A STUDY OF THE FUNCTIONAL SIGNIFICANCE OF THE
NUMBER OF NUCLEI OF OSTEOCLASTS.

IN

KIM MARY PIPER.

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The thesis examines first whether cell fusion confers a disproportionate advantage upon osteoclasts. Resorption pits produced by chick osteoclasts cultured on dentine were measured by confocal reflection microscopy and the size of each pit related to the number of nuclei of the osteoclast that had made it. A high correlation was found between the number of nuclei per cell and the volume of tissue resorbed. When the amount of tissue resorbed was expressed per nucleus, a trend for the larger cells to be less efficient was seen.

The effect of the bisphosphonate 3-amino-1-hydroxypropyldene-1,1-bisphosphonate (APD) on chick osteoclasts of known nuclear number cultured on dentine or plastic was tested. The distribution of the nuclei was similar in the control and treated osteoclasts, suggesting that there was no preferential effect of the bisphosphonate on one size of cell more than another. The bisphosphonate in the medium inhibited resorption in a dose-related manner. The largest reduction in pit size occurred when the substrate was pre-treated with the bisphosphonate. The results support the view that bisphosphonate released during resorption acts as a metabolic inhibitor.

Rat and chick bone cells were cultured on glass coverslips, the rat cells in the presence and absence of a bisphosphonate. The areas and volumes of the freeze dried osteoclasts were measured. The relationships between the number of nuclei, cell area and volume are shown, and the usefulness of the method to test for metabolic inhibition explored.

The osteoclast population garnered from a site of very active modelling and remodelling, the growing deer antler, was characterised. A high proportion of oligonuclear (2-5 nuclei) osteoclasts suggested a very active state of osteoclastogenesis.

The morphology of osteoclasts present on the parietal bone in vivo in mice and rats of different ages was investigated in order to test the hypothesis that the nuclear distribution within a population of osteoclasts indicates the state of activity of a resorption cycle. A change in both the nuclear distribution and morphology of osteoclasts with age has been shown, and the significance of this finding is discussed.
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<td>APD</td>
<td>3-amino-1-hydroxypropylidene-1,1-bisphosphonate</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CA-II mRNA</td>
<td>Carbonic anhydrase II mRNA</td>
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<tr>
<td>CPD</td>
<td>Critical point-drying</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscope</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>CT</td>
<td>Calcitonin</td>
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<td>FCS</td>
<td>Foetal calf serum</td>
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<td>FD</td>
<td>Freeze-drying</td>
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<td>FdU</td>
<td>5-fluoro-2'-deoxyuridine</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
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<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
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<td>IL-3</td>
<td>Interleukin-3</td>
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<td>Interleukin-6</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PE₂</td>
<td>Prostaglandin</td>
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<td>PTH</td>
<td>Parathyroid hormone</td>
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<td>RNA</td>
<td>Ribonucelic acid</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>TEM</td>
<td>Tandem scanning electron microscopy</td>
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<td>TRAP</td>
<td>Tartrate resistant acid phoshatase</td>
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<td>1,25(OH)₂D₃</td>
<td>1,25-dihydroxyvitamin D₃</td>
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Introduction
The skeleton has a wide variety of functions. It provides structural support for the rest of the body, acts as the site for haematopoiesis, and is a reservoir of calcium and phosphate. The skeleton comprises bone and cartilage, the bony constituent being in a constant state of both modelling and remodelling. Bone remodelling is a coupled process and is directed both by mechanical forces and humoral and local demands on the skeleton. A prolonged change in the balance between formation and resorption results in bone pathology.

There are three main cells present in the bony skeleton. The osteoblast, a cell of mesenchymal origin, lines the bone matrix and is responsible for its formation. During bone formation some of the osteoblasts become entrapped within the mineralised matrix and are known as osteocytes. These cells have long cytoplasmic processes with which they may communicate with each other, with osteoblasts, and possibly other cell types. The importance and function of this intracellular communication are not fully understood. The final cell type is the osteoclast, a cell of haematopoietic origin. It was first named by Kolliker in 1873 who recognised its role in bone degradation. The osteoclast is a highly specialised cell, being terminally differentiated and possessing a polarised morphology and highly specialised membranes. Osteoclasts are one of the few cells to be multinucleated and although much is now known about osteoclasts, there has been scant literature on the importance of multinuclearity. Throughout its life cycle an osteoclast changes in size and the number of nuclei it possesses alters.

This present research focuses on the functional significance of multinuclearity and the relevance of morphology during an osteoclast's life cycle.

**Aims.**

The specific aims of this research were:

1. to test the importance of multinuclearity in a controlled in vitro resorption assay;
2. to investigate whether a resorption inhibitor, a bisphosphonate, altered the nuclear profile or changed the importance of cell size as measured by nuclear number;

3. to examine the relationship between nuclear number and cell volume, and to determine whether a resorption inhibitor altered cell volume;

4. to establish a nuclear profile of the osteoclasts in a rapidly growing mineralising tissue and establish whether the resorptive fervour could be linked to the nuclear number distribution of the cells present;

5. to study in vivo osteoclasts at different ages to discover whether the morphology of an osteoclast is related to the number of nuclei it possesses.

6. to investigate the viability of using phase-shifting interference microscopy to study osteoclasts.
Literature Review.
Osteoclasts.

**Origin and differentiation.**

Osteoclasts are the principal, if not the only, cells responsible for bone resorption. Most osteoclasts are multinucleated cells formed by the fusion of terminally differentiated cells, the osteoclast precursors. These have been shown to take ultimate origin from the hematopoietic stem cell (Ash et al. 1980).

The differentiation and function of osteoclasts is regulated by various cytokines and the local bone microenvironment (Mundy et al. 1987). Bone marrow culture systems that form osteoclast-like cells have been developed and the induction of osteoclasts by many different cytokines has been examined. The effect of haemopoietic growth factors on osteoclast differentiation is controversial. Macrophage colony stimulating factor (M-CSF) appears to be indispensable for the proliferation of osteoclast progenitors, but sequential treatment with 1,25(OH)₂D₃ is needed for the formation of osteoclast-like cells (Tanaka et al. 1993). Other colony stimulating factors (CSFs), interleukin-3 (IL-3), granulocyte-macrophage CSF (GM-CSF) and granulocyte CSF (G-CSF) also appear to stimulate the growth of osteoclast progenitors, which differentiate into osteoclast-like cells in response to 1,25(OH)₂D₃ (Takahashi et al. 1991). Stem cell factor (SCF) appears to act on early mononuclear precursors, increasing the precursor pool for osteoclast differentiation, whilst other factors are required to complete the differentiation into mature osteoclasts (Demulder et al. 1992). Recently, small cytokines such as LD78 have been shown to increase osteoclastogenesis from rat bone marrow cells and the authors suggested that this effect is possibly obtained by increasing the frequency of osteoclast recruitment (Kukita et al. 1992). Prostaglandins have also been shown to be essential for the stromal cell induction of hematopoietic cells to form osteoclasts in the presence of 1,25(OH)₂D₃ (Collins et al. 1992).
Other workers have demonstrated, also using the marrow culture system, that osteoclasts vary in their responsiveness to osteotropic hormones at different developmental stages, osteoclast precursors from the bone marrow of foetal and new-born baboons being more sensitive to some osteotropic hormones than cells derived from adults (Takahashi et al. 1987). Jilka et al. (1992) have shown that the age-related loss of oestrogen results in a failure to adequately suppress interleukin-6 production, and causes an increase in the number of osteoclasts.

Amano et al. (1992) developed an assay system using devitilised bone for the assessment of differentiation of osteoclast progenitors. They demonstrated that interleukin-1 (IL-1), interleukin-6 (IL-6), and parathyroid hormone (PTH) increase the formation of tartrate resistant acid phosphatase (TRAP)-positive cells, numbers of resorption pits, and the area of resorption, in a dose-dependent manner. However, prostaglandin E2 (PE2) increased only the number of TRAP-positive cells.

Although much has been discovered in recent years, there are still many unanswered questions about the reason for cell fusion and the possible benefits it confers upon the osteoclast.

**Function and resorption.**

The development of the technique for isolating and culturing functional osteoclasts in vitro has enabled osteoclasts to be studied in a more controlled and experimental way. This technique was developed in slightly different ways by different groups (Hefley et al. 1982, Jones et al. 1986).

The ability of osteoclasts to migrate and resorb bone and to vacillate between the two modalities is dependant on the cytoskeleton and adhesion with the substrate. Early work using isolated osteoclasts included the study of cell-substratum interactions of cultured avian osteoclasts (Marchisio et al. 1984).
Marchisio described a form of contact, termed podosome, and suggested that this represented an osteoclast-specific adhesion structure. In 1988 Turksen et al. described the adhesion patterns of rabbit osteoclasts on glass and devitilised bone, and showed that microtubules had the same distribution on both substrates. Microfilament distribution, primarily an F-actin network, was different, however, for cells associated with resorption lacunae: these cells showed intense staining in the area of the resorption whereas cells cultured on glass showed staining at the periphery in discrete dot and rosette-like structures. Changes in the cytoskeleton during the resorption cycle in vitro were revealed by Lakkakorpi et al. (1991). During the migration phase microfilaments formed podosomes containing vinculin, talin and F-actin at the paramarginal edge of the cell. As the osteoclast entered the initial phase of resorption, the podosomes congregated at the resorption site and formed a broad ring. During resorption, vinculin and talin were present in a continuous double ring between which was a broad F-actin ring, but at the end of resorption only narrow vinculin and F-actin rings remained. Microtubules were noticed to be concentrated at the cytoplasm closest to the resorption, and the authors concluded that it was possible that these may have a role in the secretion of the protons to the extracellular compartment.

The pharmacological effects of many different reagents that affect bone resorption have been studied using osteoclasts isolated from bone. Calcitonin (CT), first discovered in 1962 by Copp and his co-workers, is a 32-amino polypeptide which regulates calcium and phosphate metabolism, its major effect being the inhibition of osteoclastic bone resorption. The effects of calcitonin have been extensively investigated in vitro systems and, more recently, the cellular signalling pathways and changes in osteoclast behaviour have been explored.

In 1988 Nicholson et al. (1988) demonstrated that forskolin acts synergistically with calcitonin in stimulating cyclic AMP production in isolated rat osteoclasts, and also augments later physiological responses to CT. Ransjö et al.
(1988) provided further evidence that cyclic AMP is the mediator for the action of CT on bone resorption. Hunter et al. (1989) investigated the effect of calcitonin on the cytoskeleton of isolated chick osteoclasts, and demonstrated that there was a marked cytoskeletal rearrangement and change in cell shape. Yumita et al. (1991) demonstrated that TRAP activity within isolated rat osteoclasts in the first 2 hours was raised above control levels, and during the subsequent 4 hours fell to below control levels. They suggested that CT firstly inhibited TRAP release and then inhibited its synthesis or increased its degradation. A similar effect was shown with forskolin and the authors concluded that the effect is likely to be mediated by cyclic AMP (Yumita et al. 1991). Calcitonin has been shown to have an effect on RNA synthesis in isolated rat osteoclasts, producing a dose responsive decrease in both total cellular RNA and RNA synthesis (Zheng et al. 1991). Other systemic regulators also have an effect on osteoclast function. Parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D₃ are stimulators of bone resorption. Holtrop et al. (1979) demonstrated a rapid activation of osteoclasts and Addison (1980) demonstrated an increase in the numbers of nuclei of osteoclasts following exposure to PTH. Oestrogen has also been shown to inhibit osteoclastic bone resorption in vitro (Oursler et al. 1991). Other locally released factors such as prostaglandins, cytokines, and growth factors have powerful effects on osteoclasts and are the subject now of a vast body of research.

