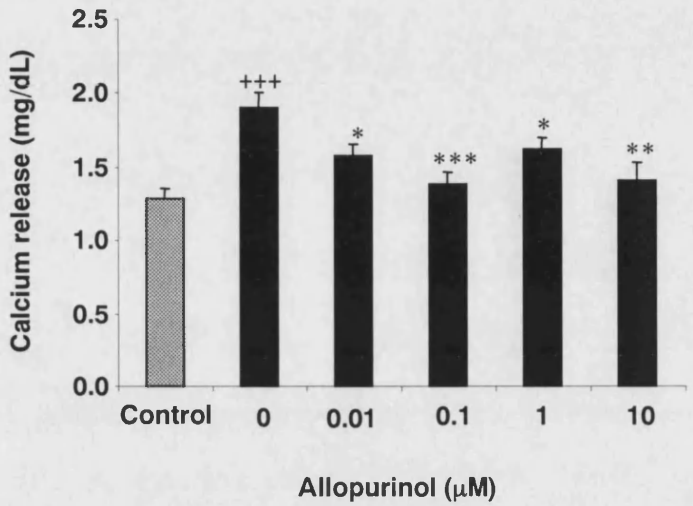


Figure 4.15. The effect of allopurinol on IL-1β (50 IU/ml)-induced mouse calvarial bone resorption. ▒ Control; ■ 50U/ml IL-1β.

Allopurinol dose-dependently inhibited IL-1β (50 IU/ml)-induced mouse calvarial bone resorption, with maximal inhibition occurring between 0.1-10μM (Fig 4.15). Similar to the TNFα-related data, allopurinol only reduced the IL-1β-induced resorption back to the basal levels. Data presented are means ±S.E.M from 4 separate experiments, where n=6 calvariae per treatment. A significant increase in resorption above control levels is shown by $^{+++}p < 0.001$. Significant inhibition of IL-1β induced resorption by allopurinol is indicated by $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4.4.2.3. The effect of BOF on IL-1β-induced bone resorption.

The tight binding XO inhibitor BOF-4272 inhibited IL-1β(50 IU/ml)-induced mouse calvarial bone resorption only at the highest concentration of 10μM (significance $^{***}p < 0.001$) (Fig. 4.16). The lower concentrations had no effect on bone resorption.

Increase in resorption over control by IL-1β was significant $^{+}p < 0.05$. The data presented are mean values ±s.t.d from 1 experiment, where n=6 calvariae per treatment.

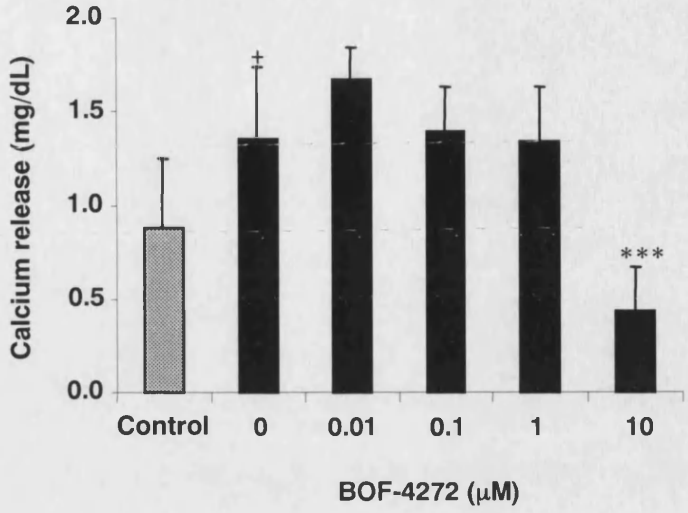


Figure 4.16. The effect of BOF-4272 on IL-1β-induced mouse calvarial bone resorption. ▒ Control; ■ 50 IU/ml IL-1β.

4.4.2.4. The effect of catalase on IL-1β-induced bone resorption.

Catalase had an inhibitory effect on IL-1β (50 IU/ml)-induced mouse calvarial bone resorption (Fig. 4.17). Although 1 IU/ml of catalase had no effect on IL-1β resorption, 10 IU/ml caused a significant inhibition of bone resorption back to basal levels ($^{***}p < 0.001$ inhibition of IL-1β-induced resorption; $^{+++}p < 0.001$ increase in resorption above control). The data presented means are ± S.E.M. of 3 separate experiments, where n= 6-8 calvariae per treatment.

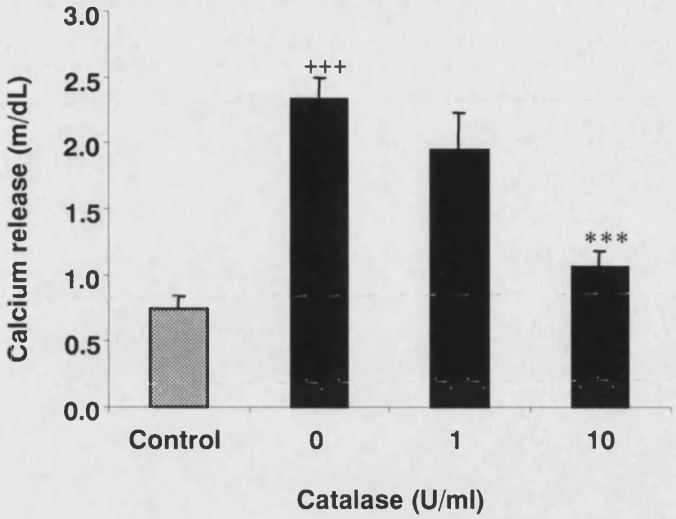


Figure 4.17. The effect of catalase on IL-1β-induced mouse calvarial bone resorption. ▒ Control; ■ 50 IU/ml IL-1β.

4.4.2.5. The effect of SOD on IL-1β-induced bone resorption.

It has previously been reported by Garrett *et al,* 1990 that superoxide dismutase (SOD) could inhibit IL-1β-induced bone resorption. On this basis, the effect of SOD on IL-1β induced resorption was also investigated in the mouse calvarial bone resorption assay system described throughout this thesis. The results of this study showed that under the conditions described, SOD had no effect on IL-1β-induced mouse calvarial resorption (Fig. 4.18). Increased bone resorptive activity by IL-1β was highly significant (***$p < 0.001$ above basal resorption). Data presented are means ±std. from 1 separate experiment, where n=4 calvariae per treatment.

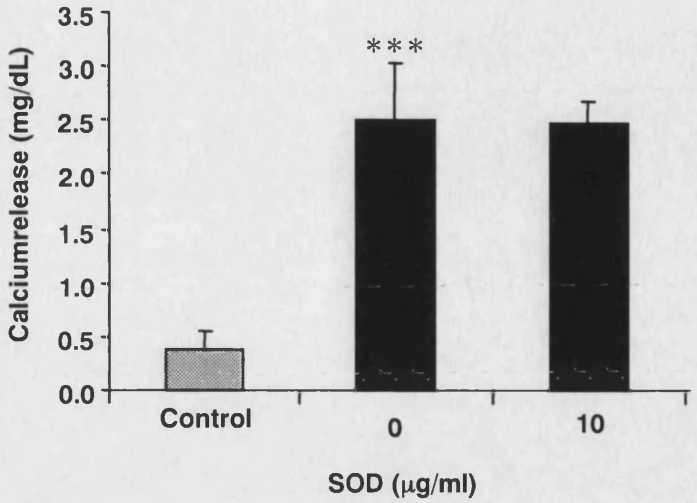


Figure 4.18. The effect of SOD on IL-1β-induced mouse calvarial bone resorption. ▒ **Control;** ■ **50 IU/ml IL-1β.**

4.4.2.6. The dose effect of IL-1β on XO activity in rat osteoblasts.

After 24hrs incubation with a range of concentrations of IL-1β (0.1-100 IU/ml) an increase in XO activity was detected using the fluorometric pterin assay (Fig. 4.19). Between 10 IU/ml and 50 IU/ml IL-1β caused significant increases in XO activity

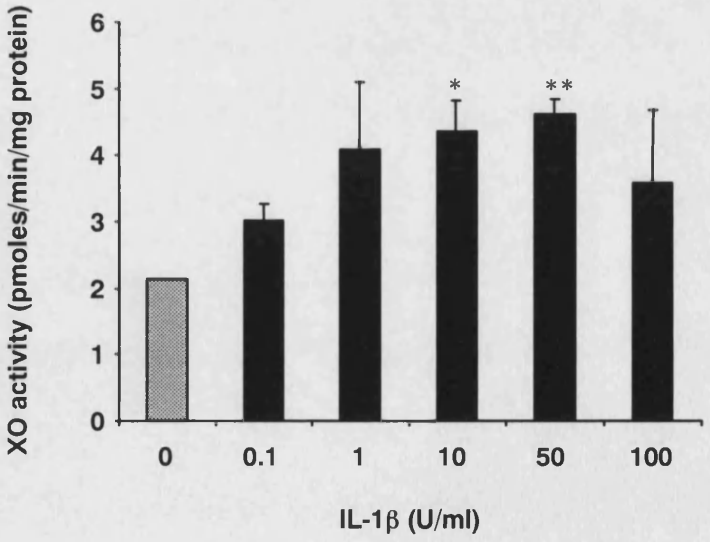


Figure 4.19. The effect of IL-1β on XO activity in rat calvarial osteoblasts.
above basal levels $*p < 0.05$ and $** p < 0.01$ respectively. Data presented are means ±S.E.M from 2 separate experiments.

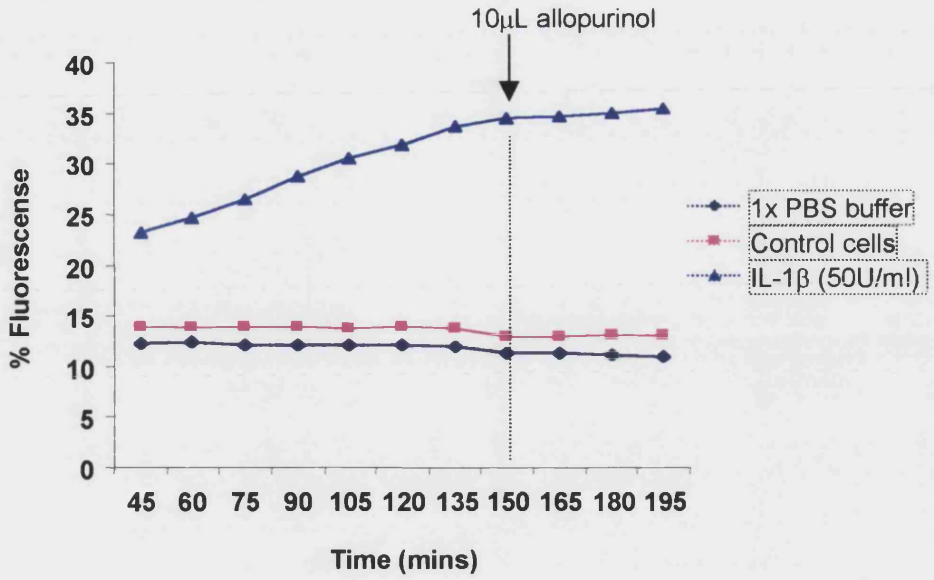


Figure 4.20. The effect of IL-1β on the XO activity in human osteoblast-like cells.

Human osteoblast-like cells grown in culture, which were assessed to be mature osteoblasts, were assayed for XO activity after treatment with IL-1β (50 IU/ml) for 24hrs. The same protocol was used as before for the RCOBs but instead of cell lysates being assessed for total protein content, HOB cultures were evaluated for cell number (see appendix III, Trypan Blue cell viability count) before being lysed. Again, following addition of 20μM pterin to the lysates, changes in fluorescence were determined over time. Fig. 4.20 shows that although no basal levels of activity were detected in unstimulated HOBs, IL-1β-treated HOBs showed some activity (0.18pmols/min/million cells). These data were from only 1 experiment, because of the large number of HOBs required for this assay. This HOB-based study was not pursued further because of a potential phenotypic change with serial passaging required to produce significant numbers of cells for such an investigation.

4.4.2.7. The effect of Il-1β on of XO protein levels in osteoblasts.

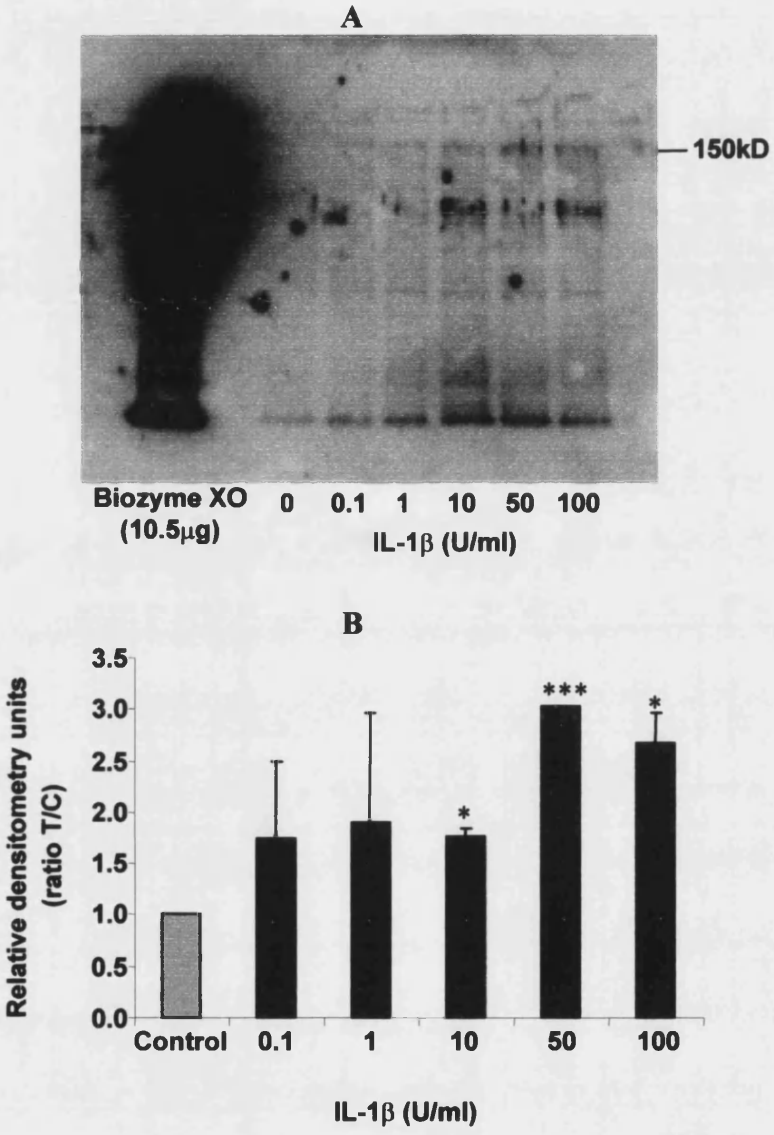


Figure 4.21. (A) A representative western blot of RCOB XO protein levels after incubation with IL-1β for 24hrs. (B) Ratio of RCOB XO protein levels in treated (IL-1β (0.1-100 IU/ml) over control levels (n=2 experiments)

In contrast to TNFα, IL-1β induced XO protein expression dose dependently in RCOBs stimulated over a 24hr period. Fig. 4.21 (A) is a representative blot of IL-1β induced XO protein expression showing an increase in the 150kD-protein band indicative of XO. Significant increases in RCOB XO protein expression were induced by 10, 50, and 100 IU/ml IL-1β (Fig. 4.21 (B). Significant increases of XO protein

expression over control levels are indicated by $*p < 0.05$; $***p < 0.001$. Data presented are means ±S.E.M from 2 separate experiments.

4.4.2.8. Summary.

We have demonstrated that IL-1β-induced resorption can be inhibited by the XO inhibitor allopurinol and by scavenging H_2O_2 by catalase. We found a dose effect increase in the activity of the enzyme when rat osteoblasts were stimulated with IL-1β. Additionally, we found that there was an increase in protein levels of the enzyme when stimulated with increasing dosages of IL-1β.

4.5. The effect of the combination of TNFα and IL-1β on mouse calvarial bone resorption.

4.5.1. Materials and methods.

4.5.1.1. The combined effect of TNFα and IL-β on bone resorption.

The optimal doses of TNFα (20ng/ml) and IL-1β (50 IU/ml) were combined and added to the mouse calvarial resorption assay to determine whether stimulation of resorption was additive or synergistic between these two cytokines.

4.5.1.2. The effect of catalase and allopurinol on TNFα and Il-1β-induced mouse calvarial bone resorption.

A dose range of allopurinol (0.01μM-10μM) and catalase (1 IU/ml & 10 IU/ml) were added to determine whether the significant inhibitory effect of allopurinol and catalase observed with these cytokines when added alone could be reproduced with the addition of the two cytokines simultaneously.

4.5.2. Results.

4.5.2.1. The effect of allopurinol on the combination of TNFα and IL-1β-induced bone resorption.

Although the basal levels of resorption were lower than in the majority of previous experiments (below 0.2mg/dL), TNFα (20ng/ml and IL-1β (50 IU/ml) still induced resorption significantly. (*p < 0.05 increase in resorption above control). A combination of these two cytokines had only a slight additive effect on the calvarial resorption. (**p < 0.01 increase in resorption above control) (Fig. 4.22). Data presented are means ±S.E.M of three separate experiments.

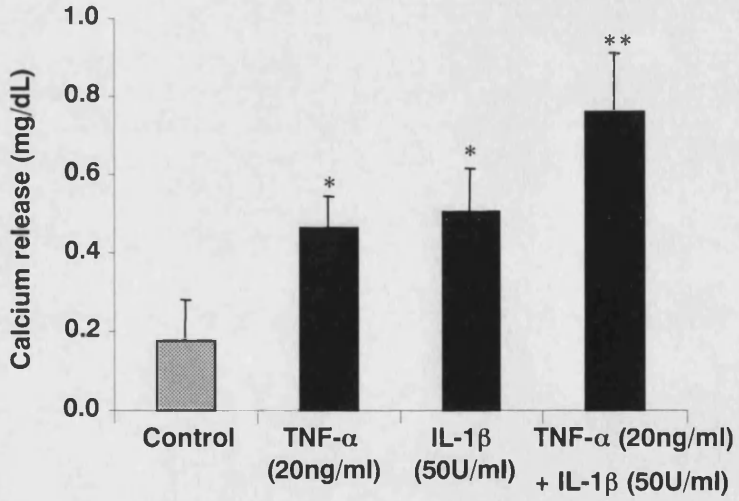


Figure 4.22. The effect of the combination of TNFα and IL-1β on mouse calvarial bone resorption.

The addition of allopurinol (0.01μM- 10μM) to the combination of TNFα and IL-1β-induced resorption (Fig. 4.23) caused an inhibition of bone resorption that was significant between doses 0.1μM and 10μM (*p < 0.05 inhibition). Allopurinol reduced the combination's resorptive effect to just above the basal level. Data presented are means ±S.E.M of three separate experiments, where n=6 calvariae per treatment.