Both the mineral and organic components of the bone matrix are degraded by osteoclasts in resorption lacunae under ruffled border membranes. In 1985, Baron et al. (1985) investigated the extracellular compartment, where bone resorption takes place, and demonstrated that this area was actively acidified, lysosomal enzymes being directionally secreted by the osteoclast into the compartment. Current concepts of osteoclastic bone resorption are illustrated in the diagram L.1.
Quantifying resorption has been the subject of much in vitro research. It is of interest to know the rate and amount of resorption, so that putative resorption inhibitors and stimulators can be evaluated in terms of the cellular response. The participation of cysteine-proteinases in the resorption of mineralised substrates was confirmed by adding specific inhibitors to this group of compounds and measuring the resultant depths, plan-areas and volumes of the resultant resorption pits using SEM stereophotogrammetry (Délaissé et al. 1987). The relevance of extracellular calcium concentration has been investigated by measuring the total area resorbed using SEM (Datta et al. 1989). The authors discovered that a rise in extracellular calcium led to an abolition of resorption. Alam et al. (1992) used SEM morphometry to measure plan area and described a decrease in osteoclast resorption in vitro following exposure of the cells to endothelin.

The changing rates of resorption of osteoclasts during culture has also been measured by Kanehisa et al. (1988). They seeded osteoclasts from new born rabbits onto translucent devitilised bone slices and evaluated the rate of resorption using SEM stereophotogrammetry. The authors concluded that osteoclasts do not resorb whilst migrating, and may resorb at different rates at different times. They confirmed that there can be multiple foci of resorption associated with one osteoclast.

As an alternative method to the isolated osteoclast resorption assay, osteoclasts may be cultured in the presence of labelled bone and the amount of the label released measured. Using this method, oestrogen has been shown to decrease resorption in avian osteoclasts (Oursler et al. 1991). Many different methods of quantifying the amount resorbed have been developed (Boyde et al. 1991), and each has its advocates and opponents. Whatever the experimental design, gleaning maximal information is prudent.
Morphology and nuclear kinetics.

Nuclear number and nuclear kinetics.

Nuclear kinetics have been studied in the osteoclast for many years and from several different approaches. One of the earliest methods used was to label bones in vivo with $[^3]$H-thymidine and examine the bones, with the osteoclasts in situ, autoradiographically. With this technique, it was demonstrated that ethane-1-hydroxy-1,1-diphosphonate given in vivo caused increases in the number of osteoclasts, nuclei per osteoclast, and total number of osteoclast nuclei (Miller et al. 1977). Rowe (1977) counted osteoclasts and their nuclei in foetal rat ulnae and radii after simple haemotoxylin and eosin staining and noted an increase in nuclear number from a mean of 3.94 to a mean of 5.9 following lipopolysaccharide treatment. Whole cell imprints have been used to study large numbers of cells, and their nuclear distribution (Addison 1980, 1978). Addison found that human odontoclasts had a mean number of nuclei of 7.8, whereas kitten osteoclasts had a mean value of 5.5. Following treatment with parathyroid extract, the mean number of nuclei per osteoclast rose to 7.0, demonstrating a change in the nuclear number in response to a humoral factor.

During egg laying, when the medullary bone is used as a source of calcium, Miller et al. (1981) found no difference in the nuclear profile of avian osteoclasts, the mean number of nuclei remaining at 3.8. In secondary haversian systems in dogs, Jaworski et al. (1981) reported the mean number of nuclei to be 9.2, with the life span of a nucleus given at 11.5 days. Space flight has been found to increase the numbers of osteoclasts in rats but there has been no data on whether it alters the nuclear kinetics (Vico et al. 1987).

Rat osteoclasts, investigated using the smear technique and tritiated thymidine, mostly have a nuclear count between 1 and 6 (Ries et al. 1987). Ries and his co-workers found that uptake of labelled nuclei was seen first between 4
and 12 hours after injection and continued for 150 hours, with the most labelled nuclei being taken up by the larger cells as determined by nuclear number. Kimmell et al. (1980), however, demonstrated labelled osteoclast nuclei after 24 hours post-labelling during longitudinal bone growth in rats and these nuclei were depleted 5 days after labelling. Age-related changes have been shown to occur in the kinetics of osteoclasts. Scheven et al. (1986) showed a morphological population shift with age of embryonic mice, from proliferating osteoclast precursors at 15 days, to multinucleated osteoclasts at 18 days. A change in the mean number of nuclei in osteoclasts of Xenopus frogs has also been demonstrated with increasing age from 4.23 in immature females to 7.35 in mature females (Shaw 1988).

Nuclear kinetics have also been studied in bone diseases resulting from osteoclast abnormalities. Miller et al. (1982) found that osteoclasts from osteopetrotic rats have very high rates of incorporation of $[^3H]$-thymidine labelled nuclei that returned to a normal level following the irradiation of the bone marrow and a spleen cell transfer. The authors suggested that the high rate of nuclear labelling was caused by the non-function of the osteoclasts.

None of the authors cited above speculates on whether or how an increase in the number of nuclei that an osteoclast possesses affects its function. It is possible that the addition of nuclei may confer some advantage on the cell by increasing its productivity or act as a survival factor, preventing apoptosis (Raff 1992).

**Morphology and differentiation.**

Mononuclear osteoclasts are the subject of much controversy. Baron et al. (1986) used a model system in the rat to show that mature osteoclasts result from the fusion of mononuclear precursors after their attachment to the local bone environment. Live bone, or live bone-conditioned medium, was said to be
essential for this process (Oursler et al. 1988). Glowacki et al. (1989) postulated that osteocalcin functions as a matrix signal for the differentiation of osteoclasts. Braidman et al. (1990) found that recruitment of osteoclasts onto the bone surface of calvaria is primarily from the osteoclast precursor pool present in the endocranial membranes and the meningeal blood vessels. Groessner-Schreiber et al. (1991) postulated that the age of the bone matrix itself is important in the differentiation of osteoclasts. The authors showed that bone particles from rats of different ages implanted onto chick chorioallantoic membrane elicited different responses depending on the age of the bone; older bone matrix producing smaller, fewer osteoclasts with less nuclei per cell.

Although bone-related mononuclear cells can be identified by staining for TRAP, there remains some question over whether they are capable of resorption. SEM and 3-D TEM reconstructions by Domon et al. (1991) suggest that they are capable of resorption and contain the structures associated with active resorption. Others have demonstrated that these cells are TRAP+, express the vitronectin receptor, and sodium pumps at high levels, and are capable of resorbing the bone matrix before fusing to become the classical multinucleate osteoclast (Prallet et al. 1992). Patterns of monoclonal antibody staining in bone biopsy specimens are similar with both mononuclear osteoclasts and mature osteoclasts (Athansou et al. 1991).

As well as changing their number of nuclei, osteoclasts are highly motile cells and as such are capable of great shape changes, both endogenously and in response to local or humoral factors. Holtrop et al. (1979) found that treatment with PTH caused a time-related increase in cell size and change in the ruffled border zone. Zambonin-Zallone et al. (1981) demonstrated that after several days on a hypocalcaemic diet, the osteoclasts of the hen medullary bone are substituted by osteoblasts at the bone surface and change their morphological features to one
suggesting inactivity. Cat osteoclasts from bone marrow cultures showed gross
morphological changes following treatment with hydrocortisone
( Suda et al. 1983 ).

Surface features of the inner aspect of the mouse parietal bone, from new-
born to adult, have been studied using SEM ( Abe et al. 1983 ). Abe suggested
that the size and distribution of the rough areas and the morphological features of
the concavities in the rough areas varied depending on the activities of the
osteoclasts. Later he also looked at the distribution and shape of the osteoclasts
on the inner surface of parietal bones of mice. He demonstrated that the
resorption surface in young mice was characterised by irregular concavities and
osteoclasts that were mainly polygonal or round in shape, whereas in the adult
mouse the concavities were elongated and shaped in a flame-like pattern, and
associated with osteoclasts of an elongated appearance. This appeared to be
correlated with the different activities of growing bones and bones where growth
had ceased ( Abe et al. 1990 ). There also appeared to be a sex-related difference,
with female mice from 7-14 weeks of age showing greater numbers of osteoclasts
and larger areas of bone resorption than males, suggesting that sex hormones
influence bone resorption ( Abe et al. 1989 ). Rat bone marrow cultures also
appear to have two distinct populations of osteoclasts: one with a smooth cellular
margin and the other with irregular spiky margin, the cells expressing the stellate
form being the more active osteoclasts ( Hata et al. 1992 ).

Whilst most investigators have studied osteoclasts from normal animals,
some have looked at cells from pathological diseases. The disease that presents
the most striking change in the morphology of osteoclasts is Paget's disease of
bone. Recently it has become possible to isolate Pagetic osteoclasts from bone
tissue and it has been demonstrated that, compared with normal osteoclasts,
isolated cells are both enlarged in size, and have an increased number of nuclei to
a mean of 33.85, with some cells having over 100 nuclei ( Basle et al. 1988 ).
Osteoclasts formed from long term Pagetic marrow cultures were found to be different from osteoclasts formed from normal marrow cultures: there was a greater formation rate, the cells were both larger, and contained more nuclei, and had a high degree of TRAP staining and showed abnormal ultrastructural features (Kukita et al. 1990). It has been suggested that IL-6 produced by the marrow or bone cells from Pagetic patients may be an autocrine or paracrine factor for Pagetic osteoclasts (Roodman et al. 1992). Osteoclasts from osteopetrotic patients also have an increased number of nuclei and greater size, although these parameters vary from mildly to markedly increased. The most striking feature of these osteoclasts is the diminished or absent ruffled border complex (Shapiro et al. 1988).

**Bisphosphonates.**

Bisphosphonates are a group of drugs used extensively for the treatment of diseases resulting in increased bone resorption. There has been a large body of work investigating their effect on bone cells both in vivo and in vitro but still the exact mechanism by which they work is not fully understood. New bisphosphonates have been developed over the years, each with a slightly different potencies, and modifications of the side groups have produced bisphosphonates far more potent than some of the original bisphosphonates (Muhlauer et al. 1991, Van der Pluijm et al. 1992). The chemical structure of the most common bisphosphonates is illustrated in Figure L.2

Difluoromethylenediphosphonate was shown to be effective at inhibiting resorption in bone organ cultures in 1983 (Rowe 1983). 3-amino-1-hydroxypropylidene-1,1-bisphosphonate was demonstrated to reduce the osteoblastic formation of bone without affecting the osteoclastic bone resorption at low doses whereas at higher doses it inhibited bone resorption and secondarily
reduced bone matrix and mineral apposition rates (Marie et al. 1985). A study using long bone explants investigated the effects of different bisphosphonates on both resorption and accession of osteoclast precursors to the mineralized matrix and concluded that whilst all the bisphosphonates reduced bone resorption at high doses, amino-bisphosphonates reduced the accession of osteoclast precursors even at low doses (Boonekamp et al. 1986).

Others have argued that the effect of bisphosphonates may be due to an inhibitory effect on the osteoclast precursor, and therefore decreased recruitment of new osteoclasts (Lerner et al. 1987). Although recent work from two separate groups has demonstrated an increase in osteoclasts following bisphosphonate treatment, one has shown that the bisphosphonate induces a prolonged stimulation of histamine which may increase the proliferation of the hematopoietic precursors (Endo et al. 1993), whilst the other group has suggested that the increase in the osteoclast precursors is due to the bisphosphonate increasing the avidity of the bone for hematopoietically derived cells (Marshall et al. 1993). Lowik et al. (1988) have suggested that the bisphosphonate interferes with a matrix factor that is essential for the attachment and differentiation of the osteoclast precursor.