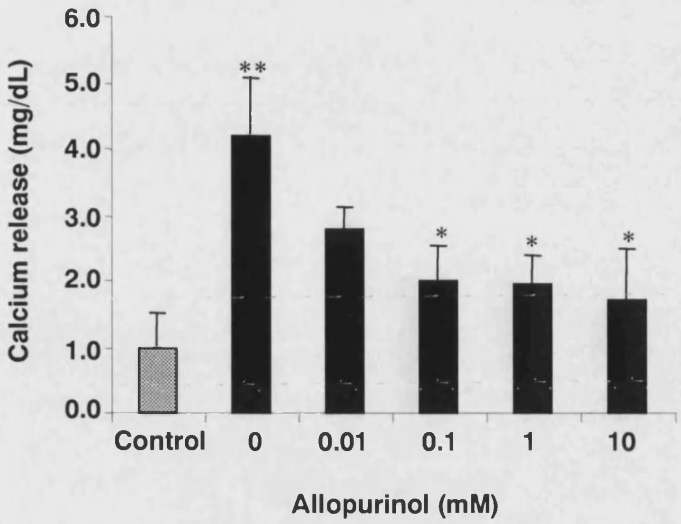


Figure 4.23. The effect of allopurinol on ■ TNFα (20ng/ml) + IL-1β(50 IU/ml)-induced mouse calvarial bone resorption.

4.5.2.2. The effect of catalase on the combination of TNFα and IL-1β-induced bone resorption.

Fig. 4.24 shows catalase to have an inhibitory effect, which is significant for the doses of 1 IU/ml and 10 IU/ml $*p < 0.05$ and $***p < 0.001$ respectively. Basal levels of resorption returned to previously reported levels in this thesis, nevertheless the combination of TNFα (20ng/ml) and IL-1β (50 IU/ml) increased resorption levels highly significantly over control ($^{+++}p < 0.001$). Data presented are means ±S.E.M from 2 separate experiments, where n=5-6 calvariae per treatment.

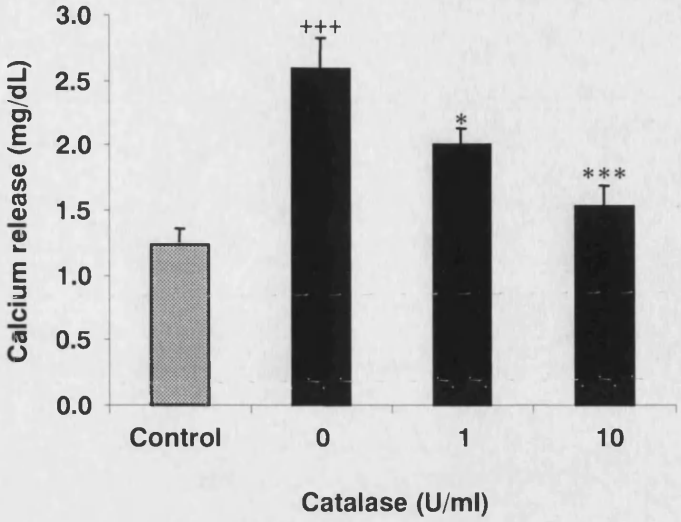


Figure 4.24. The effect of catalase on ■ TNFα (20ng/ml) and IL-1β(50 IU/ml)-induced mouse calvarial bone resorption.

4.6. The effect of XO on IFNγ inhibition of bone resorption.

4.6.1. Materials and methods.

4.6.1.1. The effect of IFNγ on mouse calvarial bone resorption.

The effect of the cytokine Interferon γ (Boehringer Mannheim, UK) on mouse calvarial bone resorption was investigated. The same protocol was followed as previously described for the TNFα and IL-1β experiments (section 4.3.1.1) replacing TNFα or IL-1β with IFNγ. Mouse hemi-calvariae were incubated with the resorption culture medium (section 4.3.1.1) which included a concentration range of IFNγ (0.025-250 IU/ml, NIBSC) for a period of 96hrs in a humidified, 5% CO_2/95% air incubator. Calcium levels in the culture medium were assayed using the cresolphthalein complexone reaction as an index of bone resorption. Initial experiments were carried out to confirm that IFNγ had an inhibitory effect on IL-1β (30 IU/ml)-induced resorption in our assay as previously reported by Ralston *et al*, 1995. For these studies, increasing doses of IFNγ were added to the IL-1β-stimulated calvariae.

4.6.1.2. Altering the inhibitory effect of IFNγ by addition of XO and reactive oxygen species inhibitors.

XO activity has been shown to be increased in human mammary epithelial cells upon treatment with IFNγ (Page *et al*, 1998). In this study, IFNγ increased XO activity by a factor of approximately 4, compared to other cytokines such as TNFα and IL-1β. Based on this observation, the XO inhibitor allopurinol (10μM) and ROS inhibitors, catalase (10 IU/ml) and SOD (10μg/ml) respectively, were included in the calvarial resorption assay (containing IFNγ 1000 IU/ml Boehringer Mannheim).

4.6.1.3. XO protein expression induced by IFNγ in rat osteoblasts.

To ascertain whether XO protein expression in RCOBs was affected by IFNγ the method previously described in section 4.3.1.5 was followed replacing TNFα with a dose range of IFNγ (1-500 IU/ml) over a 24 hr period of incubation. With the acquisition of a new mouse monoclonal XO antibody (Neomarkers, see appendix IV) protein lysates extracted from these experiments were subjected to SDS-PAGE and western blotting using this new antibody. The only changes from the method of section 4.3.1.5 is the dilution of the monoclonal antibody (1:350) and that the secondary antibody was rabbit anti-mouse horseradish peroxidase conjugate (1:1000) dilution.

4.6.2. Results.

4.6.2.1. The dose effect of IFNγ on mouse calvarial bone resorption.

IFNγ inhibited basal resorption significantly at concentrations of 0.25 IU/ml (**$p < 0.01$), 2.5 IU/ml (***$p < 0.001$), 25 IU/ml (*$p < 0.05$), 250 IU/ml (***$p < 0.001$) respectively (Fig. 4.25). Data are means ±std. from 1 experiment, where n=6 calvariae per treatment.

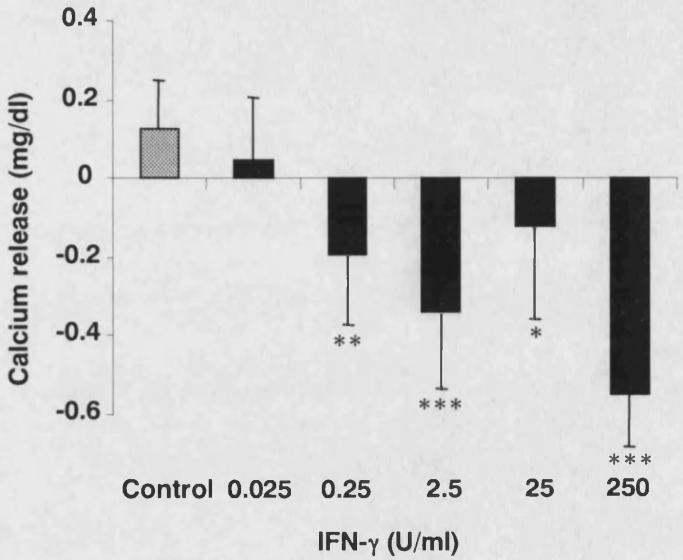


Figure 4.25. The effect of interferonγ on basal levels of mouse calvarial bone resorption *in vitro*.

4.6.2.2. The effect of IFNγ on IL-1β-induced resorption.

IFNγ as previously reported in the literature has an inhibitory effect on IL-1β (30 IU/ml)-induced bone resorption. In this study, 25 IU/ml (*$p < 0.05$) and 250 IU/ml (**$p < 0.001$) of IFNγ caused significant inhibition of IL-1β-induced resorption respectively (4.26). The data presented means ±std. of one experiment where n=5 calvariae per treatment group. IL-1 β at 30 IU/ml stimulated bone resorption significantly over the control basal levels. ($^{++}p < 0.01$).

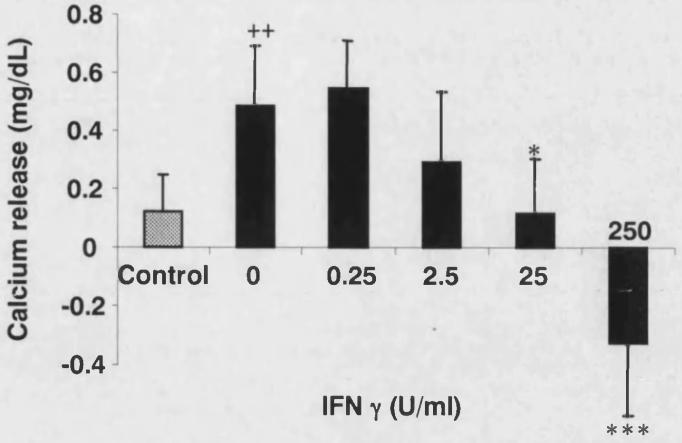


Figure 4.26. The effect of IFNγ on IL-1β (30 IU/ml) ■ induced mouse calvarial bone resorption.

4.6.2.3. The effect of SOD, catalase and allopurinol on IFNγ mediated inhibition of basal bone resorption.

The addition of the XO inhibitor allopurinol (10μM) and the superoxide scavenger superoxide dismutase (SOD) (10μg/ml) reversed the inhibitory effect of IFNγ (1000 IU/ml) (Boehringer Mannheim) on basal mouse calvarial bone resorption (Fig. 4.27). Catalase (10 IU/ml) had no effect on the inhibitory effect of IFNγ on basal levels of bone resorption $^{+++}p < 0.001$ significant inhibition of basal bone resorption by IFNγ (1000 IU/ml). A significant increase in bone resorption over IFNγ inhibition is indicated by $***p < 0.001$. Data presented are means ±s.t.d of one experiment, where n=8 calvariae per treatment.

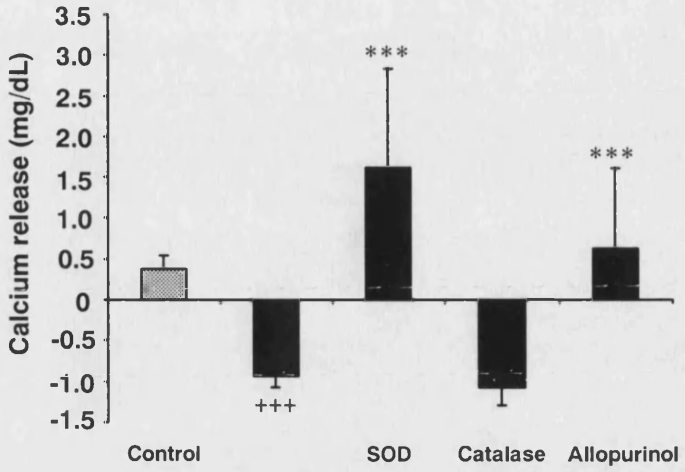


Figure 4.27. The effect of SOD (10μg/ml), catalase (10 IU/ml) and allopurinol (10μM) on ■ IFNγ (1000 IU/ml)-induced mouse calvarial bone resorption.

4.6.2.4. The dose related effect of SOD on the inhibitory effect of IFNγ on mouse calvarial bone resorption.

Reproducibility of the effect of SOD on IFNγ inhibition of basal resorption as shown in Fig.4.27 proved problematic. Further experiments, where a dose range of SOD was tested in conjunction with two different concentrations of IFNγ (100 IU/ml and 1000 IU/ml) were carried out to validate the initial observations.

Figures 4.28 A&B indicate that, the addition of increasing doses of SOD to the IFNγ incubated calvarial cultures resulted in a biphasic resorptive response in the mouse calvarial system. Fig 4.28 (B) shows that 100 IU/ml of IFNγ had a significant inhibition of bone resorption ($^{+}p < 0.05$). Addition of SOD (1μg/ml-1000μg/ml) caused a reversal of the inhibitory effect of IFNγ with 1μg/ml which steadily decreased with higher concentrations of SOD ($^{*}p < 0.05$). Fig. 4.28 (B) indicates that, where 1000 IU/ml of IFNγ resulted in a highly significant inhibition of basal bone resorption ($^{***}p < 0.001$), higher concentrations of SOD (10μg/ml) were required to reverse the inhibitory effect of IFNγ on bone resorption ($^{++}p < 0.01$).

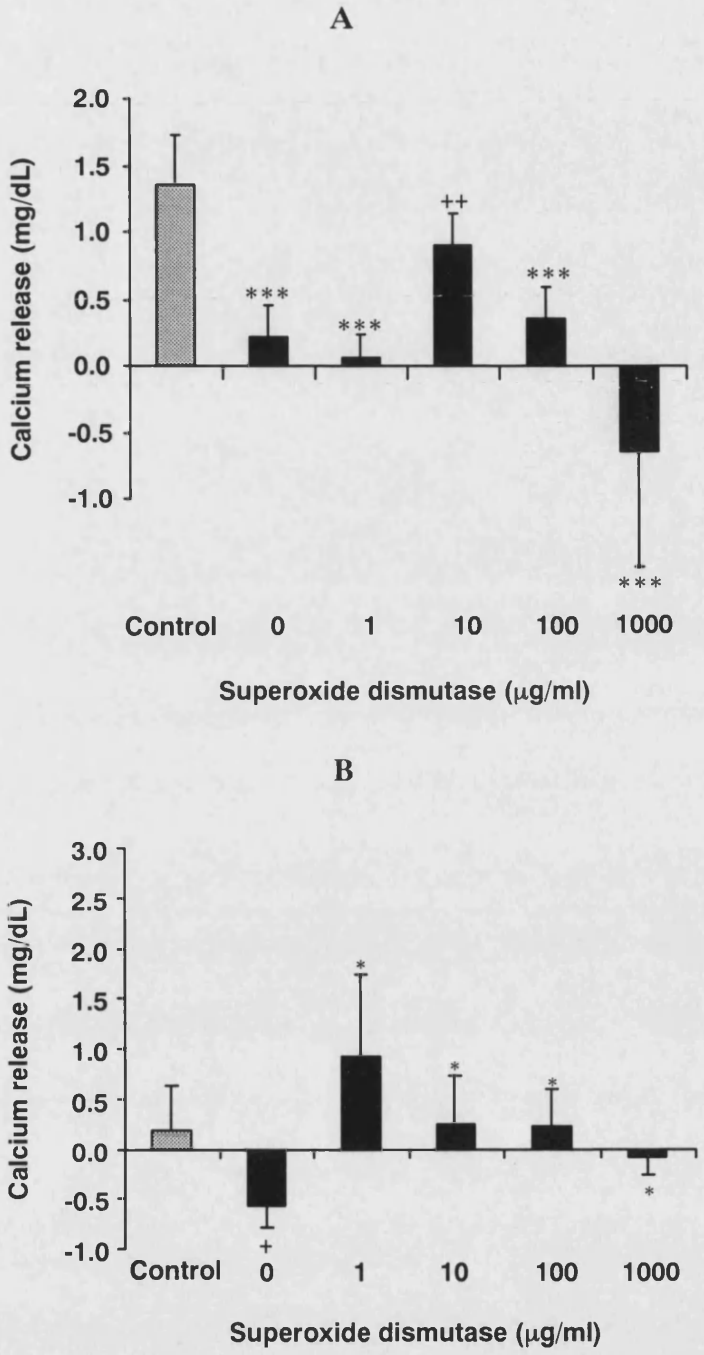


Figure 4.28. The effects of SOD (1μg/ml-1000μg/ml) on the inhibitory effect of ■ IFNγ (A) (100 IU/ml) and (B) (1000 IU/ml) on mouse calvarial bone resorption.

Again a biphasic response was evident where a reversal to IFNγ-mediated inhibitory levels were achieved. The data shown in Fig 4.28 (A & B) are means ±std. representative of experiments performed once, where n=4 calvariae per treatment for each experiment.

4.6.2.5. The effect of IFNγ on XO protein levels in rat calvarial oteoblasts.

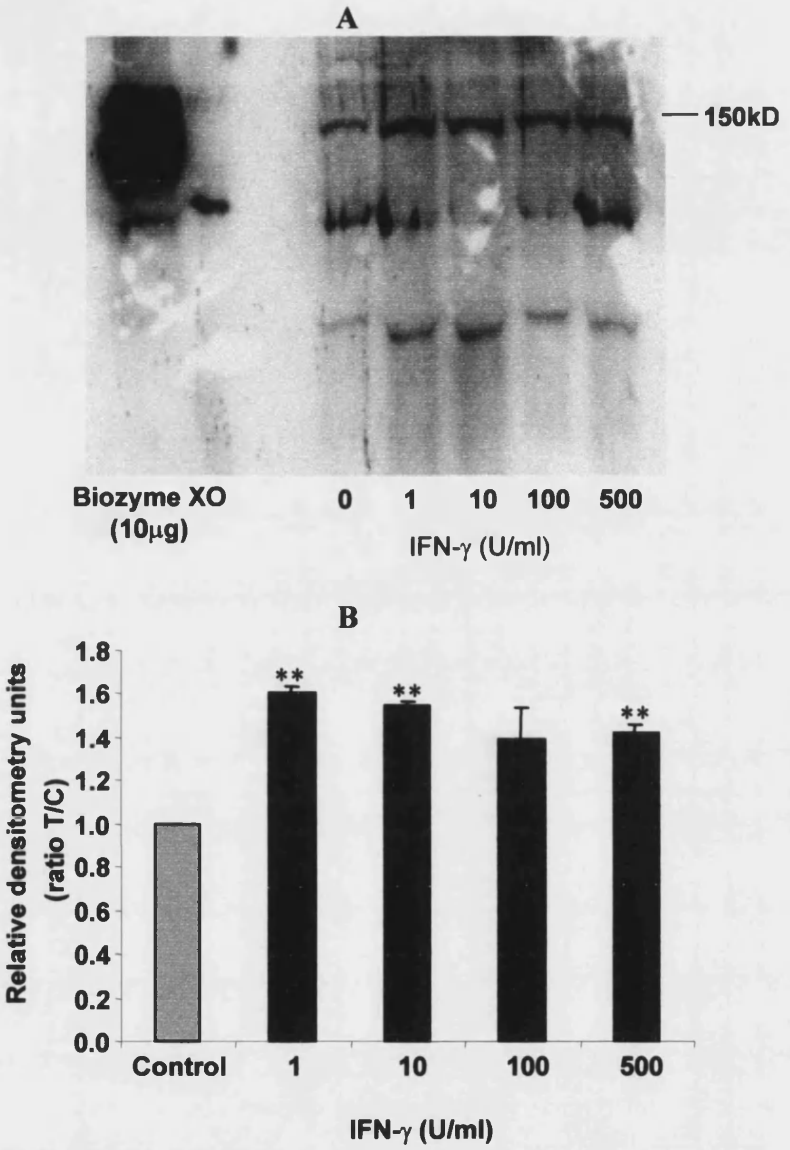


Figure 4.29. (A) A representative western blot of RCOB protein levels after incubation with IFNγ for 24hrs. (B) RCOB protein levels ratios over control levels induced by IFNγ (1-500 IU/ml). (n=2 experiments).

IFNγ (1-500 IU/ml) treatment of RCOB over 24-hrs resulted in enhanced XO protein levels. Fig. 4.29 (A) is a representative blot of IFNγ-induced XO protein levels showing an increase in the 150kD-protein band corresponding to XO. A significant increase in XO protein levels in RCOBs was achieved at 1 IU/ml IFNγ. No further increases in XO protein levels were found when increased concentrations of IFNγ (10-

500 IU/ml) were added to the RCOBs (Fig. 4.29 (B). Significant increases in XO protein over controls are indicated by $**p < 0.01$. Data are means ±S.E.M. from 2 experiments.