Apart from work investigating the accession of osteoclast precursors to the bone surface and the effect of the bisphosphonate on the ability of the osteoclast to bind to the bone mineral, there have also been studies assessing the effects of bisphosphonates on the mature osteoclast. Isolated avian osteoclasts show a decrease in metabolic activity following bisphosphonate treatment (Carano et al. 1990). Alendronate was demonstrated by Sato et al. (1991) to bind to resorption surfaces and be released during acidification of the bone mineral by the osteoclast. As the concentration of bisphosphonate released rises, it inhibits resorption and causes membrane ruffling to cease without damage to the osteoclast (Sato et al. 1991). However, other investigators have postulated that the decrease in resorption with some bisphosphonates is due to damage to the
osteoclast caused by the bone-bound bisphosphonate being released during resorption (Flanagan et al. 1991, Selander et al. 1994).

The effect of long term administration of bisphosphonate on bone biomechanics is of obvious clinical importance. The effect has been investigated by Toolan et al. (1992) using rats that had been ovariectomized. They found that the strength and area fraction of the trabecular bone in the lumbar spine were preserved without a detrimental effect on the biomechanics or morphological properties of the cortical bone (Toolan et al. 1992).

Whilst the effect on the cells is still not fully understood, bisphosphonates are being used in an ever wider variety of diseases. As well as their traditional use in Paget's disease, they are now established as a treatment for hypercalcaemia of malignancy (Kanis et al. 1991, Bonjour et al. 1991), for the treatment of osteoporosis (Papapoulous et al. 1992) and even for antiorthostatic hypokinesia (Grigoriev et al. 1992).
Fig. L.1 Current concepts of osteoclastic bone resorption: basolateral membrane (bl), sealing zone (sz) and ruffled border (rb) calcitonin receptors (ct). Evidence for the proton pump was first described by Baron et al. (1985)
Fig. 2. Chemical structures of bisphosphonates.
Chapter 1. The relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro.
Introduction.

The volume of an osteoclast varies throughout its life span and may both increase or decrease in response to local or humoral factors. There are no published data giving values for this vital parameter although one would expect that there must be some advantage conferred upon the cell by virtue of added size. At the very least, cell size may give some indication of the resorptive fervour at a particular site or time, or in response to local or humoral factors (Holtrop et al. 1979), including matrix components (Groessner-Schreiber et al. 1991). Descriptive comments on increased cell size however have been made by other investigators studying osteoclasts in both pathological (Kukita et al. 1990) and experimental conditions (Katoh et al. 1991), and also a decrease in cell size in pathological conditions has been noted (Shapiro et al. 1988).

Large cells are associated with large or small pits or with multiple small pits and small cells with small pits. The shape of a pit depends on the pattern of its growth: large pits may develop from small ones by maintaining the same overall form but increasing proportionally; or may result from the same initial plan area but radically deepening; or elongate as the cell moves over the surface. Or of course pits may vacillate between these different patterns of growth (Jones and Boyde 1988). Osteoclasts are motile cells and the complex shape of the pits is a time-related function of the osteoclast's ability to migrate and resorb, perhaps with intervening motile non-resorptive phases. At first, during the initial stages of resorption, small round pits are favoured. Later, more complex shapes are seen as a result of migration and resorption across the mineralised substrate. It therefore seems logical that the best measurement of the work done by an osteoclast must be the volume of tissue it resorbs in a given time (Boyde et al. 1987, 1991, 1992).

An osteoclast may be uninuclear (Domon et al. 1991) indeed, its origin is from a mononuclear precursor but it is most commonly recognised by virtue of its multinuclearity. The increase in cell size and nuclear number is a result of cell
fusion (Kember 1960; Young 1962; Zambonin Zallone et al. 1981; Ries et al. 1987). The importance of multinuclearity and cell fusion and the possible advantage it confers upon the cell, such as reported for multinucleated macrophages (Vignery et al. 1991), is unknown. Whether the benefit, if any, increases with each fusion or in relation to the numbers of nuclei present is at present poorly understood. The addition of new nuclei might act as a survival factor, preventing apoptosis (Raff 1992) or, conversely, an increase in nuclear number may be detrimental to the cell's efficiency, possibly beyond a certain cell size or age of its component nuclei, or as a result in the reduction of basal RNA synthesis (Zheng et al. 1991). A further possibility is that increase in cell size is neutral, cell fusion resulting from raised calcium concentration at the site of resorption and providing neither benefit nor loss.

The aim of this study was to explore the relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro.
**Materials and Methods.**

**Culture of chick osteoclasts.**

Long bones were removed from 19-day prehatch chicks, the cartilaginous epiphyses cut off, and the bones chopped in phosphate-buffered saline (PBS) with added 10% foetal calf serum (FCS). The release of osteoclasts from the bones was aided by repeatedly flushing the fragments up and down a plastic pastette. The resultant milky cell suspension was then seeded on to square sperm whale dentine slices, measuring 5mm by 5mm, each with one corner clipped to aid later relocation of the pits. The cells were allowed to settle for 45 minutes at 37°C. Each chick provided 4 aliquots for seeding, and a total of 21 chicks was used in two experimental sets for measuring the resorption pits. The dentine slices were gently washed with PBS to remove non-adherent cells and the remaining cells were cultured for 24 hours in Eagle's Minimum Essential Medium (MEM) with added 10% FCS, 2mM L-glutamine, 0.25pg/ml fungizone, 0.2U/ml penicillin and 0.2pg/ml streptomycin, in 5% CO₂ at 37°C.

In addition to the seedings on dentine, some were made on to plastic culture dishes for 6 or 24 hours and cultured in an identical way before fixation.

**Counting the nuclei of the osteoclasts and identifying the cells with their pits.**

At the end of the culture period, the dentine slices were gently washed in warmed PBS and the cells fixed in 2.5% glutaraldehyde in 0.15M cacodylate buffer at pH 7.4 for 5 minutes. The dentine slices were then washed in distilled water and air dried for 10 minutes. The cells were stained for the presence of tartrate-resistant acid phosphatase (TRAP: Sigma kit 386) and stored in 0.2M cacodylate buffer or 2.5% sodium azide in PBS until recordings of the nuclei counts and pit positions were made. Cultures on plastic were fixed and stored in the same way.

40
The locations of the pits and the nuclear counts for the associated osteoclasts on each of the numbered dentine slices were recorded using an Olympus IMT-2 microscope and a graticule lined up with one corner of the slice. Each of the slices was drawn and in this way the osteoclasts and their ascribed pits were charted in order to assist relocation of the pit once the cells had been removed. All multinucleate TRAP-positive cells with a pit that could be attributed to them were included in the study. This set included 292 osteoclasts and pits on 75 dentine slices.

The nuclei of all the multinucleate cells that were TRAP-positive were counted in the 6 and 24 hour cultures on plastic and parallel 24 hour cultures on dentine. Nuclear counts were made of 227 osteoclasts at 6 hours (on plastic) and 211 osteoclasts at 24 hours (on plastic or dentine) in this set. Therefore a total nuclear count of 730 osteoclasts was recorded in this study.

The cells were then removed from the dentine by washing with a mild detergent, 1% chlorohexidine gluconate, pH7, followed by immersion for 30 minutes in 30% H$_2$O$_2$ and the 292 pits identified again using the graticule and chart.

**Measurement of the pits.**

Measurement was carried out using a video-rate, line-confocal laser scanning light microscope (CLSM) manufactured by the Lasertec Corporation, Japan. The ILM11 instrument was developed for the semiconductor market. A He-Ne laser beam (lambda=633nm) is broadened to a line with a cylindrical lens, which is scanned down the specimen field at TV frame rate. The signal corresponding to each point in the line is directed to a single detection element in a linear diode array. Thus images are acquired at full standard TV rate. The microscope and its special Z-axis controller provide a hard-wired solution to the problem of acquiring the max and the map images during multiple frame...
acquisition. Images derive from 256 planes and are acquired in approximately 10 seconds.

Volume data from the map image (Fig. 1.1) was acquired by tracing (with a mouse and screen cursor) a line (one pixel wide) around the pit to be measured. Software, written by SIS Munster/Wurst, then found the volume enclosed within that area, deriving the height at which the surrounding reference surface was found from the values under the trace binary. The area of the pit was derived from a second screen cursor trace placed close to the edge of the pit (Fig. 1.1). False colour was added giving a pictorial indication of the depth of the pit, and three dimensional models gave further pictorial information about the shape of the pit (Figs. 1.2 and 1.3).

The depth recorded was the maximum depth of the pit. The mean depth was determined from computing volume/area.
Fig. 1.1. The map image with the line trace for volume and area.

Fig. 1.2. The same pit with false colour added.
Fig. 1.3. The same pit illustrated as a 3-D model.
Results.

The total osteoclast population.

The numbers of nuclei present in the osteoclasts seeded on to plastic culture dishes or on to the dentine slices for 6 and 24 hours are shown in the histogram (Fig. 1.4.).

All the osteoclasts in these cultures were counted, a total of 227 cells at 6 hours and 211 cells at 24 hours. There was no significant difference between the proportion of cells with different numbers of nuclei at 6 or 24 hours, although there were more highly multinucleated cells in the 24 hour group (Table 1.1).

As there was no significant difference between the two different groups (Mann-Whitney), the results were pooled. Figure 1.5 is a histogram of the distribution of the nuclei for all the osteoclasts in the study. The cumulative frequency histogram for the total number of osteoclasts is shown in (Fig 1.6). Only 11% of the cells had 10 or more nuclei and 47% had five or fewer nuclei.

There was, however, a significant difference (p=0.0004) between the numbers of nuclei/osteoclast in the 6 hour culture on plastic 227 cells and the 292 cells associated with a pit and cultured for 24 hours. This was because there were more very large cells in the latter group, and a slightly lower frequency of cells with very low numbers of nuclei with a pit. The substratum did not seem to affect the frequency of attachment of cells of different nuclear number.

The nuclei/cell in the population of the osteoclasts with measured pits.

The distribution of the cells according to their nuclear number for the 292 cells with a measured pit is demonstrated in Fig 1.7. The number of nuclei per cell ranged from 2 to 27, with a mean of 7.514 and a median of 6. Of the multinucleated osteoclasts 44% had 5 or fewer nuclei, 81% had 10 or fewer nuclei and only 11% had 15 or more nuclei.
The sizes of the pits and the nuclei per cell.

The distribution of the 262 pits where depth was measured is illustrated in Fig. 1.8. Only 262 pits had their depth measured due to an oversight on my part. The distribution of the areas and volumes of the 292 pits is illustrated in Fig. 1.9 and 1.10 respectively. The means, medians and SEM for the sizes of the 292 osteoclasts and related pits is shown in Table 1.2. The correlation matrix for nuclei/cell, pit depths, areas and volumes is shown in Table 1.3.

The results were analysed with respect to the 278 pits and cells because it is unlikely that a cell with less than 10 nuclei would have made a pit of over 10000μm³.

Whether the results are considered with respect to the total group or separately for the two experimental sets, the correlation between the number of nuclei per cell and the area of the matching pit was always higher than the corresponding correlation with volume, although both were positively correlated. The correlation between the number of nuclei per cell and the depth of its pit was always weakest.

As has been demonstrated previously, the correlation between the volume and area of the pits is high. As the size of the cells increased, the coefficient of correlation between size and volume also increased. Grouping the cells into 3 groups: 2-5 nuclei, 6-10 nuclei and more than 10 nuclei produced correlation coefficients of the number of nuclei per cell with volume of the matched pits to 0.008, 0.049 and 0.347 respectively, the two groups with the smaller osteoclasts showing no correlation between cell size and pit volume. All groups however showed a high correlation between area and volume. This reflected the much greater diversity of size in the third group that comprised 19% of the total numbers of cells.