4.6.2.6. Summary.

We have demonstrated that IFNγ has the ability to inhibit mouse calvarial bone resorption and that superoxide dismutase and allopurinol can reverse this inhibitory effect.

4.7. Discussion.

The destruction of joints by bone erosion in diseases such as rheumatoid arthritis may be facilitated by the cytokines TNFα and IL-1β which have already been established as potent inducers of bone resorption, both *in vitro* and *in vivo* (Mundy, 1993). Indeed, TNFα is currently perceived to hold a pivotal position in the hierarchy of cytokine responses in rheumatoid arthritis, as evidenced by the clinical studies of Maini *et al.* 1995. Placebo-controlled clinical trials have demonstrated the efficacy of using a chimeric anti-TNFα antibody to rapidly attenuate rheumatoid synovitis (Elliot *et al*, 1995). Some of the actions of TNFα have been shown to involve the generation of low levels of reactive oxygen species and other free radicals (Remacle *et al*, 1995). These are small, labile biomolecules, which can act as intra- or inter-cellular messengers, and appear to be involved in many physiological and pathological processes (Remacle *et al*, 1995). Select antioxidants and metal chelators can inhibit cytoxicity and gene expression in response to TNFα in numerous cell types (Terada *et al*, 1992). In addition, TNFα can upregulate mRNA expression of xanthine oxidoreductase and increases the levels of protein in renal epithelial cells (Pfeffer *et al*, 1994). Furthermore, IL-1β in conjunction with LPS can upregulate XO activity and mRNA in rat lungs (Hassoun *et al*, 1998).

We have shown that the pro-resorptive cytokines TNFα and IL-1β can upregulate neonatal rat osteoblastic XO activity, and in the case of IL-1β, increase the protein levels. Results of the studies presented in this chapter did not show TNFα to increase protein levels in osteoblasts implying that there may be a slightly different signaling mechanism for TNFα compared to IL-1β. Indeed, it has been postulated that the effect of cytokine-mediated increases in XO protein activity in epithelial cells is a

result of post-translational modification (Page *et al,* 1998). Another possibility is that a single 24-hr exposure to TNFα would not reveal any increases in XO protein earlier or later than the time point. Further experiments with TNFα investigating a kinetic profile of XO levels are therefore required in order to delineate the mechanisms of possible TNFα-mediated increase in XO levels.

It has previously been demonstrated that H_2O_2 is generated by osteoblasts upon stimulation by TNFα (Stevens *et al,* 1993). Results presented in this chapter have shown that, by blocking ROS, either by scavenging H_2O_2 with catalase, or by blocking its generation using XO inhibitors, cytokine-induced resorptive activity in mouse calvariae can indeed be inhibited. The combination of TNFα and IL-1β provided an additive effect on bone resorption. Catalase and allopurinol could inhibit this additive effect; similar to inhibitions observed when these cytokines were used on their own. These results, coupled with previously reported evidence, indicating that H_2O_2 is involved in osteoclastogenesis and increased bone resorption (Bax *et al,* 1992; Fraser *et al,* 1996; Steinbeck *et al,* 1998), allow it to be argued that XO is a source of H_2O_2 (a small labile factor) in TNFα and IL-1β- induced resorption.

In all of the resorption experiments that were carried out with TNFα and IL-1β, the inhibitory effects of allopurinol were, at best, a reversal to basal resorption levels. Although, this indicates the strong possibility of the presence of other factors working in conjunction with XO in the basal resorptive process, the observation also validates the significant role of XO in cytokine-mediated increases in bone resorption.

One of the other factors known to affect the resorptive process is nitric oxide (NO) (Ralston *et al,* 1995). In their studies, they demonstrated that low levels of NO generated by TNFα and IL-1β, could activate the bone resorptive process. However,

upon addition of IFNγ to these cytokines, high levels of NO were synergistically generated which caused an inhibition of bone resorption. Indeed, it has been shown that IFNγ can even inhibit basal levels of bone resorption in culture (Hoffmann *et al*, 1987). There is a slight discrepancy between Ralston's work and others in that IFNγ did not stimulate significant amounts of nitrite (breakdown product of NO in tissue culture medium) from calvariae (Ralston *et al*, 1995) or osteoblasts (Ralston *et al*, 1994). Whereas other groups have shown it to be significantly increased above basal levels (Hughes *et al*, 1999). This maybe due to a difference in the species and cell lines used, but the overall indication is that bone cells in response to stimulation by IFNγ produce sufficient physiological concentrations of NO. We postulate that XO and NO could be working together in the resorptive process induced by these cytokines.

One of the major mechanisms of injury associated with the production of NO is its diffusion-limited reaction with superoxide to form the powerful oxidant peroxynitrite ($ONOO^-$) (Beckman & Koppenol, 1996). The effects of superoxide are controlled by its reaction rates with possible targets. The reaction rate for the formation of peroxynitrite from $O_2^{\bullet-}$ and NO has been determined to be $6.7 \pm 0.9 \times 10^9\ M^{-1}\ s^{-1}$ (Huie & Padmaja, 1993), which has been shown to be approximately six times faster than the scavenging effects of superoxide dismutase at physiological ionic strength (Cudd & Fridovich, 1982; Rigo *et al*, 1975). *In vivo*, SOD is usually present in micromolar concentrations and therefore keeps superoxide levels low. NO concentrations required for vasorelaxation cannot out-compete SOD for $O_2^{\bullet-}$. However, in pathological conditions NO or superoxide are produced in high enough concentrations to out-compete endogenous SOD. Thus, sufficient concentrations of $ONOO^-$ can be produced (Beckman & Koppenol, 1996). Indeed the generation of high levels of $O_2^{\bullet-}$ in pathology may be

attributable to XO especially in response to IFNγ, since the XO gene contains transcriptional initiation site sequences for five IFNγ response elements (Hoidal, *et al*, 1997). Interestingly, the presence of these IFNγ responsive sites may reflect the mechanisms underlying the observations made by Page *et al*, 1998 who reported that in epithelial cells TNFα and IL-1β increase XO activity by 2 and 2.5 fold respectively whereas IFNγ impressively resulted in an 8 fold increase. These observations compound the possibility that IFNγ-induced XO could generate sufficient concentrations of $O_2^{\bullet-}$ to react with NO to produce the powerful oxidant $ONOO^-$. The latter species ($ONOO^-$) has been shown to inhibit bone resorption (Mancini *et al,* 1998). IFNγ-related data from studies discussed in this chapter substantiate the notion that when SOD (which converts $O_2^{\bullet-}$ to H_2O_2) was added to the calvarial cultures the effect of IFNγ inhibition on bone resorption was reversed. It was also found that the XO inhibitor allopurinol has a similar effect. Interestingly, a novel, developmentally regulated 150kD plasma membrane glycoprotein related Mn^{++} superoxide dismutase has been reported on osteoclasts and identified as the ligand for the osteoclast-specific monoclonal antibody (Mab) 121F (Oursler *et al*, 1991).

When a concentration range of SOD was added to the calvariae where resorption was inhibited by IFNγ, a biphasic response was observed. When higher concentrations of SOD were added, the inhibitory effect of IFNγ resumed, implying that high concentration levels of H_2O_2 were being generated which were inhibiting the resorptive process. This may partly explain the differing effects of IFNγ in *in vitro* studies compared to *in vivo* studies. *In vitro,* IFNγ has been shown to have an inhibitory effect on bone resorption especially with combinations of cytokines such as TNFα and IL-1β (Ralston *et al,* 1995). Conversely, *in vivo,* IFNγ is known to increase bone loss possibly

due to inhibition of bone turnover by inhibiting resorption as well as formation (Mann *et al*, 1994). Other studies have shown that administration of IFNγ to congenital osteopetrotic sufferers has led to an increase in bone resorption and haematopoiesis (Key *et al*, 1995).

These results complement the recent evidence that cytokine-induced bone resorption is mediated, in part, by nitric oxide (Ralston *et al*, 1995; Evans *et al*, 1996), since its biological activity is modulated by interaction with the XO product, superoxide. Considering that the osteoblast contains both inducible nitric oxide synthase (Ralston *et al*, 1994) and XO, the possible interaction of nitric oxide and superoxide maybe an important controlling factor involved in the bone resorptive process.

The studies described in this chapter demonstrate a central role for XO in cytokine-induced bone resorption and suggest that XO inhibition may suppress inflammation-related bone resorption. These studies also point out the significance of understanding how redox controlled events, such as bone resorption, are tightly regulated and that small changes in the bone microenvironment may have significant consequences in the disruption of this control, leading to pathology.

Chapter 5.

The role of XO in parathyroid hormone and 1α,25-dihydroxyvitamin D_3-induced bone resorption.

Chapter 5

5.1. Introduction.

This chapter investigates the potential significance of XO and the generation of ROS in calcitropic hormone-induced bone resorption. A number of hormones contribute to normal bone tissue activity, but the three most investigated hormones known to affect the skeleton in order to maintain the body's calcium homeostasis are parathyroid hormone (PTH), 1α, 25$(OH)_2$ vitamin D_3 (1α,25-$(OH)_2D_3$) and calcitonin (CT).

5.1.1. Parathyroid hormone.

PTH is able to maintain the body's calcium levels by exerting a direct effect on the kidney and the skeleton, and an indirect effect on the gastrointestinal tract (GI). Indirectly, PTH exerts its effect on the GI tract by triggering increases in calcium absorption through its ability to produce the active 1, 25-dihydroxyvitamin D_3 from hydroxyvitamin D_3. PTH acts directly on the kidney to facilitate greater levels of absorption of calcium and on the skeleton to elevate calcium release from bone (Fitzpatrick & Bilezikian, 1996).

PTH (1-84) which is secreted by the parathyroid glands is the predominant biologically active molecule, having a half-life in the circulation of around 3 mins. In normal settings, and due to its short half-life, about 70-90% of circulating hormone is present as inactive C-terminal fragments. The kidney and the liver then usually clear away these fragments of the hormone. The amino-terminal (N)-fragment, PTH (1-34) has a similar activity to that of the 1-84 intact PTH molecule, and is thought to be produced from the intact molecule when it undergoes hepatic proteolysis (Fitzpatrick & Bilezikian, 1996). Both *in vivo* and *in vitro,* PTH is well documented to be a potent

stimulator of osteoclastic bone resorption. Miller, 1978 and Miller *et al,* 1984 showed that PTH, *in vivo,* increases the number of osteoclasts exhibiting ruffled borders as well as increasing the size of these ruffled borders. These observations, coupled with rapid increases in osteoclast numbers 1hr after administering PTH, as well as its ability to increase the number of nuclei per osteoclast, (Addison, 1979; Tatevossian, 1973) suggest that PTH is critical for the activation of mature bone resorbing osteoclasts.

The exact mechanism(s) involved in the rapid increase of skeletal resorption by PTH are not known. However, it is deemed that structural and functional changes rapidly occur in cells on the endosteal surface in response to PTH resulting in an elevated release of calcium (Neuman *et al,* 1979; Talmage & Meyer, 1967). There appear to be two schools of thought on how PTH exerts its pro-resorptive effects on osteoclasts. Firstly, it has been shown that PTH stimulates ostoclastic bone resorption by an indirect effect, stimulating the osteoblast to release a factor or factors that activates the osteoclast (McSheehy & Chambers, 1986) and secondly, by direct activation of the osteoclast to resorb bone (Murrils *et al,* 1990; Teti *et al,* 1991). The direct action of PTH may explain the rapid increases in bone resorption when PTH is administered, but osteoblasts, or cells of a similar lineage may well mediate the prolonged effects of PTH.

ROS have been found to be elevated by PTH in cultured bone (Garrett et al, 1990), and more recently PTH has been shown to bind to a functional receptor on osteoclasts resulting in an instantaneous burst of $O_2^{\bullet-}$ (Datta *et al,* 1996). Although there is enough evidence showing that ROS is produced from osteoclasts upon stimulation by PTH, the source(s) of these ROS are yet to be fully identified.

5.1.2. Vitamin D_3

Vitamin D is a critical factor required for maintaining and developing bone, as well as playing a pivotal role in maintaining calcium and phosphorous homeostasis. Cholecalciferol is the form of vitamin D obtained when the skin is exposed to the sunlight, converting the precursor 7-dehydrocholesterol into vitamin D_3 (Norman & Collins, 1996). This pro-hormone is transported to the liver where upon it is hydroxylated at C-25, resulting in 25-hydroxyvitamin D_3. 25-hydroxyvitamin D_3 is then transported to the kidney via a circulating serum vitamin D-binding protein, where it is hydroxylated at the α position of carbon 1 of the A ring. This produces the biologically active, 1α, 25$(OH)_2$ vitamin D_3 (Christakos, 1996), which functions similarly to that of a steroid hormone (Reichel *et al*, 1989). The metabolic pathway of Vitamin D is summarised in Fig 5.1.

Once the active form has been produced it acts predominantly on three main target tissues; the kidney, intestines and the skeleton.

It is still to be determined how exactly 1α,25 $(OH)_2$ D_3 affects mineral homeostasis, but it is thought that actions on the intestine and the kidney to reabsorb more calcium and phosphorous, allow for more minerals to be incorporated into the active bone forming sites.

In vitro, 1α,25 $(OH)_2$ D_3 is a potent inducer of bone resorption (Raisz *et al*,1972). Like PTH, it is thought that its resorptive action is modulated via osteoprogenitor cells, such as osteoblast precursors and mature osteoblasts, which have receptors for 1α,25 $(OH)_2$ D_3. McSheehy & Chambers (1987) suggested that 1α,25 $(OH)_2$ D_3 might release a factor from the osteoblast that signals the osteoclast to resorb the bone matrix. The studies of Suda *et al*, 1997 supported this idea, showing that 1α,

25 $(OH)_2$ D_3-induced osteoclast development was mediated via the vitamin D receptor, which is found on the osteoblast and not the osteoclast. It is also important for the differentiation of osteoclasts to have osteoblast-osteoclast progenitor cell-to-cell interactions. Without this interaction or coupling it has been demonstrated that differentiation of osteoclasts will not take place even with the addition of 1α, 25 $(OH)_2$ D_3. (Takahashi *et al*, 1988). The possibility arises that osteoblasts produce a small labile factor(s), which can only travel small distances in the cellular environment. Reactive oxygen species have such characteristics and have already been implicated in bringing about osteoclast differentiation (Fraser *et al*, 1996; Steinbeck *et al*, 1998). As in the case of PTH, the sources of these signalling molecules are yet to be fully determined.

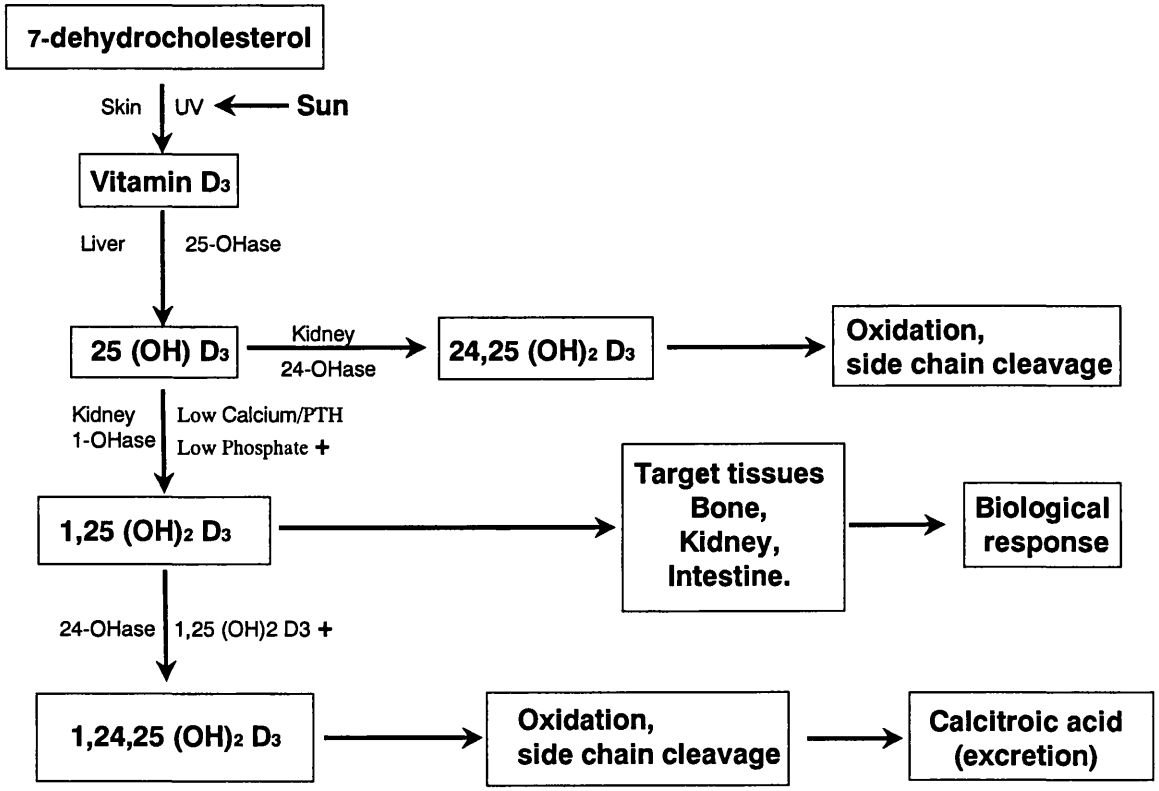


Figure 5.1. The metabolic pathway of Vitamin D.

5.1.3. Calcitonin (CT).

Mature CT is a product of procalcitonin, which is produced by the parafollicular C cells of the thyroid gland (Becker *et al*, 1996). Its effects are mainly concentrated on the kidney, the central nervous system, the respiratory tract, the gastrointestinal and reproductive systems, and bone. CT receptors have been identified to be on mature osteoclasts (Nicholson *et al*, 1986) and have been shown to have many inhibitory effects on the bone resorptive mechanism. Initially, it was found that CT when administered to laboratory animals could decrease serum calcium levels (Copp & Davidson, 1961). It was also found to inhibit the release of acid phophatase from osteoclasts (Zaidi *et al*, 1991), cell motility and cause a gradual retraction of the osteoclast (Chambers *et al*, 1986; Alam *et al*, 1993).

5.2. Aims and objectives.

- To establish the optimal dose of the calcitropic hormones PTH and 1α, 25 $(OH)_2$ D_3 for activation of mouse calvarial bone resorption.
- To study the potential role (involvement) of XO in PTH and 1α, 25 $(OH)_2$ D_3-mediated bone resorption, and the type of ROS that could play a part in hormonally mediated bone resorption.

5.3. PTH-induced bone resorption.

5.3.1. Materials and methods

5.3.1.1. The effect of PTH on mouse calvarial bone resorption.

Using the mouse calvarial assay as previously discussed in section 3.1.3. Calvariae were stimulated with increasing doses of bovine parathyroid hormone 1-34 fragment (NIBSC, see appendix II) (0.01 IU/ml-1 IU/ml). The calvariae were incubated as previously described for 96hrs in a humidified incubator with an atmosphere of 5%CO_2/ 95% air. After this period of incubation TCM samples were measured for Ca^{2+} release using the cresolphthalein complexone assay (see section 3.3.1). All the calvarial assays involving the calcitropic hormones were performed in the same manner.

5.3.1.2. The effect of allopurinol, catalase and SOD on PTH-induced bone resorption

Using the optimum dose of PTH (1 IU/ml) the XO inhibitor allopurinol (0.01μM-10μM) was added to the mouse calvarial cultures to investigate the potential inhibition of PTH-induced bone resorption. Similar experiments were carried out, where increasing doses of catalase (0.1 IU/ml-100 IU/ml) and SOD (0.1μg/ml-100μg/ml) were added to determine whether H_2O_2 or $O_2^{\bullet-}$ were involved in PTH-induced bone resorption.

5.3.1.3. The effect of diphenyliodonium chloride (DPI) on PTH-induced bone resorption.

Osteoclasts produce oxygen radicals at the bone interface (Key *et al*, 1990) that are thought to be derived from the activated NADPH oxidase enzyme (Steinbeck *et al*, 1994). Using the optimum dose of PTH on mouse calvariae we added diphenyliodonium chhloride (DPI) (ICN Flow see appendix II), a non-specific inhibitor

of flavoenzymes such as NADPH oxidase, (Robertson *et al*, 1990) to establish if NADPH oxidase is involved in the generation of ROS in PTH-mediated bone resorption.

5.3.2. Results.

5.3.2.1. The dose effect of PTH on mouse calvarial bone resorption.

PTH (0.1U/ml-1U/ml) induced bone resorption in a dose dependent manner over a 96-hour period of incubation. The increase in resorption by PTH over basal levels was highly significant ($^{***}p < 0.001$). Data presented are means ±S.E.M. of 3 separate experiments, where n=6 calvariae per treatment (Fig. 5.2).

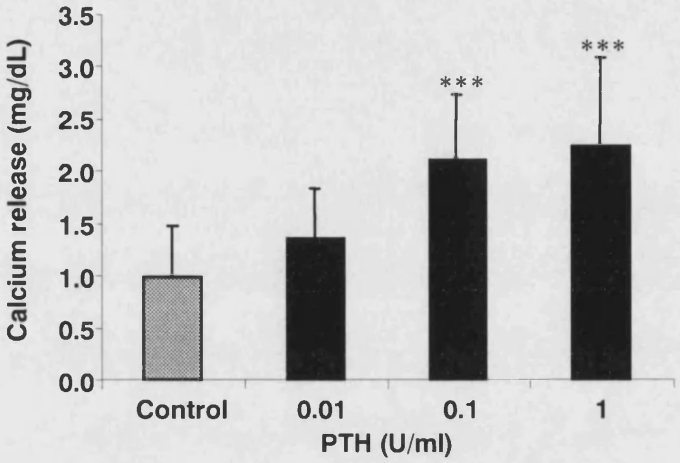


Figure 5.2. The dose related effect of PTH on mouse calvarial bone resorption.

5.3.2.2. The effect of allopurinol on PTH-induced bone resorption.

Allopurinol (0.01μM-10μM), added to the mouse calvariae that had been stimulated with an optimal dose of PTH (1 IU/ml), had no effect on TCM Ca^{2+} levels over a 96 hour period of incubation. Increases in resorption by PTH over basal levels were again highly significant ($^{***}p < 0.001$, n=6 calvariae per treatment). Data presented are means ±S.E.M. of three separate experiments, where n=6 calvariae per treatment (Fig. 5.3).

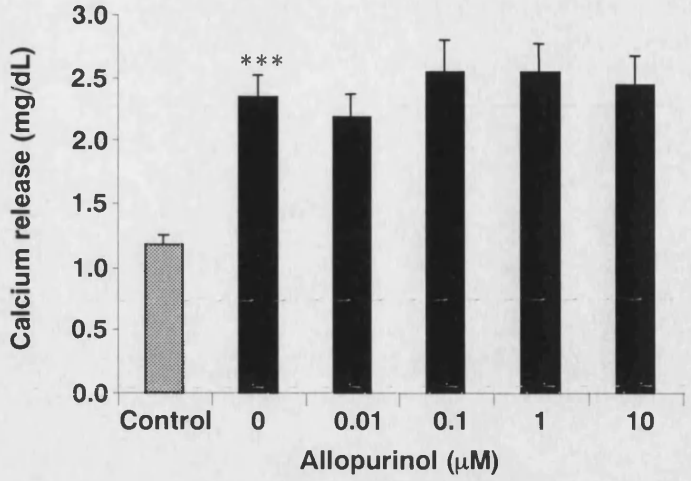


Figure 5.3. The effect of the XO inhibitor, allopurinol on PTH-induced bone resorption ▒ Control; ■ 1 IU/ml PTH.

5.3.2.3. The effect of catalase on PTH-induced bone resorption

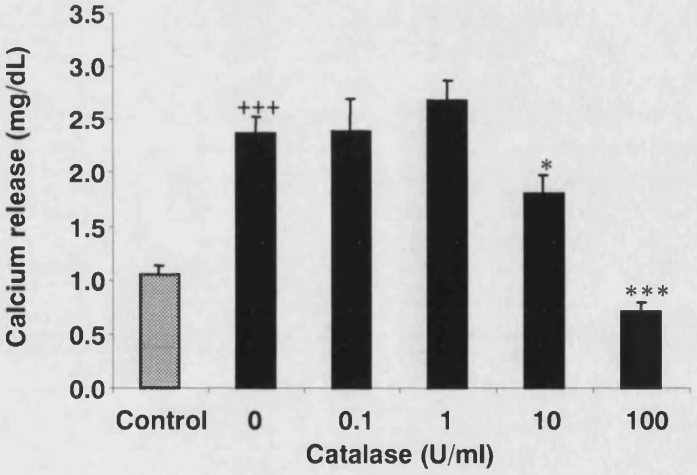


Figure 5.4. The effect of catalase on PTH-induced bone resorption. ▒ Control; ■ 1 IU/ml PTH.

Unlike allopurinol, catalase had an inhibitory effect (10 IU/ml-100 IU/ml) on PTH-induced mouse calvarial resorption over a 96-hour incubation period. Increases in resorption by PTH over basal levels were consistently significant ($^{+++}p < 0.001$). In addition significant inhibition by catalase was attained at doses of 10 IU/ml ($^{*}p < 0.05$)

and 100 IU/ml (***$p < 0.001$) respectively. Data presented are means ±S.E.M. of three separate experiments, where n = 4-6 calvariae per treatment (Fig. 5.4).

5.3.2.4. The effect of superoxide dismutase (SOD) on PTH-induced bone resorption.

Superoxide dismutase (SOD) which catalyzes the dismutation of $O_2^{-\bullet}$ to oxygen and H_2O_2 had no effect on PTH (1 IU/ml)-induced bone resorption (Fig. 5.5), indicating that the resorptive effect due to ostoblast/osteoclast signalling did not involve $O_2^{-\bullet}$ radicals. PTH showed a significant increase in resorption above basal levels ($^{+++}p < 0.001$). Data presented are means ±std. from one experiment, where n=4 calvariae per treatment.

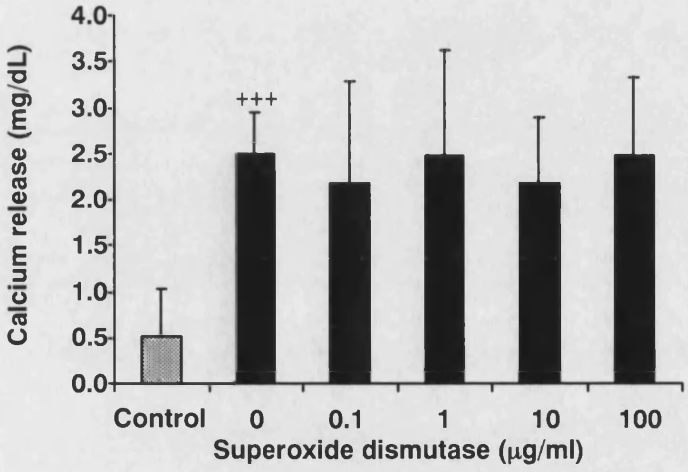


Figure 5.5. The effect of SOD on PTH-induced calvarial bone resorption. ▒ Control; ■ 1U/ml PTH

5.3.2.5. The effect of DPI on PTH-induced bone resorption.

Diphenyliodonium chloride, an inhibitor of NAD(P)H oxidase activity, had a dose-related inhibitory effect on PTH (1 IU/ml)-induced mouse calvarial resorption (Fig. 5.6). Significant inhibition was attained with 1μM (*$p < 0.05$) and 10μM (***$p < 0.001$) respectively. Significant resorption was attained above basal control levels with

1U/ml PTH. ($^{+++}p < 0.001$). Although incubation with 10μM DPI showed a slightly lower Ca^{2+} TCM reading than the control levels, this was not found to be statistically significant. Data presented are means ±std. within one experiment, where n=6 calvariae per treatment.

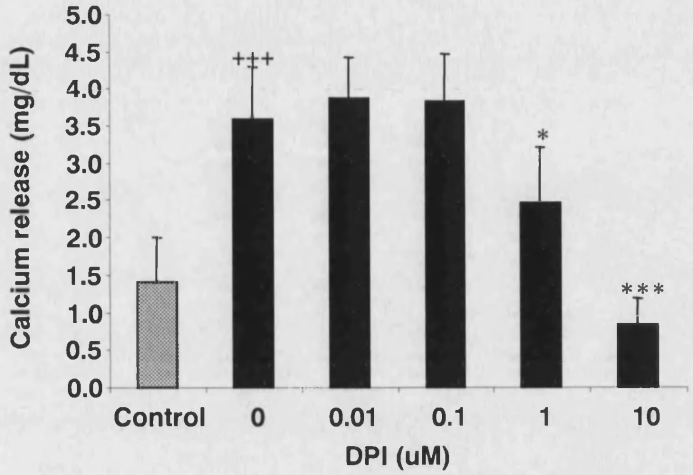


Figure 5.6. The effect of diphenyliodonium chloride (DPI) on PTH-induced mouse calvarial bone resorption. ▒ Control; ■ 1U/ml PTH.

5.4. 1α,25-$(OH)_2D_3$ induced bone resorption.

5.4.1. Materials and methods

5.4.1.1. The effect of 1α,25-$(OH)_2D_3$ on mouse calvarial bone resorption

1,25 Dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) also known as calcitriol (Sigma, UK) was added to the mouse calvarial assay in increasing doses (0.01nM-1μM) and incubated for 96hrs as previously described for PTH (section 5.3.1.1). Again the TCM was collected and analysed for calcium as an indicator of bone resorption.

5.4.1.2. Inhibition of 1α,25-$(OH)_2D_3$-induced bone resorption.

Once the optimum dose was found for the resorption of mouse calvariae, experiments were set up to find out whether XO is involved in the resorptive mechanism of 1α,25-$(OH)_2D_3$-induced bone resorption. Again in separate experiments, increasing doses of allopurinol (0.01μM-100μM), catalase (0.01 IU/ml-100U/ml) and DPI (0.011μM-10μM) were added to the TCM and after 96hrs of incubation the medium was collected and assayed for calcium release.

5.4.2. Results.

5.4.2.1. The dose effect of 1α,25-$(OH)_2D_3$ on mouse calvarial bone resorption.

Fig. 5.7 shows that stimulation of bone resorption was very potent when 1α,25-$(OH)_2D_3$ was added to the mouse calvarial cultures. 0.1nM 1α,25-$(OH)_2D_3$ caused a significant increase in bone resorption above basal levels ($*p < 0.05$) and maximal stimulation of resorption was achieved with concentrations of 10 nM-1μM ($***p <$ 0.001 increasing resorption above control levels). Data presented are means ±S.E.M. of 2 separate experiments, where n =4 calvariae per treatment.

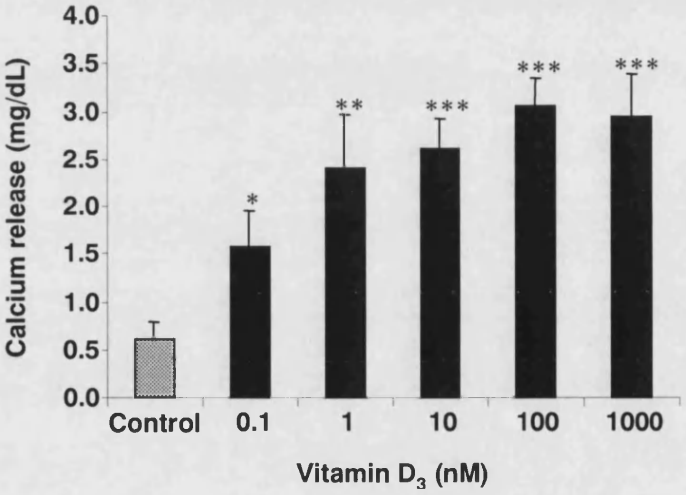


Figure 5.7. The effect of increasing doses of 1α, 25-Dihydroxyvitamin D_3 (0.1nM-1000nM) on neonatal mouse calvarial bone resorption.

5.4.2.2. The effect of allopurinol on 1α,25-$(OH)_2D_3$-induced bone resorption.

As in the case of PTH, the XO inhibitor, allopurinol had no effect on the maximal dose (100nM) of 1α,25-$(OH)_2D_3$-induced mouse calvarial bone resorption (Fig. 5.8). Data presented are means ±S.E.M. of 2 separate experiments, where n=4

calvariae per treatment. 100nM of 1α,25-$(OH)_2D_3$ induced significant bone resorption over basal levels (***$p < 0.001$).

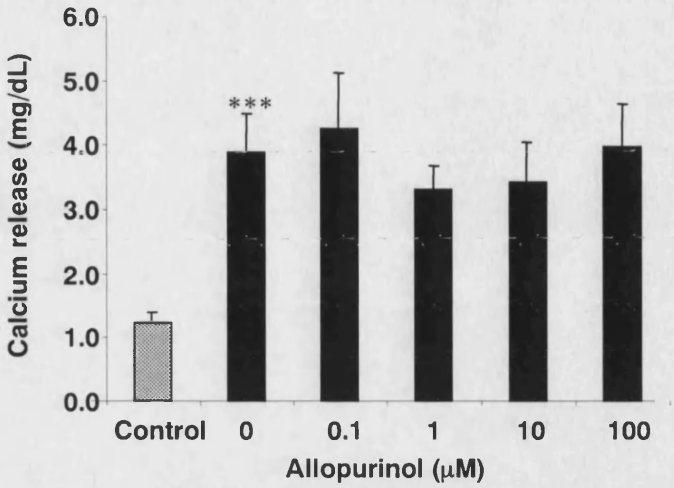


Figure 5.8. The effect of the XO inhibitor allopurinol on 1α,25-$(OH)_2D_3$ (100nM)-induced neonatal mouse calvarial bone resorption. ▒ Control; ■ 100nM 1α,25-$(OH)_2D_3$.

5.4.2.3. The effect of catalase on 1α,25-$(OH)_2D_3$-induced bone resorption

Unlike allopurinol, catalase was able to inhibit 1α,25-$(OH)_2D_3$-induced mouse calvarial bone resorption at 10 IU/ml (**$p < 0.01$) and 100 IU/ml (***$p < 0.001$) respectively (Fig.5.9). This implies that 1α,25-$(OH)_2D_3$ generates H_2O_2 from cells in the bone resorptive microenvironment which in turn activates bone resorption. 1α,25-$(OH)_2D_3$ (100nM) significantly stimulated bone resoprtion above basal levels ($^{++}p <$ 0.01). The data presented are means ±S.E.M of 3 separate experiments, where n=4 calvariae per treatment. Interestingly, 10 IU/ml of catalase significantly (**$p < 0.01$) inhibited 1α,25-$(OH)_2D_3$ (100nm) to below the basal levels of bone resorption.

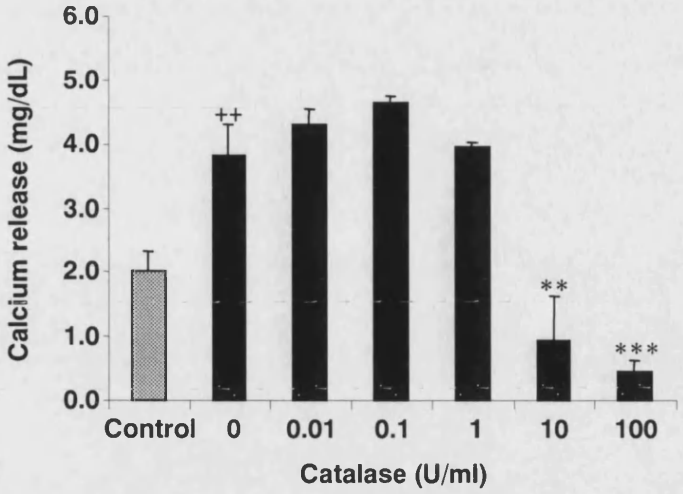


Figure 5.9. The effect of catalase on 1α,25-$(OH)_2D_3$ (100nM)-induced neonatal mouse calvarial bone resorption. ▒ Control; ■ 100nM 1α,25-$(OH)_2D_3$.

5.4.2.4. The effect of DPI on 1α,25-$(OH)_2D_3$ -induced bone resorption.

As in the case of PTH-induced resorption, DPI was able to inhibit 1α,25-$(OH)_2D_3$-induced mouse calvarial bone resorption at 1μM/ml ($^{*}p < 0.01$) and 10μM/ml

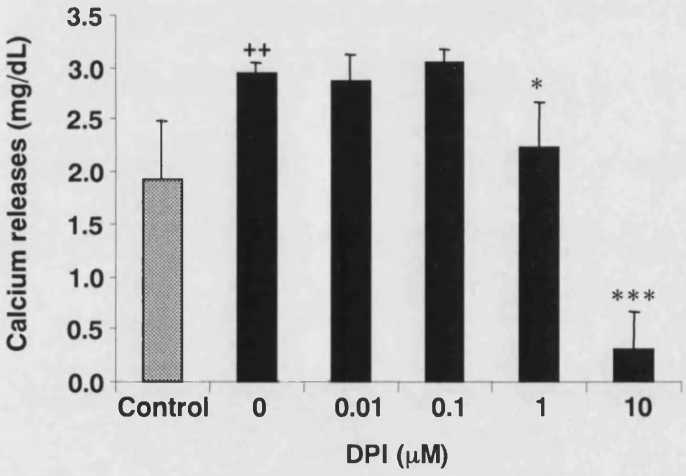


Figure 5.10. The effect of DPI on 1α,25-$(OH)_2D_3$ (100nM)-induced neonatal mouse calvarial bone resorption. ▒ Control; ■ 100nM 1α,25-$(OH)_2D_3$.

($^{**}p < 0.001$) respectively (Fig. 5.10). 1α,25-$(OH)_2D_3$ (100nm) stimulated bone resoprtion above basal levels significantly ($^{++}p < 0.01$). The data presented are means ±std. from one experiment, where n= 4 calvariae per treatment. As in the case of

catalase, 10μM DPI significantly (**p <0.01) inhibited 1α,25-$(OH)_2D_3$ (100nm)-induced resorption to below the basal levels of bone resorption.