Six random samples of 25 cells and their pits demonstrates the need for large sample sizes. The correlation for nuclei/cell and pit volume ranged from
0.108 to 0.751 and for nuclei/cell and pit area 0.060 to 0.847. Only the correlation between the volume and area of the pit remained consistently high. The mean volume resorbed/nucleus/cell was $698\mu m^3$ and the SEM was 54.5. The correlation for the pit size per nucleus are shown in Table 1.4.

There was a weak negative correlation between the volume and area resorbed per nucleus and the number of nuclei per cell. There was, however, a significant decrease in the depth of resorption per nucleus. Thus an increase in nuclear number does not appear to increase an osteoclast's resorptive activity per nucleus but seems to decrease it.

**Efficiency of resorption related to nuclear number.**

The depths, areas and volumes resorbed per cell and per nucleus were further analysed by pooling groups of osteoclasts according to their numbers of nuclei, first in 27 sets (Figs. 1.11-1.14) and then in four larger sets (Figs. 1.15-1.18 and Table 1.5). There was a clear trend for the depth, area and volume of the pit to increase with size, but for the depth, area and volume resorbed per nucleus to decrease with increasing numbers of nuclei per cell.

The cell numbers are different in Fig. 1.17 because of an oversight on my part as mentioned previously to measure the maximum depths of some of the pits. The data presented in Table 1.5 is the same as Fig. 1.15 but in addition presents the median values and the SEM.

When analysed in four sets so that there was an adequate number for statistical analysis in each group, the volume resorbed per nucleus and the area resorbed per nucleus were significantly less in the group with more than 10 nuclei as compared with the group with less than 5 nuclei (Figs. 1.15-1.16). All the sets were significantly different for the volumes and areas of the pits except for the two middle groups: these were however significantly different from the other groups. The steps for depths were not significant apart from the last step reaching
significance, but each step was significantly different from the next with regards to
depth resorbed per nucleus (Figs. 1.17 and 1.18). The statistical test used was
the Student two-sample t-test as the data once it had been grouped was parametric.
Table 1.1. *Number of nuclei in chick osteoclasts.*

<table>
<thead>
<tr>
<th>N</th>
<th>Culture</th>
<th>Substratum</th>
<th>Mean</th>
<th>SD</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>227</td>
<td>6h</td>
<td>plastic</td>
<td>6.20</td>
<td>3.47</td>
<td>18</td>
</tr>
<tr>
<td>138 (a)</td>
<td>24h</td>
<td>plastic</td>
<td>7.29</td>
<td>5.03</td>
<td>24</td>
</tr>
<tr>
<td>73 (b)</td>
<td>24h</td>
<td>dentine</td>
<td>6.11</td>
<td>3.69</td>
<td>20</td>
</tr>
<tr>
<td>211</td>
<td>24h</td>
<td>plastic/dentine</td>
<td>6.88</td>
<td>4.64</td>
<td>24</td>
</tr>
<tr>
<td>(a+b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>292 cells</td>
<td>24h</td>
<td>dentine</td>
<td>7.51</td>
<td>4.91</td>
<td>27</td>
</tr>
<tr>
<td>with pits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>730 total</td>
<td>6 or 24h</td>
<td>plastic/dentine</td>
<td>6.92</td>
<td>4.54</td>
<td>27</td>
</tr>
</tbody>
</table>
Table 1.2. The mean values of the sizes of 292 osteoclasts and related pits.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>7.514</td>
<td>6</td>
<td>0.288</td>
</tr>
<tr>
<td>Volume of pit $\mu$m³</td>
<td>4708</td>
<td>2819</td>
<td>363</td>
</tr>
<tr>
<td>Area of pit $\mu$m²</td>
<td>1015</td>
<td>700</td>
<td>57.9</td>
</tr>
<tr>
<td>Max depth of pit $\mu$m</td>
<td>6.05</td>
<td>5.87</td>
<td>0.15</td>
</tr>
<tr>
<td>Mean depth of pit $\mu$m</td>
<td>4.26</td>
<td>3.88</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Table 1.3. The correlation matrix.

Correlation matrix for the 292 cells and pits

<table>
<thead>
<tr>
<th></th>
<th>Nuclei/cell</th>
<th>Volume</th>
<th>Area</th>
<th>Max depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>0.419</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>0.530</td>
<td>0.801</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max depth</td>
<td>0.277</td>
<td>0.538</td>
<td>0.324</td>
<td></td>
</tr>
<tr>
<td>Mean depth</td>
<td>0.058</td>
<td>0.613</td>
<td>0.138</td>
<td>0.577</td>
</tr>
</tbody>
</table>

Correlation matrix for the 175 cells and pits of experiment 1

<table>
<thead>
<tr>
<th></th>
<th>Nuclei/cell</th>
<th>Volume</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>0.371</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>0.405</td>
<td>0.881</td>
<td></td>
</tr>
<tr>
<td>Max depth</td>
<td>0.273</td>
<td>0.571</td>
<td>0.391</td>
</tr>
</tbody>
</table>

Correlation matrix for the 117 cells and pits of experiment 2

<table>
<thead>
<tr>
<th></th>
<th>Nuclei/cell</th>
<th>Volume</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>0.482</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>0.737</td>
<td>0.623</td>
<td></td>
</tr>
<tr>
<td>Max depth</td>
<td>0.077</td>
<td>0.438</td>
<td>0.153</td>
</tr>
</tbody>
</table>

Correlation matrix for the 278 of the 292 cells and pits (14 cells with <10 nuclei but associated with pit's of >10000μm³ omitted)

<table>
<thead>
<tr>
<th></th>
<th>Nuclei/cell</th>
<th>Volume</th>
<th>Area</th>
<th>Max. depth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>0.517</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>0.566</td>
<td>0.865</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max depth</td>
<td>0.264</td>
<td>0.502</td>
<td>0.269</td>
<td></td>
</tr>
<tr>
<td>Mean depth</td>
<td>0.107</td>
<td>0.475</td>
<td>0.152</td>
<td>0.808</td>
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</table>
Table 1.4. The correlation for the pit size per nucleus.

<table>
<thead>
<tr>
<th></th>
<th>No of nuclei/osteoclast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume/nucleus</td>
<td>-0.119</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>-0.181</td>
</tr>
<tr>
<td>Max depth/nucleus</td>
<td>-0.589*</td>
</tr>
<tr>
<td>Mean depth/nucleus</td>
<td>-0.564*</td>
</tr>
</tbody>
</table>

*p<0.0001
Table 1.5. Work accomplished by the chick osteoclasts, grouped into four sets by their number of nuclei.

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Cells</th>
<th>Mean volume µm³</th>
<th>SEM</th>
<th>Median volume µm³</th>
<th>Mean vol/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4</td>
<td>92</td>
<td>2577</td>
<td>378</td>
<td>1650</td>
<td>984</td>
</tr>
<tr>
<td>5-7</td>
<td>89</td>
<td>3895</td>
<td>329</td>
<td>2997</td>
<td>657</td>
</tr>
<tr>
<td>8-10</td>
<td>55</td>
<td>5375</td>
<td>986</td>
<td>3583</td>
<td>597</td>
</tr>
<tr>
<td>11+</td>
<td>56</td>
<td>8849</td>
<td>1250</td>
<td>5611</td>
<td>543</td>
</tr>
</tbody>
</table>


Fig. 1.4. Histogram showing the relative frequencies of the chick osteoclasts with different numbers of nuclei in 6h cultures on plastic and 24 hour cultures on dentine and plastic, excluding those cells associated with a measured pit.
Fig. 1.5. Histogram showing the number of nuclei per cell in all the osteoclasts counted.
Fig. 1.6. Cumulative frequencies of the chick osteoclasts (n=730) with different numbers of nuclei.
Fig. 1.7. Histogram showing the distribution of the numbers of nuclei per cell for the 292 cells associated with measured pits.
Fig. 1.8. Histogram showing the maximum depths of the pits (n=262).
Fig. 1.9 Histogram showing the areas of the pits (n=292).

Areas of pits (sq microns/10)

No. of pits
Fig. 1.10. Histogram showing the volumes of the pits (n=292).
Fig. 1.11. The mean pit volumes and the volume per nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the pit volume to increase as the number of nuclei per osteoclast increases, but for the volume of the tissue resorbed per nucleus to decrease.
Fig. 1.12. The mean pit areas and the area per nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the plan area of the pit to increase as the number of nuclei per osteoclast increases, but for the plan area of tissue resorbed per nucleus to decrease.
Fig. 1.13. The mean pit maximum depth and the maximum depth per nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the maximum depth to increase as the number of nuclei per osteoclast increases, but for the depth resorbed per nucleus to decrease.
Fig. 1.14. The means of the mean pit depth and the mean depth per nucleus for the cells grouped by the numbers of nuclei that they contain. There is a trend for the mean pit depth to increase as the number of nuclei per osteoclast increases, but for the depth of tissue resorbed per nucleus to decrease.
Fig. 1.15. The relationship between the volume of the pit resorbed and the number of nuclei in the osteoclast, and the volume of tissue resorbed per nucleus and the number of nuclei per osteoclast. The numbers of cells in each group were: 2-4 nuclei (n=92); 5-7 nuclei (n=89); 8-10 nuclei (n=55); 11+ nuclei (n=56). Students t-test significance levels between steps were for volumes $p=0.009$, $p=0.16$, $p=0.03$; for volumes per nucleus no significance between the steps but $p=0.03$ between the first and last step.
Fig. 1.16. The relationship between the plan area of the pit and the number of nuclei in the osteoclast, and the plan area of tissue resorbed per nucleus and the number of nuclei per osteoclast. The numbers of cells in each group were: 2-4 nuclei (n=92); 5-7 nuclei (n=89); 8-10 nuclei (n=55); 11+ nuclei (n=56). Students t-test significance levels between steps were: for plan area p=0.0001, p=0.09, p=0.005; for plan area per nucleus no significance between the steps but p=0.002 between the first and last step.
Fig. 1.17. The relationship between the maximum depth of the pit and the number of nuclei in the osteoclast, and the maximum depth per nucleus and the number of nuclei per osteoclast. The numbers of cells in each group were: 2-4 nuclei (n=81); 5-7 nuclei (n=81); 8-10 nuclei (n=51); 11+ nuclei (n=49). Students t-test significance levels between steps were: for maximum depths no significance between the steps but p=0.019 between the first and last steps; for maximum depths per nucleus p<0.0001 between each step.
Fig. 1.18. The relationship between the mean depth (volume/area) of the pit and the number of nuclei in the osteoclast, and the mean depth per nucleus and the number of nuclei per osteoclast. The numbers of cells in the four groups were: 2-4 nuclei (n=92), 5-7 nuclei (n=89), 8-10 nuclei (n=55), 11+ nuclei (n=56). Students t-test significance levels between steps were: for mean depths no significance between the steps but p=0.17 between the first and last steps; for mean depth per nucleus, p< 0.0001, p=0.013, p=0.026.
Discussion

Sizes of osteoclasts.

The size of the osteoclast and its number of nuclei have been investigated by many groups in several different species. Counting the number of nuclei in a large and irregular cell is easier if the cell is kept whole. Whilst this is relatively simple in the in vitro system, it presents rather more of a challenge in the in vivo case. This problem has been overcome in a number of ways; Addison (1980) used bone imprinting to obtain whole osteoclasts from kittens, Ries et al. (1982) obtained rat osteoclasts from periosteal smears, Hefley et al. (1982) used enzymatic digestion to isolate foetal rat osteoclasts and Jones et al. (1986) dispersed osteoclasts from chick, rat and rabbit bones by mechanically agitating bone fragments to dislodge the osteoclasts and then allowing them to settle on culture dishes.