5.5. Discussion.

Most of the osteoclastic resorptive work involving PTH and 1α,25-$(OH)_2D_3$ have shown an indirect activation and differentiation of osteoclasts (Fuller *et al*, 1998; Owen *et al*, 1996). However, some studies have indicated that osteoclasts may possess receptors for PTH and 1α,25-$(OH)_2D_3$ (Datta *et al*, 1996; Mee *et al*, 1996) implying the possibility of direct stimulation. The calcitropic hormones such as PTH and 1α,25-$(OH)_2D_3$ are known to stimulate ROS from osteoclasts (Garrett *et al*, 1990), whereas calcitonin has been shown to have an inhibitory effect (Key *et al*, 1990; Datta *et al*, 1995). Otseoclasts have receptors for calcitonin, which when activated lead to increased levels of cytosolic cAMP and calcium via two different G proteins. This leads to inhibition of cell motility in addition to retraction of the osteoclast, resulting in inhibition of osteoclastic bone resoprtion (Zaidi *et al*, 1990). Garret *et al*, 1990 reported $O_2^{\bullet-}$ to be generated upon stimulation of osteoclasts with PTH, IL-1, TNF and 1α,25-$(OH)_2D_3$, which could be inhibited by SOD. In contrast, Suda *et al*, 1993; Fraser *et al*, 1996 and Bax, *et a*l, 1992 showed that H_2O_2 could increase osteoclast differentiation and resorption.

In Chapter 4 it was demonstrated that XO is involved in TNFα and IL-1β induced bone resorption. In our experiments with PTH and 1α,25-$(OH)_2D_3$, the addition of the XO inhibitor, allopurinol to the bone cultures did not show any inhibitory effects on bone resorption. In PTH-induced resorption, SOD was found to have no inhibitory effect either, indicating that $O_2^{\bullet-}$ was not involved in the resorptive actions of PTH. Interestingly, catalase caused a dose dependent inhibitory effect on both PTH and 1α,25-$(OH)_2D_3$-induced bone resorption indicating that H_2O_2 was still being generated in the bone microenvironment. In 1α,25-$(OH)_2D_3$,-induced resorption,

catalase at 100μM significantly inhibited resorption to below basal levels. This may be because the control resorption Ca^{2+} levels were slightly higher in comparison to the PTH experiments. Therefore, catalase at high concentrations is capable of inhibiting stimulated resorption to below basal levels. It has been shown by Suda *et al*, 1993 that catalase suppressed the formation of tartrate-resistant acid phosphatase-positive multinucleated cells (TRACP(+)MNCs) by H_2O_2 in a dose-dependent manner. In this study, the adding back of excess amounts of H_2O_2 to the culture resulted in a reversal of the inhibition by catalase.

The source of the H_2O_2, which is being induced by these hormones, remains to be established, since XO appears not to be the mechanism of production.

It is generally accepted that mature phagocytic leucocytes, some B-lymphocytes, mesangial cells and mature osteoclasts contain the enzyme NADPH-oxidase (Babior *et al*, 1973; Maly *et al*, 1989; Radeke *et al*, 1991; Steinbeck *et al*, 1994). This enzyme has been shown to generate $O_2^{\bullet-}$ at the osteoclast-bone interface (Darden *et al*, 1996) and recently Steinbeck *et al*, 1998 showed that the osteoclastic cell line HD-11EM expressed the gp91-phox cytochrome b subunit of NADPH-oxidase and produced $O_2^{\bullet-}$ in response to $1\alpha,25\text{-}(OH)_2D_3$ and PMA, a known activator of NADPH-oxidase. Direct stimulation of these HD-11EM cells with H_2O_2 led to an increase in TRAP mRNA, activity and increased numbers of multinucleated cells.

It was reported by Oursler *et al*, 1991 that osteoclasts were shown to have a 150kD plasma membrane glycoprotein related Mn^{++} superoxide dismutase which, was identified as the ligand for the osteoclast-specific monoclonal antibody (Mab) 121F. This SOD-related membrane component may serve as a signal-transducing molecule at the osteoclast surface, converting $O_2^{\bullet-}$ to H_2O_2, since its functional blockade using Fab

fragments of Mab 121F, dose-dependently inhibits osteoclastic bone particle and pit resorption activity (Collin-Osdoby *et al*, 1998).

In our calvarial resorption assays that were stimulated with PTH or 1α,25-$(OH)_2D_3$, DPI, which is a potent inhibitor of NADPH-oxidase (Cross & Jones, 1986; Robertson *et al,* 1990), was able to inhibit bone resorption. As with catalase inhibition of 1α,25-$(OH)_2D_3$-induced resorption, we found that 10μM DPI significantly inhibited resorption to below basal levels. Again this could possibly be due to the slightly higher levels of control resorption, producing false results indicating that DPI and catalase inhibit basal levels of resorption. Further experiments are needed to clarify whether this is the case or whether catalase and DPI are capable of inhibiting 1α,25-$(OH)_2D_3$-induced resorption to levels even further below basal levels.

It is thought that DPI binds to a 45kDa protein, which is believed to be the flavoprotein component of the oxidase (Yea *et al*, 1990). At higher concentrations, DPI inhibits other flavin-dependent enzyme reactions including other ROS generating enzymes such as NADH oxidase. Our results showed that SOD had no effect on PTH bone resorption while DPI had a dose dependent inhibitory effect. A possible reason for this lies in the fact that $O_2^{\bullet-}$ is produced by the osteoclasts underneath the ruffled border in a sealed zone to resorb bone matrix. As a result of this sealed zone, SOD is unable to dismutase the $O_2^{\bullet-}$. NADPH oxidase is a possible source of this $O_2^{\bullet-}$, which has been located in the osteoclasts (Steinbeck *et al,* 1994) and can be deactivated by the addition of DPI, hence the differing effects of SOD and DPI.

Xanthine oxidoreductase may not be totally ruled out as a mediator mechanism of calcitropic hormone bone-induced resorption, because it has the ability to act as an NADH oxidase enzyme. The dehydrogenase form of the enzyme (XDH), which is the predominant form in mammalian tissue, has the ability to catalyse the oxidation of

NADH by O_2 (Sanders *et al,* 1997), resulting in the subsequent generation of superoxide and hydrogen peroxide. In fact the generation of these ROS occurs a lot more quickly in this reaction than the oxidase form (Sanders *et al,* 1997). None of the classical inhibitors of XO, allopurinol, BOF-4272, amflutizole had any effect on this reaction. However, DPI did have an inhibitory effect, probably acting on the FAD site of the enzyme where NADH is known to bind (Bray, 1975; Hunt & Massey, 1997). Interestingly, it has recently been documented that patients with rheumatoid arthritis have elevated levels of circulating plasma NADH-oxidising activity of xanthine oxidoreductase (Jawed *et al,* 1997). It is a possibility that the predominant activity of XO in bone diseases such as rheumatoid arthritis uses NADH rather than classical hypoxanthine/ xanthine/ uric acid pathway, which is inhibitable by DPI but not by allopurinol.

The multi-functional xanthine oxidoreductase still may play an important part in PTH and 1α,25-$(OH)_2D_3$-induced resorption through the dehydrogenase form rather than the oxidase form of the enzyme, though, further experiments are needed for this to be determined.

Chapter 6.

Prosthetic joint loosening and the involvement of xanthine oxidase.

Chapter 6

6.1. Introduction.

Total hip replacement surgery is potentially a way of transforming a patient's life by relieving pain, disability, deformity and restoring independence to the individual. Europe's health services could be on the verge of financial ruin in the near future because sufferers at risk of hip and spine fractures, deformities and chronic pain will become far more prevalent as people live longer. It is predicted that one in eight Europeans over 50 will suffer a spine fracture, and one in three women, and one in nine men over 80 will experience a hip fracture because of osteoporosis (Whitehouse, 1998). With annual hip fractures in the EU expected to more than double over the next 50 years-from 414,000 to 972,000 (Whitehouse, 1998) reliable, long lasting prostheses appear to be very important for both patient and the financial stability of these health services.

A major problem of total hip replacement surgery is the failure of the implant, necessitating revision surgery. In the UK alone, 11,000 revision operations are undertaken each year, with aseptic loosening being the most common cause of early failure (Corbett *et al*, 1998). Additionally, revision surgery has a lower rate of success in comparison to primary replacements, adding to the socio-economical burden of prosthetic joint replacement.

The loosening of the prosthetic joint occurs in the absence of any infection. Once the loosening is established, it may be visualised by X-ray analysis, where it is represented as areas of translucency compatible with bone resorption (Corbett *et al*, 1998). Although this provides us a relatively early indication of joint loosening it still not early enough to prevent the aggressive osteolysis that appears to occur early on at the bone-prosthesis interface. Much interest has been directed at the bone/prosthesis interface at the cellular level in an attempt to characterise the mechanisms underlying

the pathogenesis of aseptic loosening. Although its still uncertain what causes the loosening, some recent evidence has shown that the adverse tissue response is due to wear particles (Murray and Rushton, 1990; Amstutz *et al*, 1992).

Macrophages and fibroblasts are the predominant cells that are found immediately adjacent to an orthopaedic implant, which is aseptically loosened (Revell *et al*, 1997). These cells make up one of the main features of a loosened prosthesis; a pseudosynovial membrane which lines the prosthesis surface. (Boynton *et al*, 1995; Goldring *et al*, 1983; Lalor and Revell, 1993). This membrane becomes laden with particulate material derived from the articulating surfaces (Revell *et al*, 1997). Other wear debris may result from marginal impingement of the acetabular cup in hip joints, where large amounts of this particulate material finds its way deep into the bone around the implant (Revell *et al*, 1997).

With the onset of what many people would term a chronic inflammatory reaction in prosthetic aseptic loosening, evidence has shown that the fibrous tissue of the interface, contains many inflammatory mediators and cytokines, such as TNFα, IL-1β and PGE_2 (Sedel *et al*, 1992; Jiranek *et al*, 1993). These have all been implicated as strong modulators of bone resorption (Bertolini *et al*, 1986; Gowen *et al*, 1983; Dewhirst *et al*, 1985; Kaji *et al*, 1996). Additionally, it has been determined *in vitro* that isolated macrophages and pseudomembrane cells secrete IL-1β, TNFα and PGE_2 and stimulate bone resorption as a result of wear particle stimulation (Murray and Rushton, 1990; Westacott *et al*, 1992; Hukkanen *et al*, 1995; Algan *et al*, 1996). These proinflammatory cytokines may induce the expression of iNOS to produce large quantities of NO, which in low concentrations can stimulate bone resorption (Ralston *et al*, 1995). Interestingly, high concentrations of NO were found to inhibit bone resorption (Ralston *et al*, 1995). It seems unlikely that a relatively weak oxidant like

NO would be cytotoxic, or have an inhibitory effect on cells in the bone environment. However, it does have the ability to react with superoxide anions to form a very strong oxidising agent, namely peroxynitrite ($ONOO^-$) (Beckman and Koppenol, 1996). Peroxynitrite is known to cause oxidation of cell membrane lipids, DNA bases and modification of tyrosine in proteins to generate nitrotyrosine, leaving a footprint *in vivo.* This knowledge is increasingly being employed in immunohistochemical methods for the detection of ONOO activity (Beckman and Koppenol, 1996).

Hukkanen *et al,* in 1997, showed that iNOS, COX-2 and 3-nitrotyrosine were present in macrophages in the interface membrane of aseptic loosened joints. NO in high enough concentrations has been shown to out-compete SOD for $O_2^{\bullet-}$ (Beckmann & Koppenol, 1996). NO can also reduce the activity of superoxide dismutase (Joe & Lokesh, 1997) leading to the accumulation of the superoxide anion with which it can easily react. An environment can thus be created for the production of the strong oxidising agent, peroxynitrite. Additionally, peroxynitrite has been implicated as an important modulator of cyclooxygenase activity in inflammatory cells linking NO and $O_2^{\bullet-}$ to increased prostaglandin synthesis (Landino *et al,* 1996). The majority of studies in this respect have been directed to inflammatory mediators, such as cytokines, prostanoids, NO, and peroxynitrite, which are believed to be involved in the aseptic loosening of joint prostheses. There have been indications that the superoxide anion is involved in these processes, yet very little work has been done to identify the sources of $O_2^{\bullet-}$.

Shortly after joint replacement, the environment in and around the prostheses is predicted to be fairly ischaemic until the vasculature returns. One would infer that the cells in this microenvironment would be deemed hypoxic. Xanthine oxidase (XO), a ubiquitous three centred redox-enzyme, known for its ability to generate superoxide and

hydrogen peroxide, as well as being transcriptionally and functionally induced by cytokines and hypoxia, would be a suitable candidate for a source of superoxide anion involved the pathogenesis of aseptic prostheses loosening.

6.2. Aims and objectives.

To identify whether xanthine oxidase protein is expressed immunohistochemically in specimen tissue samples that were obtained from patients undergoing revision of total hip arthroplasties.

6.3. Materials and methods.

6.3.1. Isolation and culturing of human osteoblasts.

Human osteoblasts were isolated and cultured as previously described in section 2.3.2 with slight modifications. Briefly, acetabular reamings were collected from patients undergoing primary, but mainly revisions of hip replacements in sterile pots containing HBSS. The isolation technique from the reamings was slightly modified according to the cell type requiring isolation. All isolations were carried out in a Class II sterile cabinet. The reamings were placed in a 100mm-diameter dish and washed 2-3 times with HBSS to remove any blood. The method of Gallagher *et al*, 1998 was used to isolate human osteoblasts, and the extraneous fibrotic tissue was used to isolate fibroblasts.

6.3.2. Isolation and culturing of human osteoclasts.

Osteoclasts were isolated from the fibrotic and bone fragments of the acetabular reamings. Briefly, acetabular reamings were washed twice with PBS to remove most of the hematopoietic tissue, but some extraneous fibrotic tissue remained attached to the bone. These were explanted in 7mls of medium at 37°C in a humidified atmosphere of 95% air, 5% CO_2. The cultures were left for 7 days, and after this period of incubation the tissue culture medium (TCM) was carefully replenished, as not to disturb the explants. From this point on the TCM was changed every three to four days thereafter.

6.3.3. Identification of osteoblasts and osteoclasts.

Osteoblasts isolated from these explants were characterised by the extent of alkaline phosphatase positivity (see section 2.3.5). Osteoclasts were identified by the presence of tartrate resistant acid phosphatase (TRAP) activity as described previously

in section 4.4.1.3 with a slight modification to the method in that, Fast BB blue salt was replaced with Fast violet red BB salt.

6.3.4. Immunolocalisation of XO in human osteoclasts.

Th procedure described in section 2.3.5 was followed for the immunodetection of XO in of osteoclasts. In this procedure cells were grown, fixed and stained for XO on 100mm dishes (Sterilin), since it proved to be difficult to dislodge the cells from the plastic dish by Trypsin/EDTA (1x) and scraping of the cells resulted in cell death. Following immunocytochemistry cells were photographed using a Nikon Diaphot TMD® inverted microscope and a Nikon F-601 automatic focus camera.

6.3.5. Immunohistochemical analysis of the acetabular reamings.

Acetabular reamings were collected as previously described in section 6.3.1 fixed in formal saline and processed as described in section 4.4.1.3.

6.4. Results.

6.4.1. Osteoblasts from acetabular reamings in cell culture.

Fig. 6.1 shows (A) human osteoblasts cultured from acetabular reamings from which all extraneous fibrotic tissue was removed. High proportions of these isolated cells were positive for alkaline phosphatase activity in the early passages (B).

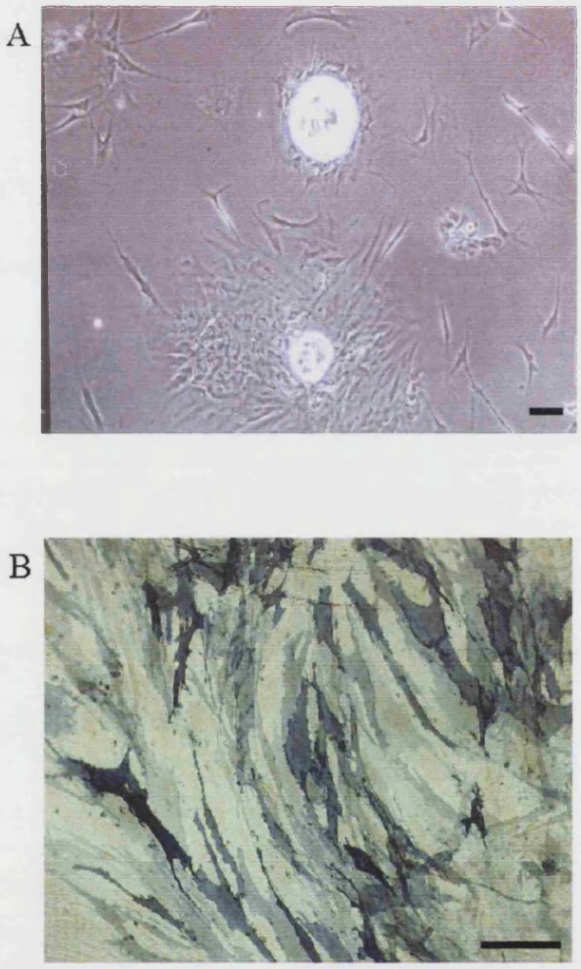


Figure 6.1. Photomicrographs depicting (A) human osteoblasts migrating out of a bone explant from acetabular reamings and (B) staining positively for alkaline phosphatase activity. Bar represents 50μm.

6.4.2. Osteoclasts from acetabular reamings in cell culture.

In the bone explants where the fibrotic tissue was not fully removed osteoclasts were noted to migrate out of the explant onto the 100mm plastic culture dishes.

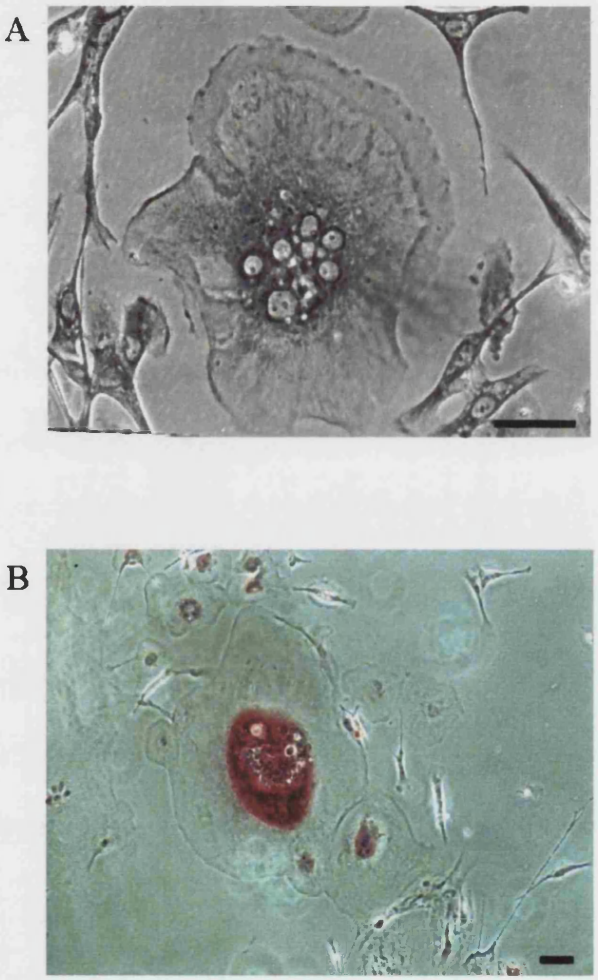


Figure 6.2. Photomicrographs of (A) an osteoclast isolated from explants of acetabular reamings in culture and (B) an osteoclast staining positively for TRAP (red) activity. Bar represents 50μM.