The relative frequencies of the numbers of nuclei in osteoclasts from different animals are quite similar, and not very dissimilar from counts from human or kitten odontoclasts (Addison 1978). The majority (81%) of the osteoclasts in the present study had ten or fewer nuclei; in Addison's study 80% of human odontoclasts fell into this group.

In 1980, Addison was able to demonstrate a change in the nuclear frequency of feline osteoclasts during a 6 hour experiment between control animals and animals treated with parathyroid extract, although the shift in the cumulative frequency curve was small. The addition of extra nuclei does not necessarily parallel the activation of the cell, for example, by PTH (Holtrop et al. 1979). Perhaps cell fusion may enhance the activity of the cell in its own right. Clearly the supply of preosteoclasts will affect the renewal rate of nuclei in osteoclasts, and the frequency distribution. The addition of new nuclei may act in some way as a survival factor, preventing programmed cell death (Raff 1992),
but there is obviously a limit to the numbers of times this may apply in the normal cell. Pathological conditions such as Paget's disease appear to disregard this rule as huge osteoclasts are produced both in vivo (Basle et al. 1988) and in vitro (Kukita et al. 1990).

Mononuclear osteoclasts or preosteoclasts (Athanasou et al. 1991) were excluded from the analysis as it was extremely difficult to identify a pit under these cells in the relatively short culture period, although there is now growing support that these cells are capable of resorption (Hattersley et al. 1989, Domon et al. 1991). This claim has sometimes been based merely on the fact that there are small resorption pits. This is unsafe as even large osteoclasts are capable of making multiple, tiny pits. However some cultures with mononuclear osteoclasts are reported to make pits (Domon et al. 1991) and it has also been demonstrated that in long-term marrow cultures where new osteoclasts are generated many small pits can be demonstrated (Jones et al. 1986).

The way in which in vivo the addition of new nuclei to a multinucleated cell takes place is not clear. The addition of new nuclei may be the result of the fusion of one mononuclear cell to another or to a multinucleated cell or may be the result of two multinucleated cells fusing or both situations may occur. The production of gigantic cells in pathological conditions may be as a result of abnormal fusion between multinucleated osteoclasts, or may be due to the increased production of the mononuclear pool and their subsequent addition to the multinucleated cells or to nuclei not being lost from osteoclasts as is thought to occur (Johnson et al. 1964). Oversize osteoclasts that develop in long term marrow cultures are a result of the success of preosteoclast production and are capable of producing huge single-scoop pits (Jones et al. 1986).

The possibility that fusion of osteoclasts or preosteoclasts with osteoclasts occurred in the culture period was not excluded in this study. The significant difference between the numbers of nuclei per osteoclast in the 6 hour culture
period on plastic and the cells associated with a pit and cultured for 24 hours may be explained by the fact that there were larger cells in the latter group and a slightly lower frequency of cells with low numbers of nuclei associated with a pit. This may be due in part to the difficulty of reliably recognising a small cell with a tiny pit beneath it. However, this did not shift the cumulative frequency curves to a significant amount.

There are changes in the nuclear distribution of osteoclasts depending on the age of the animal (neonate, juvenile, adult, aged) and at different stages in the resorption activation cycle, with smaller osteoclasts predominating at early stages in the resorption cycle and younger ages of the animal. In this experiment, however, I used the same ages of chicks, although each one may have been in a slightly different stage of its resorption activation cycle.

Sizes of resorption pits.

The correlation between pit size and osteoclast size may be higher than determined if the time taken to make each individual pit were known and taken into account. A 24 hour culture period was chosen for the experiment for two reasons: firstly, in order to have a reasonable sample size and secondly in order to minimise the number of osteoclasts making several pits. Clearly, not all the osteoclasts would have started resorbing at the same time, some would have made more than one pit, and others would have finished resorbing before the end of the culture period. Also it is possible that an occasional osteoclast may have been wrongly 'accused' of making an adjacent pit. The rate of resorption may vary during the making of one pit (Jones et al. 1988) and two pits that are made simultaneously by the same cell may have different rates of resorption (Kanehisa et al. 1988).

There did not appear to be any enhancement of resorption associated with the addition of extra nuclei beyond that merely due to an increase in nuclear
number and size. In fact the converse appeared to be true. There was a trend for the volume of mineralised tissue destroyed per osteoclast nucleus to decrease with increasing nuclear number. This result is consistent with the data of Zheng and colleagues (1991) who demonstrated that oligonuclear (2-5 nuclei) rat osteoclasts showed greater uptake of tritiated uridine than osteoclasts with more nuclei and concluded that these cells had higher rates of RNA synthesis. The finding that oligonuclear osteoclasts resorb more dentine per nucleus could therefore be a direct result of increased production of proteases and other enzymes involved with proton secretion per nucleus in these cells. However large osteoclasts although not gaining any functional advantage with the addition of new nuclei, may have an added advantage by means of their size alone, as an increase in cell cytoplasm may allow greater membrane storage, and faster availability of ruffled membrane. The size of the larger osteoclast also increases its potential for lobopodial and satellite extension and hence its possible sphere of, and potential for, resorptive activity. This may give a larger osteoclast a functional advantage (Jones et al.1994). The plan area of the pit largely represents the size of the cell whereas the volume:area ratio (mean depth) gives a greater indication of cell efficiency.

This study illustrated the importance of measuring all the available parameters, which would ideally include active resorption time for each cell. It emphasised that the sample must be of a reasonable size to avoid incongruous results. To look only at pit numbers or area resorbed, whilst gaining a good indication of the achievement of the cell population, is to risk missing some of the most exciting data.
Chapter 2. The effect of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (APD) on the resorptive function of osteoclasts of known nuclear number.
Introduction.

Bisphosphonates have been developed over the past two decades as a class of drugs which suppress bone turnover. The exact mechanism by which they work to decrease resorption is still poorly understood. There is still much controversy over the effect of bisphosphonates on the osteoclast precursors, with some groups purporting to have seen a reduced recruitment of the osteoclast precursor (Lerner et al. 1987, Boonekamp et al. 1986), whilst others have described an increase in the recruitment of osteoclast precursors (Endo et al. 1993, Marshall et al. 1993).

Others have studied the effect of bisphosphonates on the mature osteoclast, in a variety of different ways. Evidence has been put forward to suggest that bisphosphonates decrease the metabolic activity of osteoclasts (Carano et al. 1990), affect the osteoclasts' ability for membrane ruffling and the production of the proton pump (Zimolo et al. 1994) and resorption, albeit not permanently (Sato et al. 1991), or permanently damage the osteoclast (Flanagan et al. 1991). There have also been investigations into the ability of osteoclasts to bind to the bone mineral in the presence of a bisphosphonate (Lowik et al. 1988).

Despite the fact that the exact mechanism by which the bisphosphonates work is still not known, they have a wide ranging clinical use in the treatment of conditions resulting in ectopic calcification and increased bone resorption. They are currently used in the treatment of Paget's disease (Smith et al. 1971), hypercalcemia of malignancy (Kanis et al. 1991, Bonjour et al. 1991), osteoporosis (Papapoulos et al. 1992) and in the prevention of bone loss associated with microgravity (Grigoriev et al. 1992).

This study set out to investigate firstly the effect of 3-amino-1-hydroxypropylidene-1,1-bisphosphonate (APD) on the nuclear profile of an osteoclast population in vitro, and secondly to measure the resorptive efficiency of individually characterised osteoclasts in the presence and absence of the
bisphosphonate. APD was chosen for this study because it is a bisphosphonate that is now in wide clinical use and was made available to me by Ciba-Geigy (Basle).
Materials and Methods.

Culture of chick osteoclasts.

Osteoclasts were obtained from 19 day pre-hatch chick long bones in the manner I have described in chapter 1. Briefly, the long bones of one chick were chopped in phosphate-buffered saline (PBS) with 10% foetal calf serum (FCS) added. The osteoclasts were released from the bone fragments by repeatedly flushing them up and down in a plastic pipette. The resultant milky white cell suspension was then seeded onto one of four sperm whale dentine slices measuring 5mm by 5mm, derived from a 1cm square slice to minimise substrate differences. One slice from each set of four was pre-soaked in 10^{-6}M APD for 48 hours. Prior to seeding, the APD solution was withdrawn and replaced three separate times with 1mm of PBS.

Following seeding, cells were allowed to settle for 45 minutes at 37°C before washing gently in PBS to remove non-adherent cells. The dentine slices were then cultured for 24 hours in Eagle's medium (MEM) containing 10% FCS, 2mM L-glutamine, 0.25μg/ml fungizone, 0.2U/ml penicillin and 0.2μg/ml streptomycin, in 5% CO₂ at 37°C.

In each experiment the control and pre-treatment slices were cultured in the control medium (without added bisphosphonate). The other two slices received medium with added APD at 10^{-6}M or 10^{-8}M. Seedings were also made onto plastic culture dishes for 24 hours and cultured under identical conditions, receiving either the control medium, or the medium with added 10^{-6}M or 10^{-8}M APD. Both experiments were repeated a total of 45 times.

Counting the nuclei of the osteoclasts, and identifying the cells with their pits.

At the end of the culture period, the dentine slices and tissue culture dishes were gently washed in PBS and the cells fixed in 2.5 % glutaraldehyde in 0.15M
cacodylate buffer at pH 7.2. The cells were stained for the presence of tartrate-resistant acid phosphatase (TRAP). The numbers of nuclei of all the TRAP-positive multinucleate cells were counted on the tissue culture dishes.

Locating the pit in dentine and counting the number of nuclei of the associated osteoclast were carried out as described in chapter 1. All TRAP-positive multinucleate cells with a pit clearly ascribed to them were included in the study. The cells were removed from the dentine slices by washing with a mild detergent, 1% chlorohexidine gluconate, pH 7, followed by immersion in 30% H₂O₂. Total pit numbers were counted on all the specimens using a Bausch and Lomb reflected light microscope.

**Measurement of the pits.**

The measurements of the pits were carried in the same way as described in chapter 1 but with a few minor alterations. An updated version of the video-rate, line-confocal light microscope manufactured by the Lasertec Corporation, Japan, was used, called the ILM12. The difference in the method was that only one screen cursor placed around the edge of the pit was used. The software derived a trace binary outside the pit to determine the height of the surrounding surface, and derived the volume beneath the surface, calculating the area at the same time. The depth recorded was the maximum depth of the pit.
Results.

The nuclear number distribution

The nuclei of 2420 osteoclasts cultured on tissue culture dishes were counted, comprising 826 control cells, 794 cells at APD $10^{-6}$M and 800 cells at APD $10^{-8}$M. There was no significant difference within a 24 hour culture period between the cells of the treatment groups and the cells of the control group, with regard to their size (as measured by the number of nuclei). This is illustrated in Figure 2.1.

Hence there was no evidence to suggest that the presence of the bisphosphonate affected the fusion or fission of the osteoclasts, or preferentially targeted a particular cohort of cells during the 24 hour culture period of this experiment.

The number of pits.

The total pit numbers on the sperm whale dentine slices varied between the groups (Table 2.1)

There were fewest pits in the group where the dentine slices were pre-treated with the APD, and fewer in the group that received $10^{-6}$M APD in the medium compared to the control group. The highest numbers of pits were in the group that received the APD at a concentration of $10^{-8}$M. The numbers of pits suitable for inclusion in this study ranged from 10% in the pre-treatment group to 15% in the control group. There was a reduction in the percentage of osteoclasts associated with multiple resorption foci with $10^{-6}$M APD in the medium, and only one osteoclast in the pre-treatment group had multiple foci of resorption.
The sizes of the pits.