These cells had the classical characteristics of osteoclasts with numerous nuclei and ruffled borders (Fig. 6.2A), and stained positively for tartrate resistant acid phosphatase enzyme (TRAP) activity, one of the standard markers of osteoclasts. In these results, TRAP staining appeared to be localised around the nuclei (Fig. 6.2B).

Interestingly, bone chips from these reamings which had the fibrotic tissue removed, produced very few osteoclasts in culture and subsequently none in later passages. However, when both were placed in culture many ostoeclastic cells were obtained, even after re-explanting the tissue 6 times.

6.4.3. Immunolocalisation of XO in human osteoclast and osteoblast like cells.

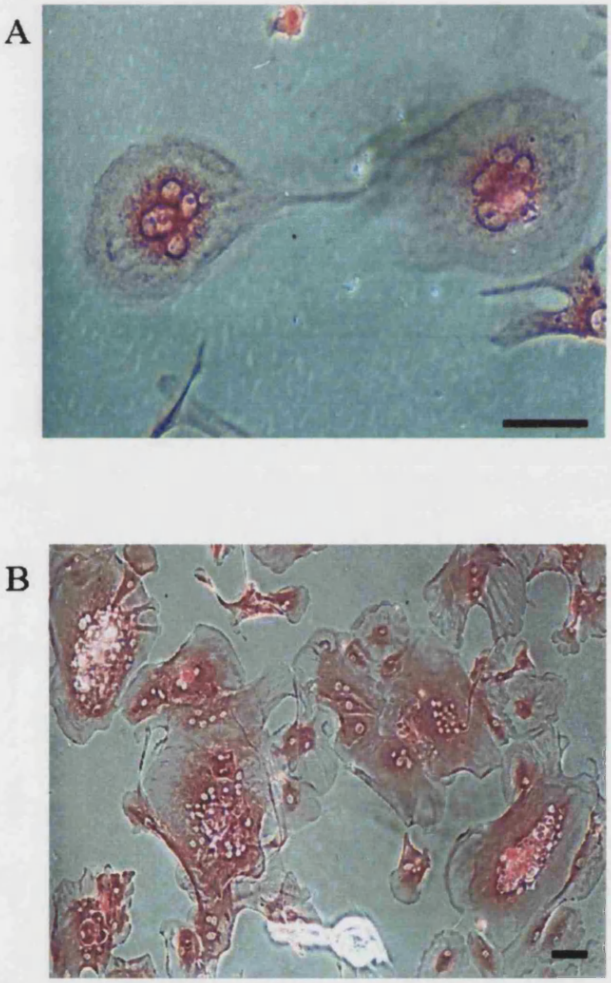


Figure 6.3. Photomicrographs (A & B) depicting osteoclasts in culture staining positively for xanthine oxidase (red). Bar represents 50μm.

Osteoclasts from these acetabular reamings stained positively for xanthine oxidase. As in the case of human osteoblasts, the staining of XO was localised around the many nuclei of the osteoclasts (Fig. 6.3 A & B)

6.4.4. The histology of bone/fibrotic tissue from acetabular reamings.

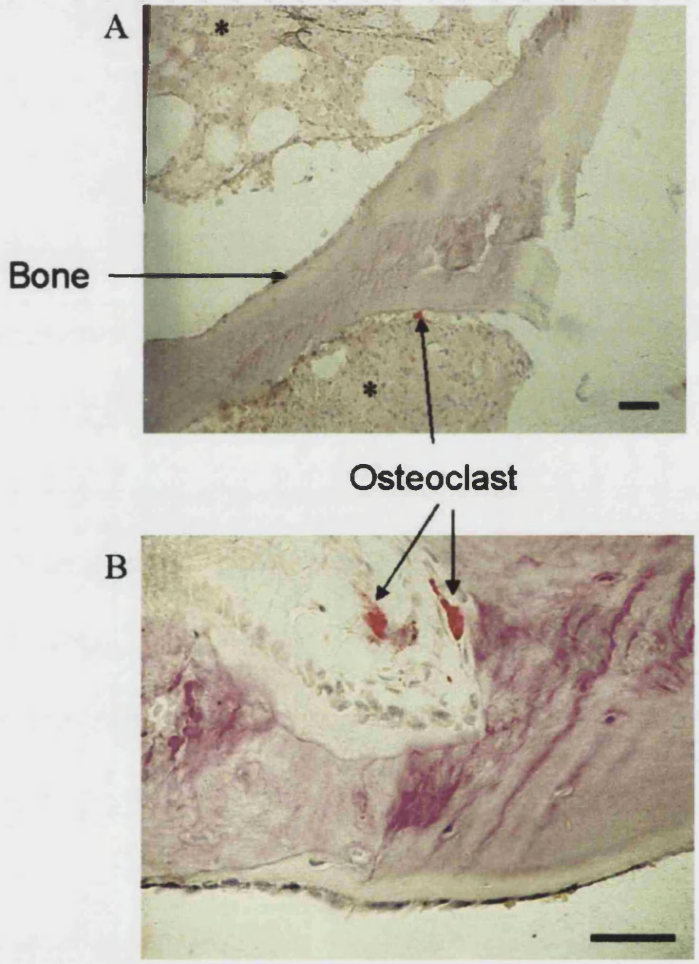


Figure 6.4. Photomicrographs depicting (A) the histological profile of a section from reamings of bone & fibrotic tissue (B) osteoclasts in an active area of bone destruction. Osteoclasts were identified as staining positively for TRAP activity (red). Mayer's haematoxylin was used as a counterstain for nuclei which also stains bone a blue/purple colour. Bar represents 50μm

Sections (8μm thick) were cut from fixed and processed acetabular reaming samples and stained for TRAP activity. The two photomicrographs on the previous page show the histology of some of the acetabular reaming samples obtained from revision of hip replacements. Note in Fig. 6.4 (A) that the fibrotic tissue (*) in some regions is directly attached to the bone (bone depicted by the blue/purple colouring due to Mayer's haematoxylin stain). The TRAP positive (red stain), possibly osteoclastic cells were localised to the interface between the fibrotic tissue and bone. Fig 6.4 (B) photomicrograph depicts two osteoclasts in an area of bone that appears to be undergoing resorption.

6.4.5. Immunolocalisation of XO in acetabular reamings.

Some of these acetabular reaming samples were fixed, processed, sectioned (8-10μM) and stained for the presence of xanthine oxidase. Strong XO staining was found (red) in regions where the fibrotic tissue was adjacent to the bone (blue). It is unlikely that they are osteoblastic lining cells because the region stained for XO is far too large for this bone cell type to occupy. Fig. 6.5 (B) shows that only regions adjacent to or at the bone stain positively for XO, in contrast to regions further away from the bone where XO immunoreactivity was relatively weak

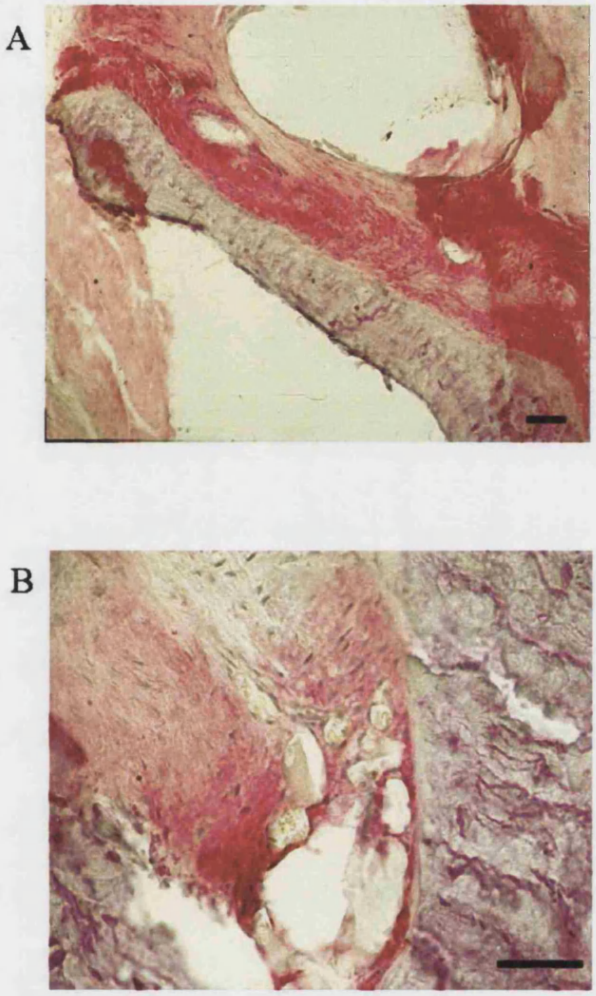


Figure 6.5. Photomicrographs (A & B) identifying positive XO staining (red) localised to the fibrotic tissue adjacent to the bone (blue). Bar represents 50μm.

Fig 6.6 (A & B) show that some of the fibrotic tissue staining for XO seems directional. Note in Fig. 6.6 (A) the distance of the nearest vasculature bed (*) from the region of bone. XO appears to be positively staining the fibrotic tissue (probably consisting mainly of the fibroblastic cell type) in an area where bone is about to be resorbed or is in the process of being resorbed. Fig 6.6 (B) shows the specificity of XO staining, quite clearly indicating that the fibrotic region, adjacent to bone is staining positively for XO.

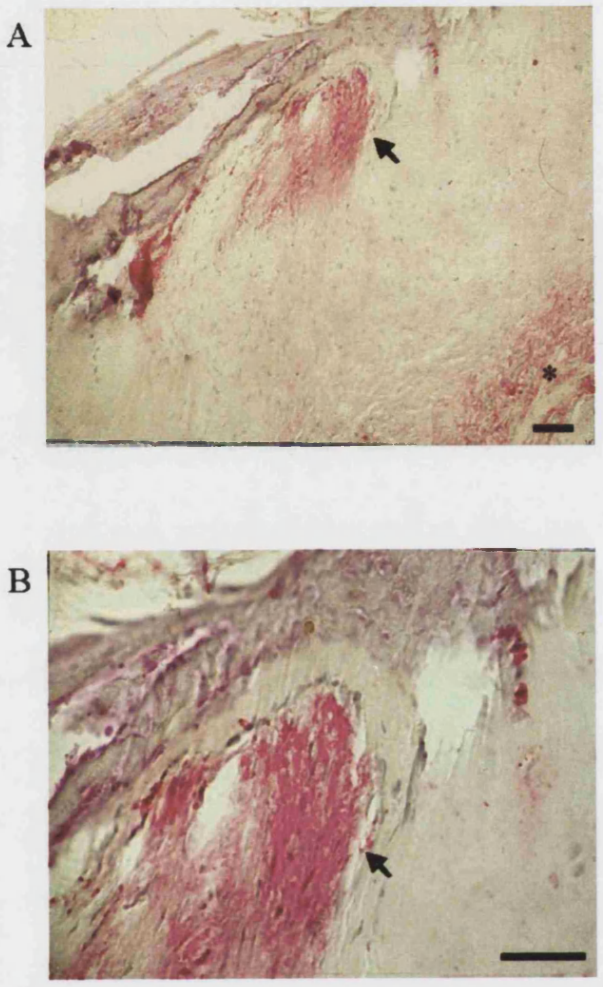


Figure 6.6. Photomicrographs (A & B) depicting a small region of fibrotic tissue staining positively for XO (red) which seems to be specific to areas (arrows) adjacent to bone (blue). Positive XO staining was found in blood vessels (*) that were some distance from the bone fibrotic tissue interface. Sections were counterstained with Mayer's haematoxylin to depict blue nuclei and bone. Bar represents 50μm.

6.5. Discussion.

In the samples of acetabular reamings from patients undergoing revision of total hip athroplasties, multi-nucleated giant cells (MNGC's) generated from the fiborotic/bone tissue stained positively for TRAP indicating their osteoclast-like nature. These osteoclast-like cells had the phenotypical characteristics of many nuclei and in some isolated cells, a ruffled border. Interestingly, neither Trypsin/EDTA nor non-enzymatic cell dissociation solutions could dislodge these cells from the plastic culture dish even after prolonged incubation times. These observations confirm what Chambers, 1979 described. He demonstrated that osteoclasts isolated from New Zealand white rabbits were found to be resistant to removal from glass coverslips by trypsin compared to the fibroblastoid cells that were growing in co-culture with these osteoclasts. This indicates that a tight sealing zone on the osteoclast brought about the strong attachment to the plastic/glass, a similar process that occurs with osteoclasts attaching to bone *in vivo*. Scraping these cells off the plastic resulted in cell death. It was therefore not feasible to place these cells on bone slices to check their osteolytic activity. It was concluded that the cells isolated and cultured from these reamings were highly likely to be human osteoclasts. But further characterisation is needed with the identification of the vitronectin or calcitonin receptors.

Re-plating of the explants resulted in the generation of more osteoclasts. It was found that human osteoclasts could be generated up to about the 6^{th} re-plating of the explant, although numbers of osteoclasts were reduced with each successive re-explanting. It was noted that hardly any osteoclasts were obtained in culture when bone explants were cultured alone. With the fibrotic tissue left attached to the bone chips it was found that osteoclasts could be generated and isolated.

It has been shown that, MNGC's associated with mineralised bone implants express little, if any, TRAP reaction product, where TRAP is highly expressed by osteoclasts in situ (Kelly *et al*, 1991). This is not to say that MNGC's cannot resorb bone. In fact macrophages and MNGC's, both containing wear debris, found next to bone have been reported to have the ability to resorb bone (Revell *et al*, 1997; Kadoya *et al*, 1996).

Aseptic loosening of total hip prostheses is believed to be a result of adverse tissue responses to particles derived from frictional wear of the prosthetic components, whereby accelerated bone loss occurs as a consequence of cytokines and prostanoids released by macrophages activated by the phagocytosis of these particles. This may well be a response that is delayed because loosening is often initiated before any evidence of wear, and the process is more protracted than might be expected with cytokine-induced resorption. Reactive oxygen species may well start the problem from the very onset of implantation because it has been shown that when the methylmethacralate is initiated with benzoyl peroxide and accelerated by N,N-dimethylaniline, free radicals were detected several days after polymerisation, and were shown to be cytotoxic to Saos2 osteoblast-like cells (Moreau, 1998). Furthermore, in a related situation, the loss of bone and cartilage in rheumatoid arthritis (RA) by activated fibroblastic cells, cytokines do not appear to be the mediators of bone resorption. The evidence for this is threefold. Firstly, in a model system in SCID mice where RA fibroblasts invade human cartilage, transfection of IRAP (IL1 receptor antagonist) into the invading fibroblasts did not prevent erosion but did inhibit chondrocytic chondrolysis (Muller-Ladner *et al*, 1997). Secondly, in RA patients with HIV, when terminal loss of T-cells occurred and all immune and cytokine activity had ceased (essentially the patients were in remission) bone resorption was still continuing (Muller-

Ladner *et al*, 1995). Thirdly, cysteine proteinase cathepsin K mRNA (expressed by osteoclasts and is pivotal to osteoclast bone resorption) has been found to be expressed in the synovium of patients with RA at sites of synovial fibroblast bone destruction (Hummel, *et al*, 1998)

Recently, similar bone resorbing, fibroblastic cells have been found in the pseudosynovial membrane expressing the membrane-type-1 matrix metalloproteinase (MT1-MMP) (Pap *et al*, 1998). This may indicate that cytokines are not the major mediators of bone loss around loosening prostheses (Sainsbury *et al*, 1998). In the sample reamings that were obtained and sectioned, it was found that sections of fibrotic tissue dramatically stained positively for XO in regions that could well be deemed to possess this pseudosynovial membrane. Thus providing a source of ROS from these fibroblasts to facilitate resorption of bone, similar to the process described for osteoclasts in the resorptive lacunae of bone (Key *et al*, 1994)

It has been suggested that a hypoxia-driven mechanism may facilitate this early form of loosening. The interface region can be profoundly hypoxic for several reasons. Primary reaming mimics complete medullary ischaemia leaving total dependence on periosteal perfusion resulting in a centripetal gradient of increasing hypoxia (particularly in younger patients where periosteal perfusion is less well developed) (Bridgeman and Brookes, 1996). Complete anoxia is described as a situation in any cell more than 150μm from its nearest vascular supply (Helmlinger *et al*, 1997). In addition, a vascular supply is difficult to find in the tissues extracted on revision surgery. It seems reasonable to suggest therefore, that these prostheses may well create a suitably hypoxic environment. The data presented in this chapter may allude to this hypothesis in that the fibrotic tissue (probably consisting of fibroblasts) in some of the reaming sections that were a great distance from a vascular supply stained positively for XO. On

the other hand, the fibrotic tissue nearer the vascular bed did not, indicating a potential reason for their "transformed" appearance.

As previously discussed, hypoxia is a stimulus for the upregulation of xanthine oxidase (XO) (Poss *et al,* 1996). It has recently been shown that NADH (raised in hypoxia (Obi-Tabot *et al,* 1993)) acts as an electron donor at the FAD center of XO, resulting in superoxide generation in the presence of oxygen (Zhang *et al,* 1998). Additionally, when oxygen levels fall, the enzyme becomes capable of generating NO from nitrates and nitrites (Millar *et al,* 1998). Interestingly, physiological levels of mechanical strain can induce nitrite production (Pitsillides *et al,* 1995). Therefore, at certain levels of hypoxia, XO can simultaneously generate superoxide and NO, which, by interaction, gives the enzyme the potential to produce the bioactive species peroxynitrite. A marker for peroxynitrite, nitrotyrosine, has been localised, not only in iNOS-positive macrophages but also in the sublining layer of the periprosthetic membrane (Hukkanen *et al,* 1997) where fibroblastic osteolysis is seen to occur. In some of our acetabular reaming sections we have clearly shown that fibrotic regions particularly those adjacent to bone surfaces, show strong XO expression (immmuno-reactivity). These fibrotic regions may well contain "transformed-appearing" fibroblasts, which express VCAM-1 and cathepsin K which are capable of osteolysis (Sainsbury *et al,* 1998). The observation that XO is present in the acetabular reamings especially adjacent to the bone/fibrotic interface and that osteoclast-like cells appear to be generated quite readily which also express XO, indicates that the source of superoxide anion in aseptic joint loosening could be XO.

Patients, who will experience premature loosening, will ultimately have in the early stages, vascular insufficiency dictating that the bone/prosthesis interface is a chronically hypoxic environment. As a consequence of this we speculate that the

upregulation of xanthine oxidase, which generates NO and $O_2^{\bullet-}$ in such a hypoxic environment leading to the formation of the damaging peroxynitrite metabolite (Millar *et al*, 1998). In combination, these factors can act to phenotypically transform fibroblasts to osteolytic cells and activate osteoclasts. Thus, osteolysis of the interface of the bone and prosthesis by these transformed fibroblasts is detrimental to the bone remodelling units that are normally active at these sites. This may be one of the mechanisms that result in premature joint prosthesis loosening.

Chapter 7

Conclusions and further work.

There are several stages in the process of osteoclastogenesis and activated bone resorption at which oxygen free radicals may have an important effect. The generation of ROS may be of particular importance in bone resorption that occurs in association with inflammatory diseases. This thesis was aimed at establishing the sources of ROS generated in the bone microenvironment, particularly those, which are influenced by the proinflammatory cytokines TNFα and IL-1β.

Since the 1950's ROS have been implicated in a wide range of diseases, but particular interest has been directed to the process of aging (Harman, 1983). In 1968 findings were published showing that free radicals could be generated upon mechanical stimulation of bone (Marino & Becker *et al,* 1968). However, it was not until the late 1980's when nitric oxide was discovered to be the factor responsible for vascular smooth muscle relaxation (Ignarro *et al,* 1987; Palmer *et al,* 1987), that free radicals came to the forefront of cell biology. From then, scientists discovered that free radicals not only played a significant role in cell signal transduction and differentiation (Allen *et al,* 1988; Beckman *et al,* 1989), but when produced in abnormal concentrations, were found to contribute to the pathology of many diseases.

It is widely accepted that osteoclasts are derived from haematopoietic stem cells, which are from the same lineage as monocytes and macrophages (Scheven *et al,* 1986). When activated, monocytes/macrophages release several active oxygen species (H_2O_2 singlet oxygen $OH^{\bullet}$ and hydroxychloride) that are responsible for killing microorganisms (Iyer *et al,* 1961; Holmes *et al,* 1967; Johnston *et al,* 1975). Morover, ROS are also involved in the functioning of differentiated osteoclasts (Garrett *et al* 1990; Key Jr. *et al* 1990; Okabe *et al* 1990). Other groups then identified, that one of these species, H_2O_2 could directly stimulate osteoclastic bone resorption and increase osteoclastogenesis (Bax *et al,* 1992; Fraser *et al,* 1996; Steinbeck *et al,* 1998).

Similarly, it was found that osteoblasts stimulated with the cytokines TNFα and IL-1β could also generate NO (Damoulis & Hauschka, 1994; Ralston *et al*, 1994), the latter was found to have pro-resorptive effects on bone (Ralston *et al*, 1995).

Contrasting reports have clouded the identification of the ROS that are important for resorption. Garrett *et al* (1990), reported that $O_2^{\bullet-}$ was involved in bone resorption which could be inhibited by SOD, whereas, Key *et al* (1990), found that SOD had no inhibitory effect on bone resorption. Likewise, Fraser *et al* (1996), reported similar observations with SOD, but found H_2O_2 to be the significant intermediate in osteoclastic bone resorption.

Both intermediates may have a role to play in osteoclastic bone resorption. Superoxide is known to be generated by the osteoclast especially under the ruffled border when it is resorbing bone. As the osteoclast forms a tight seal to the bone surface the SOD molecule may not be able to access the area under the osteoclast and prevent osteoclastic bone resorption. Also $O_2^{\bullet-}$ may have an indirect effect on bone resorption, since osteoclasts have been identified as having a developmentally regulated, 150kD plasma membrane glycoprotein related manganese SOD molecule, that can dismutate $O_2^{\bullet-}$ to H_2O_2 (Oursler *et al,* 1991). H_2O_2 appears from the literature to be the critical ROS intermediate in bone resorption. It's not surprising that H_2O_2, the chemical that can turn a brunette into an instant blonde, which reacts strongly with everything, and can easily penetrate cell membranes, has the ability to transform inactive osteoclast precursors into multinucleated bone destroying cells. Additionally, the role of hydrogen peroxide cannot be underestimated, as it has been demonstrated to be a critical component in the signal transduction pathways for other differentiation factors of osteoclasts such as macrophage colony stimulating factor (M-CSF). Indeed, it has been shown that TGFβ can upregulate M-CSF gene transcription via H_2O_2 activation of NF

Kappa B (Hong *et al*, 1997). Conversely, catalase has been recently shown to be adsorbed onto calcium hydroxyapatite (Barroug *et al*, 1998). This could provide a regulatory switch off mechanism for H_2O_2 after the activation of the osteoclast to resorb bone.

As it was alluded to in the introduction chapter, a plethora of factors contribute to bone resorption, exerting their osteoclastic resorptive effects through osteoblasts and stromal like cells. Initially, cytokine induced resorption was thought to be mediated by prostaglandins (Tashjian *et al*, 1987) although there was subsequent publications which contradicted this work (Thomson *et al*, 1987; Lerner & Ohlin, 1993). As ROS are involved in activating bone resorption then what are their sources? Osteoclasts have been reported to have NADPH oxidase (Steinbeck, *et al*, 1994), although, so far, no NADPH oxidase has been identified in osteoblasts. NADPH oxidase might be involved in ROS generation by the osteoclast, but it still cannot explain the signalling mediated through the osteoblast, when induced by the inflammatory cytokines TNFα and IL-1β, or the calcitropic hormones PTH and $1\alpha,25\text{-}(OH)_2D_3$.

It was Stevens *et al* (1991), who showed that XO was localised to the synovium of joints. On the basis of the hypothesis put forward by McCord in 1985 in which XO was proposed to be involved in inflammatory diseases and hypoxic/reperfusion mediated pathologies. It became apparent that in the inflamed joint, where pro-inflammatory cytokines are elevated and the ultimate manifestation of the disease is bone erosion, XO could be responsible for generating ROS, ultimately leading to the activation of the bone erosive process.

The above observations led to the hypothesis that cytokine modulation of bone resorption was mediated via ROS, generated by xanthine oxidoreductase. The studies described in this thesis were based on this hypothesis. The results described in this

thesis substantiated the observations from other groups that H_2O_2 stimulates mouse cavarial bone resorption. It is also confirmed that XO is localised not only to the osteoblast but also to osteoclast-like cells. In chapter 4 we established that the pro inflammatory cytokines, TNFα and IL-1β stimulate XO activity in RCOB's and increase mouse calvarial bone resorption. It was observed that resorption induced by these cytokines was inhibited by the XO inhibitors allopurinol and BOF 4272 and by catalase which dismutases H_2O_2 to O_2 and H_2O. Data that was not included in this thesis confirmed that at the transcriptional level, TNFα and IL-1β significantly increased RCOB XO mRNA.

As described in chapter 5 the hormones PTH and 1α,25 $(OH)_2$ D_3 were shown to mediate their resorptive effects via the production of hydrogen peroxide. However, upon addition of the XO inhibitor allopurinol, no change was observed in the resorptive effects of PTH and 1α,25 $(OH)_2$ D_3. The possibility therefore arises that other ROS generating enzymes such as NADPH oxidase are activated in this process. This possibility was confirmed by the observations, which showed inhibition of PTH and 1α,25 $(OH)_2$ D_3-induced resorption with DPI, a non-specific inhibitor of FAD sites in these enzymes. However, the role of XO in hormone-induced resorption may not be totally ruled out because of the unique properties this enzyme possesses. The human form of XO has been demonstrated to be 95% demolybdo and has a low activity towards purine substrates (Godber *et al*, 1997). Of the three redox-active centers on XO, the active site in the human form of XO is believed to be the FAD site, where NADH oxidation leads to the generation of ROS. Generation of ROS through the NADH oxidase activity of XO could therefore have an effect on basal levels of resorption. This may provide an explanation as to why allopurinol had no effect on

PTH and 1α,25 $(OH)_2$ D_3-induced resorption, and why allopurinol could only inhibit resorption induced by TNFα and IL-1β back to basal levels

Other factors such as the prostaglandins, cannot be ruled of these processes, because they too, in conjunction with NO and ROS, could affect bone resorption. Indeed, it has recently been hypothesised that PGE_2 is the mediator that induces the osteoclast differentiation factor (ODF) in osteoblasts when stimulated with IL-17 (Kotake *et al*, 1999). ODF is a recently identified novel member of the TNF ligand family, which is expressed as a membrane-associated protein by osteoblasts/stromal cells. The soluble form of this factor has been demonstrated to induce the differentiation of osteoclast precursors into mature osteoclasts (Jimi *et al,* 1999). Osteoprotegerin (OPG) can inhibit osteoclast recruitment by binding to the OPG-ligand, the ODF found on osteoblasts (Brandstrom *et al,* 1998). Jimi *et al,* 1999 demonstrated that osteoclast-like cells that had been formed in cocultures of osteoblasts and bone marrow cells expressed mRNA of the receptor activator of NF-kB (RANK), a receptor of ODF. This suggests that ODF-induced activation of NF-kB in pre-osteoclasts is a significant factor in the differentiation to the mature osteoclast. Additionally it is well documented that the cyclooxygenase and lipoxygenase pathways of arachidonic metabolism can activate osteoclastic bone resorption, but, it is has been recently demonstrated that the cytohrome P-450 pathway also contributes to Il-1β induced osteoclastic bone resorption (Choo *et al,* 1999).

We speculate that these activation pathways may involve ROS and RNS. Menadione and paraquat (redox-cycling compounds) are readily reduced by flavoproteins such as NADPH:cytochrome P-450 reductase to free radicals. They in turn reduce O_2 to produce $O_2^{\bullet-}$ and reform the menadione and paraquat compounds (Cheeseman, 1995). As already discussed in previous chapters NF-kB can be activated

by ROS, in particular by H_2O_2 (Schreck, *et al*, 1991). Indeed, it is interesting that Hall *et al*, 1995 found that NAC can inhibit the activation of NF-kB by ROS *in vitro*. They subsequently found that NAC inhibited osteoclastic bone resorption when added early to the bone slice assay (<3hrs), but not when added later (6hrs) at a time when active bone resoprtion was taking place. This indicates that ROS and the activation of NF-kB play a role in the differentiation and activation of osteoclasts rather than the actual process of resorbing bone.

All these mechanisms involve the coupling of osteoblasts/stromal cells and osteoclast/osteoclast-precursors for the activation of the resorptive process. Direct activation of the osteoclast cannot be discounted, since it has recently been shown that PTH can stimulate the osteoclast directly to produce superoxide anions and subsequently resorb bone (Datta *et al*, 1996).

The inhibition of bone resorption by compounds such as the bisphosphonates (BP) may exert their mechanistic effects by partially acting as anti oxidants. BPs are characterised by their Phosphate-Carbon-Phosphate (P-C-P) central structure. This P-C-P structure helps in the binding of the substance to the exposed bone mineralised matrix, around resorbing osteoclasts. The BPs are taken up by the osteoclasts, disrupting the sealing zone, the cytoskeleton and the ruffled border (Rogers *et al*, 1997). The molecular/biochemical mechanisms by which bisphosphonates inhibit osteoclast activity still remain unclear. Some evidence has pointed to the fact that the BPs can induce apoptosis in osteoclasts depicting distinct changes in cell and nuclear morphology (Hughes *et al*, 1995). Other evidence has suggested that they may direct their influence on the generation of ROS and RNS. Inhibition of protein-tyrosine phosphatases (PTPs) has been shown to abolish bone resorption induced by PTH (Krieger & Tashjian, 1983). Skorey *et al*, 1997 demonstrated that to inhibit PTPs a

combination of H_2O_2, calcium and the bisphosphonate alendronate are required. Although not conclusive TRK-530, a newly synthesised bisphosphonate, dose-dependently inhibited superoxide production from PMNs and osteoclast pit formation stimulated by 1,25-dihydroxyvitamin D_3. However, incadronate and risedronate had a strong inhibitory effect on pit formation, but no anti-oxidative activity, indicating that TRK-530 maybe unrelated to its anti oxidant properties (Tanahashi *et al,* 1998). Due to the functional groups that can be attached to these BPs, these groups may determine the differing functions and efficacy in bone resorption of these compounds. Additionally, tiludronate dose-dependently inhibited both cytokine and NO secretion from activated macrophages (Monkkonen *et al*, 1998).

Manipulation of the redox state in the bone microenvironment, by either inhibitors or inducers of bone resorption is a significant consideration in developing therapeutic drugs for bone destructive diseases. With the presence of XO and iNOS in osteoblasts and the subsequent generation of their respective metabolites by TNFα IL-1β and IFNγ, differing resorptive effects may result during the development of inflammatory disorders such as RA. Like slight changes of pH, fluctuations in the production levels of ROS and RNI can alter bone resorption activity. This is evident from the observations made by Ralston *et al* (1995), who reported that cytokine induced NO causes a biphasic effect on mouse calvarial bone resorption. Studies described with IFNγ in this thesis provide an insight into the modulation of bone resorption through these pathways. IFNγ (a known inducer of NO and $O_2^{\bullet-}$ in bone cells) caused a significant inhibition of resorption not only to basal levels but also to levels comparable to IL-1β induced resorption. However, when SOD was added to these cultures, a

reversal of the inhibitory effects of IFNγ was observed, indicating that effects were not solely due to NO.

NO and ROS can simultaneously be produced by the pro inflammatory cytokines in the bone microenvironment. Bone resorption therefore, can either be stimulated or inhibited depending on the concentrations of NO, ROS or type of ROS ($O_2^{\bullet-}$ H_2O_2) produced. If high concentrations of NO or $O_2^{\bullet-}$ are generated, which can out-compete cellular SOD, the highly oxidising species, peroxynitrite, can be generated. This could act as a failsafe mechanism to prevent aggressive osteolysis by inhibiting the resorptive process and prevent the recruitment and differentiation of preosteoclasts. Conversely, ONOO- has been shown to inactivate tissue inhibitor metalloproteinase-1 (TIMP-1) (Frears *et al*, 1996). As stated previously, XO is localised in osteoclasts, where NADPH oxidase (Steinbeck *et al*, 1994) and iNOS (Brandi *et al,* 1995) are also present. Collectively, these observations point to the possibility that $ONOO^-$ generation under the osteoclast in the Howship's lacunae may cause the degradation of the bone matrix.

The ability to suppress erosions in inflammatory diseases such as rheumatoid arthritis remains a much-debated topic but, at best, present therapies have a minimal anti-erosive effect. Steroids may suppress erosive development in the short term but the effects are not dramatic and are suggested to be independent of an effect on synovitis (Kirwan, 1995). For other disease modifying agents such as gold, penicillamine and methotrexate the anti-erosive effects, when started late, are very poor. Treatment with second line drugs is now advocated early following the onset of disease. Despite much rhetoric, there are however, still no data to suggest that early aggressive therapy will halt erosion development. Although a clear effect on synovitis from non-steroidal cyclo-oxygenase inhibitors has been reported (Choy, 1997), it is evident that such drugs

neither halt erosions nor suppress their development. Given the apparent relationship of synovitis and erosions, and the ability of these compounds to partially suppress synovitis, the failure to show any effect on the erosive process requires an explanation. Is it because the effect of the anti-inflammatory compound is required to be much more dramatic, in which case steroids should have a very demonstrable effect? Or is it because there is something about the pathological basis of synovitis that is not influenced by these drugs.

It is possible that the inflammatory cytokines, are not the factors involved solely in creating these bone erosions. In the rheumatoid joint, cycles of ischaemia and reperfusion prevail. This is due to an inadequate perfusion of the highly metabolically active synovial tissue and the facility for intra-articular pressure-induced fluctuations in blood supply (Blake *et al,* 1989). Also, osteoblasts have been shown to generate ROS when subjected to cycles of ischaemia and reperfusion (Gasbarrini *et al,* 1997). Therefore, hypoxia and ischaemia/reperfusion events are important regulatory factors in RA pathology. Additionally, the bone-prosthesis interface in patients undergoing hip replacements can be profoundly hypoxic due to the fact that the primary reamings mimic medullary ischaemia leaving total dependence on periosteal perfusion resulting in a centripetal gradient of increasing hypoxia (Bridgeman and Brookes, 1996).

Hypoxia is a stimulus for the upregulation of XO (Poss *et al,* 1996) and can induce increases in GAG's (Levene *et al,* 1982) to which XO has been shown to avidly bind (Radi *et al,* 1997). As described in chapter 6 samples from hip replacement revisions showed localisation of XO to the acetabular reaming fibrous tissue that lines the bone. In addition, XO has been demonstrated to be localised to endothelial cells of the synovium (Allen *et al,* 1987; Stevens *et al,* 1991). On such basis it can be suggested that the inflamed joint and the tissue surrounding the periprosthetic hip are undergoing

cycles of hypoxia reperfusion, leading to increased XO activity that facilitates the increased production of ROS, leading to aggressive osteolysis.

Some preliminary studies were undertaken to investigate the effect of hypoxia on bone resorption. The results of these preliminary studies showed that basal levels of mouse calvarial bone resorption were reduced significantly. Upon stimulation of these calvariae with IL-1β bone resorption occurred, albeit at only half the value of resorption attributed to bone calvarial resorption induced by IL-1β under relatively more oxygenated conditions. Clearly, ROS could not be involved in stimulating this resorption because oxygen is required for this active process. Recently, a novel mechanism has been described for XO in which NO can be generated from nitrite under hypoxic conditions (Millar *et al,* 1998). Initial results implied that this could be the case, because addition of increased concentrations of allopurinol to the TCM totally abolished IL-1β-induced resorption under hypoxic conditions. Further work is needed to confirm these initial observations.

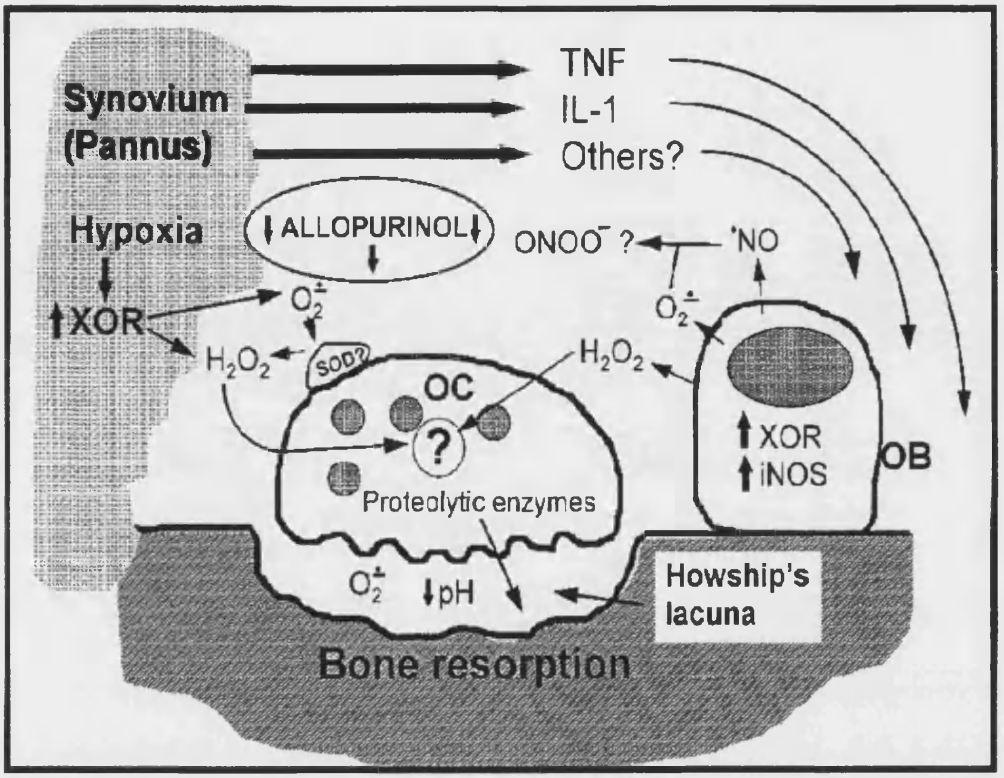


Figure 7.1. This schematic diagram shows the oxidative influences on rheumatoid bone erosions. We propose that a possible source of these oxidants, which exert their effects on osteoclasts (OC) could be provided by xanthine oxidoreductase (XOR) from osteoblasts (OB) and cells within the synovium.