The maximum depths, areas and volumes of all the pits that could be ascribed to a particular osteoclast were measured. The mean values and the standard error of the means are illustrated for the volumes, areas, and depths resorbed per cell and the volumes, areas, and depths resorbed per nucleus in Figures 2.2-2.4. The numbers in each group are as follows: control group n=57, $10^{-8}$M APD group n=60, $10^{-6}$M APD group n=43 and APD pre-treatment group n=14.

The mean and median value for both the areas and volumes decreased in a dose-related manner, although there was a small but non-significant increase in the maximum depths of the pits and the number of nuclei of the osteoclasts in the group cultured in the presence of $10^{-6}$M APD in the medium. The largest reduction in all the measured parameters occurred in the group where the dentine slice was pre-treated with the bisphosphonate at $10^{-6}$M. The depth, area and volume resorbed per nucleus also decreased in a dose-related manner. The median values and the Mann-Whitney significance levels are illustrated in Table 2.2. Mann-Whitney was chosen as the statistical test because the data was non-parametric.

The results were also analysed excluding the cells to which more than one pit was ascribed. The means and medians of the volumes of the pits, and the volume resorbed per nucleus showed a dose-related decrease. The largest and most significant reduction was in the pre-treatment group. This data is shown in Table 2.3.

In addition, the first 150 pits encountered on a raster search in each of the different groups were analysed in order to exclude any bias associated with the preselection of a pit with an associated osteoclast. The means and medians of the volume and area of the pits are demonstrated in Table 2.4. The same trend was found, with the volumes and areas decreasing in a dose-related manner. The
largest and most significant decrease occurred in the pre-treatment group. The mean values for the measurement of the 150 pits were smaller in all the groups than those for the preselected pit cell pairs in the corresponding group. This may be due to the fact that it is extremely difficult to see small pits beneath a densely stained osteoclast, and hence cells associated with larger pits tended to be selected.

The proportion of the cells with 10 or more nuclei in the 10^{-6}\text{M} APD group (34.9\%) was twice as high as in the control group (15.8\%) and the 10^{-8}\text{M} APD groups (15.0\%). These values are different from the data shown in Figure 2.1 that represents the osteoclast population seeded onto tissue culture dishes where the proportion of cells with 10 or more nuclei in the control group is 27.4\%, 10^{-8}\text{M} APD 28.5\% and 10^{-6}\text{M} APD 23.82\%. This would influence the results if the activity decreased with increasing cell size. Therefore, I analysed the data in the 10^{-6}\text{M} APD group in three distinct subsets, those cells with 2-5 nuclei (n=11), 6-9 nuclei (n=17) and those with 10 or more nuclei (n=15). Although the volumes resorbed per cell were significantly higher for the larger cells (1289\text{um}^3, 1323\text{um}^3, and 3465\text{um}^3 median values for the three subsets respectively), the volumes resorbed per nucleus were not statistically different in the subsets (353.5\text{um}^3, 256\text{um}^3, and 369.3\text{um}^3 respectively). The statistical test used was the Mann-Whitney test.

Correlations of nuclear number and pit size.

The correlation between the number of nuclei of an osteoclast and the size of its resorption pit/pits are given in Table 2.5.

Larger cells made larger pits, whether or not APD was added to the medium or used in the pre-treatment of the dentine slice. The correlations between cell size and the volumes and areas of the pits are stronger than those for pit
depths in all the groups. The correlation coefficients for the sizes of the pits and
the number of nuclei were always lower with the APD in the medium.

The correlations between the areas and volumes resorbed per nucleus are
poor in all the sets except for the volume per nucleus in the pre-treatment group.
The depth resorbed per nucleus, however, was strongly negatively correlated with
the number of nuclei of the cell in all the groups. Thus larger cells made relatively
shallower pits.

There was no significant difference between the volumes of tissue resorbed
per nucleus by small (2-5 nuclei) and large (6+nuclei) osteoclasts in the control
and APD in the medium groups, although larger cells made larger pits.
Table 2.1. The total numbers of pits and the numbers of osteoclast-pit sites measured.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10⁻⁸M APD</th>
<th>10⁻⁶M APD</th>
<th>Pre-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total pits</td>
<td>543</td>
<td>578</td>
<td>483</td>
<td>178</td>
</tr>
<tr>
<td>No. of ocs with pits</td>
<td>57</td>
<td>60</td>
<td>43</td>
<td>14</td>
</tr>
<tr>
<td>No. of pits measured</td>
<td>81</td>
<td>82</td>
<td>57</td>
<td>17</td>
</tr>
<tr>
<td>% of ocs with &gt; 1 pit</td>
<td>24.6</td>
<td>23.3</td>
<td>16.3</td>
<td>7.1</td>
</tr>
</tbody>
</table>
Table 2.2. The sizes of the pits of known numbers of nuclei.

<table>
<thead>
<tr>
<th>Median value</th>
<th>Control</th>
<th>10^-8M APD</th>
<th>10^-6M APD</th>
<th>Pre-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=57</td>
<td>n=60</td>
<td>n=43</td>
<td>n=14</td>
<td></td>
</tr>
<tr>
<td>Nuclei/cell</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Depth (max)μm</td>
<td>5.94</td>
<td>5.11</td>
<td>6.22</td>
<td>2.71**</td>
</tr>
<tr>
<td>Depth/nucleus</td>
<td>1.02</td>
<td>0.82</td>
<td>0.73</td>
<td>0.54**</td>
</tr>
<tr>
<td>Area μm²</td>
<td>802</td>
<td>563</td>
<td>430</td>
<td>382**</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>134</td>
<td>82*</td>
<td>60**</td>
<td>62*</td>
</tr>
<tr>
<td>Volume μm³</td>
<td>3426</td>
<td>1895</td>
<td>1542</td>
<td>727**</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>552</td>
<td>323*</td>
<td>244**</td>
<td>130**</td>
</tr>
</tbody>
</table>

* p<0.001, **p<0.0001 values significantly different from the control values
Table 2.3. The sizes of the pits made by the cells associated with a single pit.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$10^{-8}$M APD</th>
<th>$10^{-6}$M APD</th>
<th>Pre treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>43</td>
<td>46</td>
<td>36</td>
<td>13</td>
</tr>
</tbody>
</table>

**Volume:**

<table>
<thead>
<tr>
<th></th>
<th>mean μm$^3$</th>
<th>$SEM$</th>
<th>median</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4749</td>
<td>806</td>
<td>3235</td>
<td></td>
</tr>
<tr>
<td>$10^{-8}$M APD</td>
<td>4052</td>
<td>1087</td>
<td>1894</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$M APD</td>
<td>2937</td>
<td>675</td>
<td>1439</td>
<td></td>
</tr>
<tr>
<td>Pre treatment</td>
<td>1005</td>
<td>263</td>
<td>701**</td>
<td></td>
</tr>
</tbody>
</table>

**Volume/nucleus:** mean 610.4, $SEM$ 57.2, median 486

<table>
<thead>
<tr>
<th></th>
<th>mean</th>
<th>$SEM$</th>
<th>median</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>509.7</td>
<td>93.5</td>
<td>329.2</td>
<td></td>
</tr>
<tr>
<td>$10^{-8}$M APD</td>
<td>410.7</td>
<td>96.7</td>
<td>215.3*</td>
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</tr>
<tr>
<td>$10^{-6}$M APD</td>
<td>144.1</td>
<td>26</td>
<td>109.3**</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.001 **p < 0.0001 significantly different from control values
**Table 2.4. The sizes of 150 pits randomly selected.**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$10^{-8}$ M APD</th>
<th>$10^{-6}$ M APD</th>
<th>Pre treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=150</td>
<td>n=150</td>
<td>n=150</td>
<td>n=150</td>
<td></td>
</tr>
<tr>
<td><strong>Volume:</strong> mean</td>
<td>1586</td>
<td>1749</td>
<td>1198</td>
<td>402.7</td>
</tr>
<tr>
<td>$\mu$m$^3$ :SEM</td>
<td>178</td>
<td>265</td>
<td>239</td>
<td>71.1</td>
</tr>
<tr>
<td>median</td>
<td>699</td>
<td>589</td>
<td>463*</td>
<td>161.5**</td>
</tr>
<tr>
<td><strong>Area:</strong> mean</td>
<td>422.8</td>
<td>358</td>
<td>333.87</td>
<td>172.9</td>
</tr>
<tr>
<td>$\mu$m$^2$ :SEM</td>
<td>42.8</td>
<td>41.8</td>
<td>51.2</td>
<td>25.7</td>
</tr>
<tr>
<td>median</td>
<td>252.5</td>
<td>168</td>
<td>170</td>
<td>95**</td>
</tr>
</tbody>
</table>

*<p0.001 **p<0.0001 significantly different from control values
Table 2.5. Correlations between the number of nuclei of a cell and the size of its resorption pit/s.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$10^{-8}$M APD</th>
<th>$10^{-6}$M APD</th>
<th>Pre treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth (max)</td>
<td>0.331</td>
<td>0.312</td>
<td>0.189</td>
<td>0.427</td>
</tr>
<tr>
<td>Depth/nucleus</td>
<td>-0.605</td>
<td>-0.516</td>
<td>-0.627</td>
<td>-0.429</td>
</tr>
<tr>
<td>Area</td>
<td>0.810</td>
<td>0.734</td>
<td>0.429</td>
<td>0.671</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>0.286</td>
<td>0.053</td>
<td>-0.044</td>
<td>0.231</td>
</tr>
<tr>
<td>Volume</td>
<td>0.774</td>
<td>0.574</td>
<td>0.291</td>
<td>0.694</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>0.237</td>
<td>0.112</td>
<td>-0.098</td>
<td>0.468</td>
</tr>
</tbody>
</table>
Fig. 2.1 Number of nuclei in chick osteoclasts cultured on plastic dishes in control medium, or with medium $10^{-8}$M or $10^{-6}$M APD added to the medium. The addition of APD did not alter the distribution of nuclei.
Fig. 2.2a The mean and SEM for the volume resorbed in each of the groups. The total numbers of pits measured in each of the groups respectively were: 543 pits (control), 578 pits (10^{-8}M APD), 483 pits (10^{-6}M APD), 178 pits (pre-treated).
Fig. 2.2b. The mean and SEM for the volume resorbed per nucleus in each of the groups. The volume resorbed per nucleus corrects for variation in cell size (number of nuclei), the cells in the group 10(-8)M APD having more larger cells.

<table>
<thead>
<tr>
<th></th>
<th>Volume resorbed per nucleus (cu microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>600</td>
</tr>
<tr>
<td>10(-8)M APD</td>
<td>500</td>
</tr>
<tr>
<td>10(-6)M APD</td>
<td>400</td>
</tr>
<tr>
<td>Pre-treated</td>
<td>300</td>
</tr>
</tbody>
</table>
Fig. 2.3a The mean and SEM for the area resorbed in each of the groups.
Fig. 2.3b The mean and SEM for the area resorbed per nucleus for each of the groups.
Fig. 2.4a The mean and SEM for the maximum depth resorbed for each of the groups.
Fig. 2.4b The mean and SEM for the maximum depth resorbed per nucleus for each of the groups.
Discussion.

This study has shown that the presence of APD in the medium during the culture period diminishes the size of the pit made by the osteoclast. This is best demonstrated when the data is expressed as the volume, area, or depth resorbed per nucleus for each osteoclast, as this tends to correct for the variation in cell size. This study confirms previous work describing a dose-related decrease in resorption measured by area or the numbers of pits at the individual cell level, even at low concentration of the bisphosphonate (10⁻⁸M APD) (Sato et al. 1990). In addition, my study shows that this is true for the volume of tissue resorbed.

There was no significant shift in the nuclear number distribution in this experiment analogous to that reported to have occurred in vivo following treatment with, for example, parathyroid extract (Addison 1980) or the bisphosphonate ethane-1-hydroxy-1,1-diphosphonate (Miller et al. 1977). This would be predicted if there were no preferential effect of the bisphosphonate on one cell size more than another, as the time course for this experiment was kept short (24 hours) in order to minimise the possibility of the cells migrating away from the pits that they had created. There would have been limited opportunities for cell fusion either between the mature osteoclasts or between precursor cells from the bone marrow and mature osteoclasts. The bisphosphonate may have prevented the attachment of cells from the bone marrow to the mineralised substrate, a step that some groups consider essential for their differentiation (Lowik et al. 1988, Boonekamp et al. 1986). However, there has been some evidence to suggest that osteoclast recruitment in mice increases following bisphosphonate treatment in vivo (Marshall et al. 1993), with new nuclei being added to pre-existing osteoclasts, and an increase in the numbers of osteoclasts (Endo et al. 1993). It is unclear from the experiments of Marshall whether the precursor cells from the mice were attached to the bone or not before fusing with
the resident osteoclasts. The fusion of the cells in their experiments however, was not affected by the presence of the APD. The duration of these in vivo experiments suggests that one would expect no change to occur within the 24 hour period of this experiment, and the response of the haemopoetic bone marrow in vivo is due to the change in the avidity of the APD bone for haematologically derived cells and not to the presence of the bisphosphonate per se (Marshall et al. 1993).

There has been some evidence to suggest that bisphosphonates have a degenerative effect on osteoclasts (Marshall et al. 1990, Flanagan et al. 1991), altering their morphology. The large reduction in the pit numbers, areas, volumes and depths in the pre-treatment group in this study would seem to support this view, as the accumulation of the APD in the surface layer of the dentine would be maximal in this group and its release during resorption would be increasingly detrimental to the cells as the concentration increased in the sub-cellular area. Calcitonin, itself an inhibitor of resorption, also protects osteoclasts from the effect of bisphosphonates (Flanagan et al. 1989, 1991, Boonekamp et al. 1986). Therefore, it is not surprising that there was the least percentage of cells associated with more than one pit, and the smallest pits, in the pre-treatment group, as also shown with a variety of other bisphosphonates (Selander et al. 1994).

The correlation coefficients for the numbers of nuclei in a cell and pit size pose an interesting question. The correlations may be weaker in the $10^{-6}$M APD group because the cells respond unevenly to the APD, but without regard to their size. There seems to be a dose-related trend for the relationship between cell size and pit size to be less with APD in the medium. This is not a feature of the pre-treatment group, where the reduction in pit size is greatest. A possible explanation for this may be that in the pre-treatment group APD does not affect the cell until resorption is well under way, and then as the concentration of APD rises below the resorbing cell, it does so overwhelmingly and resorption ceases abruptly. Thus
the relationship between the cell size and resorptive activity is not disrupted in the same way as a more gradually increasing suppression with APD in the medium, where the correlation between pit size and cell size falls as the drug takes effect.

These results show that there is a progressive loss of efficiency of resorption, as measured by the volume of tissue resorbed per osteoclast nucleus, with increasing bisphosphonate concentration in the medium. This supports the hypothesis of Carano et al. (1990) that bisphosphonates act as metabolic inhibitors, altering the protein synthesis of the cell. Other groups, using rat bone derived osteoclasts, have reported that there is an increase in pit numbers with low concentrations of bisphosphonates in the medium (Sato et al. 1990). I also found a slight increase in pit numbers in the $10^{-8}$M APD group, and this would offset to some extent the reduction in the mean volumes of the individual pits. Despite this, however, there was a clear reduction in the measured pit parameters in this group, emphasising the need to measure as many parameters as possible to allow a full picture of resorptive activity to be established (Boyde et al. 1991, 1992).

This work has confirmed the finding described in chapter 1, that larger cells make larger pits. However, I did not find a reduction in resorptive efficiency, measured as the volume of tissue resorbed per nucleus, in the larger cells compared with the smaller cells in any of the groups. This may be because the total numbers were small, and the range limited, with few cells at either end of the spectrum. There was, however, a strong negative correlation between the maximum depth resorbed per nucleus and the number of nuclei in the osteoclast.
Introduction.

The work capacity of each osteoclast is related to its size, for which the best measurement must be its total volume, that is the sum of its nuclear material, synthetic apparatus and secretory organ. Determining the volume of a complex shape is difficult, especially on a resorbed, rough substrate, but the measurement of cell volume on a flat substrate is feasible.

Attempts to measure changes in osteoclast size have been made by many workers investigating the effects of different reagents. Most groups have measured areas of spread cells, either by using time-lapse video recordings of live osteoclasts in bone cell cultures, using a system for digitising the cell outlines and hence calculating the spread area (Allam et al. 1993, 1992, Chambers et al. 1986); or by using fixed and air-dried cells and digitising the cell outline (Ransjö et al. 1988, Nicholson et al. 1988 Chambers et al. 1984). Estimation of the volume change of rat osteoclasts, fixed in situ, as a result of treatment with PTH has been calculated using digitised images of TEM sections from treated and control specimens (Holtrop 1979). Domon and Watika (1991) extended this method to cultured cells and calculated the volume of resorbing osteoclasts from serial TEM images.

There are innate problems with fixing the cells for measurement. Cells take time to die, and might change shape and size whilst they do. They also bleb and loose wet cell volume (Boyde et al. 1972). Allowing them to air-dry produces volume shrinkage that occurs on drying, although cell area may not change because of the adhesion to the substrate. It is not known whether this affects osteoclasts with different numbers of nuclei equally because measurements are lacking for multinucleate cells.

Digitising the outlines of the time-lapse video recordings using phase-contrast microscopy of living cells is a lengthy procedure. There is also some difficulty experienced with visualising and tracing the cell outline from the phase-
contrast video images which may lead to inaccuracies, and reports are often based on a very small sample size, (e.g. n=5, Alam et al. 1992). Furthermore, the approach is limited by phase halo effects which provide poor discrimination of the true location of the cell periphery (Boyde et al. in press). Perhaps the most accurate and simplest method is to capture images of the interface of the cell with a flat surface, such as a glass coverslip, using confocal reflection microscopy (Jones et al. 1990). The measurement of spread area, however, will not necessarily demonstrate changes in the metabolic activity of the osteoclasts in response to different reagents: this can only be evaluated by measuring the volume of an osteoclast.

As most resorbing osteoclasts are multinucleate, and the nuclei are not necessarily of equal age or size, obtaining the nuclear:cytoplasmic volume ratio is very difficult and it is perhaps more useful to ascertain the nuclei count:whole cell volume ratio.

The aims of this study were: to examine the relationship between the number of nuclei of an osteoclast and its volume, to compare the volumes of chick and rat osteoclasts, and to determine whether cell volume changes in the presence of the bisphosphonate APD (3-amino-1-hydroxypropylidene-1,1-bisphosphonate).
Materials and Methods.

Culture of osteoclasts.

Osteoclasts were obtained either from the long bones of 19 day prehatch Cobb White chicks or from 4 day old Sprague-Dawley rat pups in a similar manner to that described in chapter 1. Briefly, the long bones were excised, and their shafts, freed of periosteum, were chopped in Eagle's Minimum Essential Medium (MEM) with added 10% foetal calf serum (FCS). The resultant cell suspension was seeded on to sterile 18mm square glass coverslips which were chosen to allow a raster search to be carried out.

In experiment 1 both rat and chick cells were used. The coverslips with the droplets of seeded cells were placed in sterile plastic 35mm petri dishes, allowed to settle, and overlaid with the above medium after 15 minutes and cultured in 5% CO$_2$ at 37°C for 4 hours till the cells were well adherent and spread sufficiently to allow easy counting of the osteoclast nuclei. In experiment 2, only rat cells were used, and the cells were seeded and cultured in the presence or absence of 10$^{-6}$M APD in the medium. The coverslips were placed in 35mm plastic petri dishes in identical conditions to the previous experiment for either 4 or 6 hours.

Fixation and drying of the cells.

At the end of the culture period the coverslips were washed gently in warmed (37°C) phosphate-buffered saline (PBS) to remove non-adherent cells and then fixed for 24 hours in 2.5% glutaraldehyde in 0.15M cacodylate buffer (initially pre-warmed to 37°C).

In experiment 1 the chick cells were either critical point-dried or freeze-dried. Rat cells in both experiments were freeze-dried.
For critical point-drying (CPD), the cells were dehydrated in ethanol, which was substituted with CCL\textsubscript{2}FCCL\textsubscript{2} prior to CPD from CO\textsubscript{2}. For freeze-drying (FD), the coverslips were immersed for 1 hour in chloroform water (Boyde et al. 1969). Immediately prior to quenching in freon (CCL\textsubscript{2}F\textsubscript{2}) at -155°C, the gross excess moisture was removed by touching the side of each coverslip on to a piece of filter paper. The coverslips from one experiment were placed together in a specimen holder that had been pre-cooled in liquid nitrogen. Freeze-drying was conducted in an Edwards Speedivac freeze-dryer at -60°C for 24 hours. The freeze-dryer was gradually warmed up to room temperature at the end of the 24 hour period to avoid condensation when air was admitted.

All the coverslips from one experiment were dried in the same holder at the same time to minimise differences in cell shrinkage during the drying procedure. After drying, both the critical point-dried and the freeze-dried cells were coated by vacuum sublimation of gold to provide a reflective surface.

**Counting the nuclei of the osteoclasts and measuring their cell volume.**

The coverslips were attached to a glass slide, with the coated cells uppermost, and the cells measured using a video-rate, line-confocal laser scanning microscope, the 1LM12 manufactured by the Lasertec Corporation, Japan. Each osteoclast that was encountered on a raster search was firstly analysed to see whether it was suitable for inclusion in this study. The criteria used were: the nuclei must be clearly visible so that they could be accurately counted; the cell body free from any overlying debris that would influence measurement of cell volume, and also the osteoclast had to be an adequate distance from other cells or debris to allow a trace binary to be derived to determine a reference surface (Boyde et al. in press).

Once a suitable osteoclast was encountered, the nuclei were counted and recorded using phase contrast optics (20/0.20 objective). The cell was then
measured using a 40/0.95 lens and the He-Ne laser beam as described in chapter 1. Volume and area data were derived from the map image using the dedicated software, by tracing a screen cursor carefully around the periphery of the cell. The software derived a trace binary encircling the cell to determine the height of the reference surface and then derived the volume above the surface and the cell area.

A map image of an osteoclast with false colour added is illustrated in Fig. 3.1
Fig 3.1 A map image of an osteoclast with false colour added
Results.

Experiment I.
The difference in cell size with respect to freeze drying and critical point drying.

The means, medians and SEM.s for the measured parameters for the 67 freeze-dried chick osteoclasts and the 10 critical point dried chick osteoclasts are given in Table 3.1. The volumes of the freeze-dried cells were significantly larger than those of the critical point-dried cells, p=0.0045, and the volume/nucleus p<0.0001 (Mann-Whitney), but there was no significant change in the cell area.

The difference in cell volumes as a result of the drying methods is shown in Figure 3.2. Where the slope of the line is given by the equation y=f(x) +c, the freeze-dried cells have f=389 and the critical point-dried cells have f=221. The volume of each cell may be calculated by determining the nuclear number of the cell and multiplying this value by the f value.

Comparison of freeze-dried rat and chick osteoclasts.

A total of 50 freeze-dried rat osteoclasts, seeded for 4 hours prior to fixation, were measured in the same way as the 67 chick osteoclasts that had been freeze-dried. The data for the sizes of the rat osteoclasts is presented in Table 3.2 and the data for the chick osteoclasts is presented in Table 3.1.