Results of the studies relating to the role of XO in bone resorption, together with previously published work point to the increasingly accepted belief that NO and ROS could work simultaneously in the bone microenvironment. In support of this, the osteoblast contains both iNOS and XOR, and that these enzymes may be differentially controlled or expressed by a complex assortment of "bone resorptive" factors. The levels and the type of ROS or RNI produced will ultimately affect the coupling of the osteoblast and the osteoclast in bone resorption (Fig. 7.1). As bone remodelling has to be tightly regulated it's not surprising that these small labile, short-lived metabolites play an integral part in these processes. However, altered environmental conditions,

such as the increase in elevated cytokines in the inflamed joint or the hypoxic invading pannus, can result in the uncoupling of the system leading to pathological, erosive situations such as rheumatoid arthritis.

In summary, the elevation of pro-inflammatory cytokines in bone destructive diseases such as RA mediate their pro resorptive effects via the generation of ROS from xanthine oxidase. Variation in the levels of production of ROS and RNS in the bone microenvironment can mediate disruption of cellular interactions, either at the level of the intrinsic antioxidants or at the level of antioxidant/oxidant-responsive transcription factors. This can lead to the disregulation of the physiologically co-ordinated processes of bone remodelling. Such disturbances in this finely tuned phenomenon of oxidant/antioxidant balance may be the underlying reason for both chronic inflammation and uncontrolled bone resorption in rheumatoid arthritis. With bone resorption so closely linked to bone formation multiple therapies including manipulation of the redox environment maybe required to offset the altered bone remodelling cycles that lead to crippling bone deformities.

Chapter 8.

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Chapter 9

Appendices.

Chapter 9.

Appendix I Basic materials for tissue culture.

Human, Rat, Saos-2 & MG63 osteoblasts.

Human osteoblasts were isolated from specimens that were obtained from a variety of orthopaedic procedures, mainly from patients undergoing total hip and knee replacements. Some tissue samples came from revisions of hip replacements.

Rat calvarial osteoblasts were isolated from male wistar rats obtained from Harlan Olac, UK.

Saos-2 Human osteoblast cell line. (European collection of animal and cell cultures, Porton Down, UK. Cat. No. 89050205).

MG-63 Human osteoblast cell line. (European collection of animal and cell cultures, Porton Down, UK. Cat. No. 86051601).

Hanks balanced salt solution (HBSS) 1X.

This contained $Ca^{2+}$and Mg^{2+}, sodium bicarbonate and phenol red. It was sterile and endotoxin free. (Gibco, Cat. No 2402 091).

Foetal calf serum (FCS).

This was heat-inactivated at 56°C for 30mins before being transferred to the medium. FCS was stored at -20°C. It was purchased from Globepharm, Surrey, UK.

Antibiotics.

Penicillin (10,000IU/ml) and streptomycin (10,000µg/ml) (Gibco, Cat. No 15140-114).

Osteoblast culture medium (human and rat).

10% heat–inactivated FCS, Dulbecco's modified eagles medium (DMEM) with added L-glutamine (Gibco, Cat. No. 31885-023), 100 IU/ml penicillin, 100µg/ml streptomycin (Gibco, Cat. No 15140-114) and L-Ascorbic acid (Sigma. Cat No. A 1417).

1x Phosphate Buffered Saline

w/o Ca^{2+} & Mg^{2+}. (Gibco. Cat. No.14190-080).

Chapter 9.

Collagenase solution for isolating osteoblast from neonatal rat calvaria.

Collagenase Type II (Sigma. Cat. No C-6885).

1mg/ml solution was made up in 1x PBS and sterile filtered using a 0.22μm filter (Sartorius. Cat. No. 16534K).

Appendix II. Materials for mouse calvarial resorption.

Animals.

Swiss albino CD-1 5 day old neonatal rat pups were obtained from Harlan Olac, UK

5 day old, MF-1 neonatal rat pups were bred at the University of Bath. Original mice were obtained from Harlan Olac, UK.

Mouse calvarial tissue culture medium.

5% heat-inactivated FCS. (Globepharm, Surrey, UK).

CMRL 1066 medium (Gibco Cat No. 21530-019).

2mM L-glutamine. (Gibco Cat. No. 25030-024).

100 IU/ml penicillin and 100μg/ml streptomycin (Gibco, Cat. No 15140-114).

Cytokines.

TNFα human, recombinant (E. coli) 10μg (1ml) (Boehringer Mannheim, UK. Cat No. 1371 843).

IL 1β human recombinant (E. coli) 100,000U (2μg) (1ml) (Boehringer, UK. Cat No. 1457 756).

IFNγ human, recombinant (E. coli) 100,000U (1ml) (Boehringer, UK. Cat No. 1040 596).

Hormones.

1α,25-Dihydroxycholecalciferol (1α,25-Dihydroxy-vitamin D_3; Calcitriol). (Sigma Cat. No. D-1530).

Parathyroid hormone (bovine 1-34 fragment) 503U lyophilised powder. (NIBSC. Cat. No.82/512)

Xanthine oxidase inhibitors.

Allopurinol (Sigma Cat. No. A 8003)

BOF-4272 (Otsuka Pharmacetical Factory, Inc, Japan. Gift from Dr. Nishino).

Diphenyliodonium chloride (DPI). ICN Flow, UK. Cat. No. 150976).

Reactive oxygen species inhibitors.

Catalase (Sigma. Cat. No. C-8531).

Superoxide dismutase (Zn^{2+}/Cu^{2+}) (Boehringer, UK. Cat. No.567680)

N-acetyl-L-Cysteine (Sigma. Cat. No. A 8199)

Calcium detection reagents.

IL Test™ Calcium kit. (Instrumentation labs, UK. Cat. No. 181619-80)

Ca^{2+}detection kit (Sigma. Cat. No 587-M).

Calcium standard solution. (Sigma, Cat. No. 360-5).

1ml disposable cuvettes (Kartell). (BDH. Cat No. 307/3802/04)

1.5m tubes (Alpha labs. Cat. No. CP5518).

Reagents & equipment for the analysis of pyridinium crosslink

Hydrochloric acid (Sigma Cat. No. H7020).

Disodium tetraborate (Merck Cat. No. 102674E).

Gilson Aspec X1Li solid phase extraction system (Anachem Ltd, Luton, UK)

Isolute Confirm HCX SPE cartridges (Jones chromatography Ltd, Hengoed, UK).

Sodium formate (Merck Cat. No. 153213Y).

Heptafluorobutyric acid (Sigma Cat. No. H7133).

Shimadzu LC6A pump (Dyson Instruments, Tyne and Wear, UK)

Jasco FP920 Fluorescense detector (Jasco UK Ltd, Great Dunmow, UK)

Techsphere ODS column (HPLC Technologies Ltd, UK)

Acetonitrile HPLC (Merck Cat No. 152515P).

Appendix III. Materials for XO activity (pterin assay)

Cell dissociation solutions.

Trypsin\EDTA 10X (Sigma. Cat. No. T 4174).

Non enzymatic cell dissociation solution 1X (Sigma. Cat. No. C5914).

Lysing buffer.

50mM KPO_4 pH 7.4 (Sigma. Cat.No. P0662).

1mM EDTA (Promega, UK. Cat.No. H503a).

1mM PMSF (Sigma. Cat. No. P-7626).

Pepstatin A (1μg/ml) (Sigma. Cat. No. P4265)

Antipain (1μg/ml) (Sigma. Cat. No. A-6191).

Leupeptin (1μg/ml) (Sigma. Cat. No. L-2023).

XO activity (pterin) assay.

Isoxanthopterin (2-Amino-4, 7 -dihydroxy-1,3,5,8 tetraazanapthalene; 2-Amino-4,7-dihydroxypteridine) (Sigma. Cat. No. I 7388)

Pterin (2-amino-4-hydroxyperidine) (Sigma. Cat. No. (P 1132)

Methylene blue (Sigma Cat. No. MB-1).

Xanthine oxidase (bovine buttermilk) (Biozyme. Cat. No. XO2) 1.0-1.5U/mg protein.

Bio Rad protein assay Standard II.

Bio-Rad, UK. Cat. No.500-007.

Followed manufacturer instructions in determining protein concentrations in samples.

Appendix IV. Materials for immunohistochemical, immunocytochemical and activity identification of xanthine oxidase.

Trypan Blue exclusion-viability of cells.

Cells that had been trypsinised or non-enzymatically dissociated from the plastic culture ware, were counted before seeding onto LabTek® slides (see appendix VI). 100μL aliquots of cell solutions were thoroughly mixed with an equal volume 0.4% Trypan blue (Sigma. Cat. No. T-8154) and an aliquot of this were placed on an improved Neubauer haemacytometer by a capillary action underneath a coverslip. Total cells were counted within the $0.1mm^3$ boxes of the haemacytometer using a low powered transmitted light microscope. All cells (blue and clear) were counted in the counting chambers and divided by four. This number was then multiplied by $1x10^4$ to obtain the original cell number/ml before dilution in Trypan blue. Total cells = cells/ml x the original volume of the cell solution. Viable cells were calculated by repeating the above procedure but only counting the cells that excluded the Trypan blue dye, (clear cells). Cell viability was expressed as a percentage of total number of cells. *i.e.* viable cells (stained)/ total cells (stained & unstained) x 100

Histochemical identification of alkaline phosphatase. (ALP).

Tris –HCL. (Sigma Cat. No. T-3253).

Napthol AS MX Phosphate. (Sigma Cat. No. N-5000).

Dimethyl formamide. (Sigma Cat. No. D-4254).

Fast blue BB salt. (Sigma Cat. No. F-3378).

Materials for tartrate resistant acid phosphatase (TRAP) staining of osteoclasts.

Napthol AS-MX phosphate. (Sigma. Cat. No. N-5000).

Dimethyl formamide. (Sigma. Cat. No. No. D-4254).

Sodium Acetate pH 5. (Sigma. Cat. No. 386-3)

Fast red violet BB salts. (Sigma. Cat. No. F-3381).

Fast blue BB salts (Sigma. Cat. No. F3778).

Sodium tartrate. (Sigma. Cat. No. S-4797).

Blocking buffer.

Marvel® dried skimmed milk.

Tween 20 (polyethylene sorbitan monolaurate) (Sigma. Cat. No. P1379

1 X PBS (Oxoid Ltd, Basingstoke, UK Cat. No. BR14a)

4% formal saline. (BDH. Cat. No. 361367L).

Antibodies for staining XO

Rabbit anti-xanthine oxidase (bovine buttermilk) polyclonal antibody (Chemicon International. Cat. No. AB1242) 10mg/ml.

Mouse monoclonal antibody. XO Ab-2 (Neomarkers, USA. Cat. No. MS-474-P1).

ABC-Alkaline phosphatase conjugate (AP) complex kit. Including biotinylated goat anti-rabbit antibody. (Vector Labs, UK. AK-5001).

Fast™ Fast red TR/ Napthol AS-MX Tablet set. (Sigma. Cat. No. F-4523).

Dewaxing slides in xylene an IMS.

Xylene (BDH, Cat. No. 30578564) 2 x 5mins. IMS (BDH, Cat. No. 30244) 2 x 5mins.

Rehydrate in 1 x PBS for 5mins.

Poly-l-lysine coated slides.

Pol-l-lysine (Sigma. Cat. No. P1524). 0.05-0.1% Poly-l-lysine MW>35000 in DDW.

Smear on glass microscope slide and leave to dry (Huang *et al,* 1983)

Microscope slides (BDH. Cat. No. 406/0184/02)

Appendix V. Materials for western blotting.

Lysing buffer.

50mM Tris-HCL pH 6.8 (Sigma Cat. No. T3253).

100mM DTT (Promega Cat. No. V3151).

2% SDS (Sigma Cat. No. L-3771).

20% Glycerol (Sigma Cat. No. G7893).

2x Loading buffer.

50mM Tris-HCL pH 6.8 (Sigma Cat. No. T3253).

100mM DTT (Promega Cat. No. V3151).

2% SDS (Sigma Cat. No. L-3771).

20% Glycerol (Sigma Cat. No. G7893).

Added to this is 0.01% bromophenol blue (Promega Cat. No. H5011).

Rainbow markers.

High molecular weight range (14300-220000). (Amersham, UK. Cat. No. RPN 756)

Resolving gel (8%) (10mls)

Acrylamide 40% (Anachem Cat. No. 20-2400-05). 2mls.

1.5M Tris/base pH 8.8 (Promega Cat. No. H5131). 2.51mls.

SDS 20% w/v (Sigma Cat. No. L-3771). 50µL.

Ammonium persulphate (AMPS) 10% w/v (Sigma Cat. No. A9164). 100µL.

Temed (Promega Cat. No. V3161). 7.5µL.

Made up with 5.33mls H_2O.

Stacking gel (4%) (3.3mls).

Acrylamide 40% (Anachem Cat. No. 20-2400-05). 0.33mls.

1.0M Tris/base pH 6.8 (Promega Cat. No. H5131). 0.42mls.

SDS 20% w/v (Sigma Cat. No. L-3771). 16.7µL.

AMPS 10% w/v (Sigma Cat. No. A9164). 33μL.

Temed (Promega Cat. No. V3161). 4μL.

Made up with 2.49mls H_2O.

Running Buffer.pH 8.3.

25mM Tris/base (Promega Cat. No. H5131).

190mM Glycine (Sigma Cat. No. G4392).

0.001% SDS (Sigma Cat. No. L-3771).

Made up to 1000mL H_2O.

Blotting/transfer buffer.

25mM Tris/base (Promega Cat. No. H5131).

190mM Glycine (Sigma Cat. No. G4392).

20% Methanol (BDH/Merck Cat. No.101586B).

Made up to 1000mL H_2O.

Washing buffer.

1x PBS (Oxoid Cat. No. BR14a)/0.5% Tween 20 (Sigma Cat. No. P1379).

Blocking buffer.

5% Non fat dried milk-"Marvel®" in 1x PBS/0.5% Tween 20

Nitrocellulose paper-hybond-C super (Amersham Life Sciences RPN 203G)

Whatman paper (Whatman International Ltd, Maidstone, UK Cat. No. 3017915)

Amersham ECL detection kit.

(Amersham, UK. Cat. No. RPN 2106)

The principle is based on enhanced chemiluminescence, which is achieved by performing the oxidation of luminol by the HRP/hydrogen peroxide system in the presence of chemical enhancers such as phenols. This increases the output of light by approximately 1000 fold.

The detection is summarised in the diagram below.

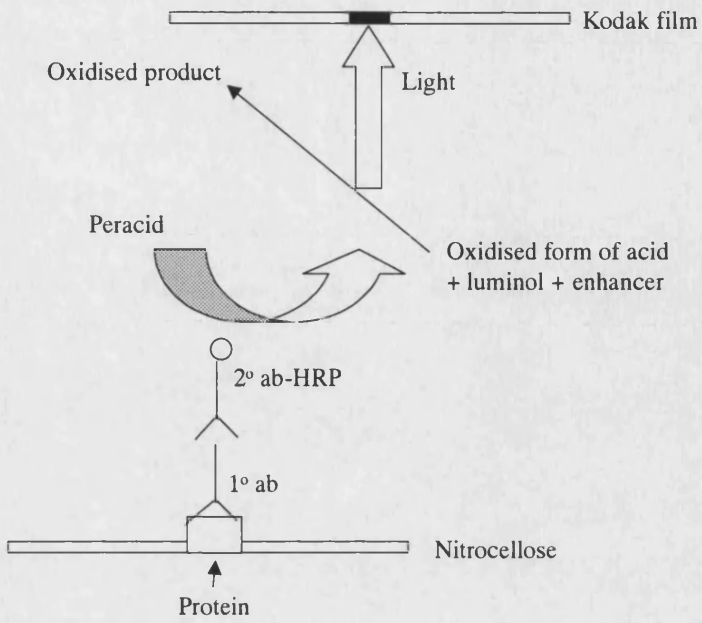

Figure 9.2. The ECL detection system

Comassie blue staining of gels.

Stain [45% (v/v) methanol, 10% (v/v) acetic acid and 0.1% Comassie Brilliant blue] (1hr)

De-stain [5% (v/v) methanol and 7.5% (v/v) acetic acid] 3hrs, x 3 changes.

Comassie Brilliant Blue (Sigma Cat. No. B-0149

Kodak X-OMAT AR auto rad film (Amersham Life Sciences, UK Cat. No. V8532665)

Appendix VI. Notes on statistical analysis.

Throughout this project, the two-tailed Student's t-test (Microsoft Excel statistical package) was used to determine significant differences between experimental and control groups of mouse calvarial bone resorption. Likewise, any of the inhibitory effects of such compounds such as allopurinol, catalase, SOD etc on cytokine or hormonal induced bone resorption was also analysed for significant differences using the two-tailed Student's t-test.

A two-tailed correlation analysis using GraphPad Prism statistical package (Graphpad Software, Inc, San Diego, USA) was used to determine whether there was a significant correlation between calcium release into the TCM from mouse calvariae with increased changes in pH levels of the medium. Also the same statistical analysis was used to determine whether calcium release from mouse calvariae in culture significantly correlated with the release of pyridinoline crosslinks.

Appendix VII. Equipment hardware used.

Monarch 2000 centrifugal analyser

Instrumentation Labs, Warrington, UK.

Tissue culture plasticware. (Fahrenheit, UK, unless stated).

T-25 0.22μm filtered capped flasks. (Falcon. Cat. No. 3108).

T-75 flasks 0.22μm filtered capped flasks. (Falcon. Cat. No. 3110).

6 well plates (Falcon. Cat. No. 3046).

10ml pipettes (Falcon. Cat. No. 7551)

8 well LabTEK® slides (Nunc. Cat. No. 1-77402K).

15ml polypropylene tubes (Falcon. Cat. No. 2070)

50ml polypropylene tubes (Falcon. Cat. No. 2096)

Gas cylinders.

95%air / 5% CO_2 were Purchased from the British Oxygen Company (BOC, Surrey)

Class II microbiological safety cabinets.

Model No. M20229. (MDH. Model No. M20229, Andover, UK).

Tissue culture incubators.

95%air / 5% CO_2 (Jouan, Techgen, UK).

Bench top centrifuge

Denley, UK BS400.

Automated autorad developer

Fuji RG II X-ray film processor (Fuji photo film company, Ltd, Japan)

F4500 fluorescence spectrophotometer.

Hitachi Scientific Instruments, Wokingham, Surrey.

Shandon Hypercenter II tissue processing apparatus.

The procedure for processing tissue samples into wax blocks.

	Temp (°C)	Immersion time
Formalin (10%)	RT	10mins
Alcohol (70%)	RT	30mins
Alcohol (80%)	RT	1hr
Alcohol (95%)	RT	1hr
Alcohol (absolute)	RT	1hr
Alcohol (absolute)	RT	1hr
Alcohol (absolute)	RT	1hr
Xylene	RT	1hr
Xylene	RT	1hr
Xylene	RT	1hr
Wax	60	1hr
Wax	60	1hr

Alcohol = Industrial methylated spirits