The rat osteoclasts were significantly larger cells in all the measured parameters except the median number of nuclei per cell where they had the same value as the chick cells. The mean number of nuclei per cell was larger in the chick cells (5.28±0.54 compared with 4.14 ±0.27 SEM for the rat cells), due to the inclusion of a number of chick osteoclasts with more than 12 nuclei. The significant difference for the cell volume was p=0.0012, cell area p=0.0044 and for both the volume and area per nucleus p<0.0001 (Mann-Whitney). The correlation coefficients for the areas, volumes and the numbers of nuclei for rat
and chick cells were all high. Scatterplots and correlation coefficients are shown in Figures 3.3-3.8.

**Experiment 2.**

**Effect of APD on the size of rat osteoclasts.**

Rat osteoclasts were seeded and cultured for a period of 4 or 6 hours. A total of 40 osteoclasts in the 4 hour control group and 48 osteoclasts in the 4 hour APD-treated group were measured. In the group cultured for 6 hours a total of 56 osteoclasts in the control group and 48 osteoclasts in the APD-treated group were measured.

The means, medians and SEM.s for the control and APD-treated osteoclast for both culture times are illustrated in Table 3.3-3.6. Both groups showed a significant reduction in the volume/nucleus p=0.01 and volume/area p=0.01 for the cells cultured for 4 hours and volume/nucleus p=0.001 and volume/area p=0.0001 for cells cultured for 6 hours.

There was no significant difference between the 4 hour group and the 6 hour group in either the control or APD-treated osteoclasts. The two groups were thus pooled to provide a larger sample size providing a total of 96 control osteoclasts and 96 osteoclasts treated with 10^{-6}M APD. The means, medians and SEM.s for the control and APD-treated osteoclasts are given in Table 3.7 and 3.8 respectively.

There was a significant reduction in the volumes, volumes per nucleus, and the volume:area ratio of the osteoclasts cultured with APD (p=0.0023, p=0.0003 p<0.0001 respectively; Mann-Whitney).

There was no significant difference in the area or area per nucleus and the median number of nuclei in both groups remained the same. The mean number of nuclei for the control rat osteoclasts was 4.97 (+0.31 SEM.) compared with 4.93 (+0.32SEM.) for the APD-treated group. The correlation coefficients in both
groups remained high. These and scatterplots are illustrated graphically Fig. 3.9-
3.14. The regression lines are compared for cell areas, volumes, and volume:area
ratios in Figs. 3.15-3.17.

The sizes of the control freeze-dried rat cells in experiment one (n=50) and
experiment two (n=96) were compared. There was no difference in the numbers
of nuclei, volumes, or volumes per nucleus of the two groups, but the areas, areas
per nucleus and volume:area ratios of the cells were significantly different
(p=0.0009, p=0.0009 and p=0000 respectively, Mann-Whitney), reflecting
different degrees of spreading of the cells on the substrates in the two series.
<table>
<thead>
<tr>
<th>Table 3.1. The sizes of the chick osteoclasts.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td><strong>Critical point dried osteoclasts (n=10)</strong></td>
</tr>
<tr>
<td>Nuclei/cell</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
</tr>
<tr>
<td>Volume/nucleus</td>
</tr>
<tr>
<td>Area/nucleus</td>
</tr>
<tr>
<td><strong>Freeze dried osteoclasts (n=67)</strong></td>
</tr>
<tr>
<td>Nuclei/cell</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
</tr>
<tr>
<td>Volume/nucleus</td>
</tr>
<tr>
<td>Area/nucleus</td>
</tr>
</tbody>
</table>
Table 3.2. The sizes of the rat osteoclasts.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/ cell</td>
<td>4140</td>
<td>4.00</td>
<td>0.274</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
<td>2354</td>
<td>1954</td>
<td>245</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
<td>2929</td>
<td>2667</td>
<td>249</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>539</td>
<td>520.2</td>
<td>32.4</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>708.3</td>
<td>699.7</td>
<td>40.9</td>
</tr>
</tbody>
</table>
Table 3.3. The sizes of the 40 control rat osteoclasts cultured for 4 hours.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>4.125</td>
<td>4</td>
<td>0.273</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
<td>2595</td>
<td>2131</td>
<td>268</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
<td>4206</td>
<td>4239</td>
<td>378</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>593.3</td>
<td>567</td>
<td>37.8</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>992</td>
<td>988</td>
<td>59.3</td>
</tr>
</tbody>
</table>

Table 3.4. The sizes of the 48 APD-treated rat osteoclasts cultured for 4 hours.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>4.771</td>
<td>4.00</td>
<td>0.438</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
<td>2335</td>
<td>1978</td>
<td>251</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
<td>4295</td>
<td>3548</td>
<td>461</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>488.9</td>
<td>445.3</td>
<td>34.9</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>896.7</td>
<td>873.9</td>
<td>49.2</td>
</tr>
</tbody>
</table>
Table 3.5. The sizes of the 56 control rat osteoclasts cultured for 6 hours.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>5.571</td>
<td>4.50</td>
<td>0.485</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
<td>2945</td>
<td>2319</td>
<td>276</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
<td>4770</td>
<td>3938</td>
<td>424</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>534</td>
<td>479.1</td>
<td>30.6</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>880.1</td>
<td>751.4</td>
<td>46.2</td>
</tr>
</tbody>
</table>

Table 3.6. The sizes of the 48 APD-treated rat osteoclasts cultured for 6 hours.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>5.083</td>
<td>4.00</td>
<td>0.461</td>
</tr>
<tr>
<td>Volume $\mu m^3$</td>
<td>2097</td>
<td>1764</td>
<td>236</td>
</tr>
<tr>
<td>Area $\mu m^2$</td>
<td>4084</td>
<td>3542</td>
<td>402</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>390.5</td>
<td>415.4</td>
<td>22.4</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>816.3</td>
<td>740.7</td>
<td>46.2</td>
</tr>
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</table>
### Table 3.7. The sizes of the 96 control rat osteoclasts.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
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</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>4.969</td>
<td>4.00</td>
<td>0.313</td>
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<tr>
<td>Volume $\mu m^3$</td>
<td>2799</td>
<td>2193</td>
<td>196</td>
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<tr>
<td>Area $\mu m^2$</td>
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<td>3986</td>
<td>293</td>
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<tr>
<td>Volume/nucleus</td>
<td>558.7</td>
<td>520.7</td>
<td>23.9</td>
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<tr>
<td>Area/nucleus</td>
<td>926</td>
<td>875</td>
<td>36.8</td>
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### Table 3.8. The sizes of the 96 APD-treated rat osteoclasts.

<table>
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<tr>
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<td>4.927</td>
<td>4.00</td>
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<tr>
<td>Volume $\mu m^3$</td>
<td>2216</td>
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<tr>
<td>Area $\mu m^2$</td>
<td>4190</td>
<td>3542</td>
<td>304</td>
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<td>Volume/nucleus</td>
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<td>428.9</td>
<td>21.8</td>
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<tr>
<td>Area/nucleus</td>
<td>856.5</td>
<td>803</td>
<td>33.8</td>
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</table>
Fig. 3.2 Comparison of chick osteoclast cell volumes following freeze-drying (squares) and critical point-drying (circles).
Fig. 3.3. Scattergram and correlation coefficient for the relationship between nuclear number and volume for the rat osteoclasts.
Fig. 3.4. Scattergram and correlation coefficient for the relationship between nuclear number and area for the rat osteoclasts.
Fig. 3.5. Scattergram and correlation coefficient for the relationship between cell volumes and areas of the rat osteoclasts.
Fig. 3.6. Scattergram and correlation coefficient for the relationship between nuclear number and volume for the chick osteoclasts.
Fig. 3.7. Scattergram and correlation coefficient for the relationship between nuclear number and area for the chick osteoclasts
Fig. 3.8. Scattergram and correlation coefficient for the relationship between cell volumes and areas of the chick osteoclasts.
Fig. 3.9. Scattergram and correlation coefficient for the relationship between nuclear number and volume for the control rat osteoclasts.
Fig. 3.10. Scattergram and correlation coefficient for the relationship between nuclear number and area for the control rat osteoclasts.
Fig. 3.11. Scattergram and correlation coefficient for the relationship between cell volume and area of the control rat osteoclasts.
Fig. 3.12. Scattergram and correlation coefficient for the relationship between nuclear number and volume for the APD-treated rat osteoclasts.

<table>
<thead>
<tr>
<th>Nuclear number</th>
<th>Cell volume (cu microns)</th>
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</thead>
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<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2000</td>
</tr>
<tr>
<td>2</td>
<td>4000</td>
</tr>
<tr>
<td>3</td>
<td>6000</td>
</tr>
<tr>
<td>4</td>
<td>8000</td>
</tr>
<tr>
<td>5</td>
<td>10000</td>
</tr>
</tbody>
</table>

\( r = 0.856 \)

\( n = 96 \)
Fig. 3.13. Scattergram and correlation coefficient for the relationship between nuclear number and area for the APD-treated rat osteoclasts.
Fig. 3.14. Scattergram and correlation coefficient for the relationship between cell volume and area of the APD-treated rat osteoclasts.
Fig. 3.15. A comparison of the regression lines for nuclear number and volume of the control and APD-treated rat osteoclasts.
Fig. 3.16. A comparison of the regression lines for nuclear number and area of the control and APD-treated rat osteoclasts.
Fig. 3.17. A comparison of the regression lines for the relationship of cell volume and area of the control and APD-treated rat osteoclasts.
Discussion.

Drying Methods

The relative shrinkages of the cell volumes in this experiment for the freeze-dried and critical point-dried cells are in good agreement with previous data for cellular tissues (King 1991). The freeze-dried volume would be approximately 80% of the wet volume of the cell, and the critical point-dried cells 40% of the total volume of the cell (Boyde 1978, Boyde et al. 1981). On air-drying even from non-polar solvents, cells shrink 80% by volume. Thus solvent evaporation-drying is the least satisfactory method for preparing cells for volume measurement, although it has been used for estimating osteoclast areas (Ransjö et al. 1988, Nicholson et al. 1988, Chambers et al. 1984). Thus freeze-drying must be the technique of choice. The ratio of volumes after CPD and FD (FD:CPD 2.35) is relevant to all biological microscopy. These values are in agreement with those reported for bulk test specimens composed of uniform cellular tissues with a low collagen content. This indicates that the volume of living cells may be calculated by multiplying the FD volumes by 1.25.

Wheatley et al. (1987) found that the wet weight/dry weight ratio and protein/unit mass of cell remained constant throughout the HeLa cell cycle. There is no reason to believe that the ratio would change with cell fusion in osteoclasts but this determination has not been made.

The consistency of the results in the two sets of control, freeze-dried, rat osteoclasts with respect to the volume and volume per nucleus values suggests that the nuclear count is a valid index of the size of an osteoclast within similar cell populations. The difference in the areas, and areas per nucleus, can be explained by a variation in spreading in the two experiments: this also results in a difference in the volume:area ratio. Comparing the sizes of chick cells, assuming a 20% reduction in volume on drying, with the sizes of resorption pits made under
similar culture conditions during the first 24 hours after seeding (chapters 1 and 2), it is apparent that chick osteoclasts can destroy more than their own volume of dentine per day. This has been previously reported, with a chick osteoclast in its second day of culture destroying 128% of its own volume in 18 hours (Jones et al. 1988).

Species differences.

These results show that rat osteoclasts are larger than chick osteoclasts with the same number of nuclei. Previously it has been noticed that although rat cells adhered and spread faster than the chick cells, they made smaller pits within a 24 hour culture period than either rabbit or chick osteoclasts, and it has been suggested that the rat osteoclasts were tardy in commencing resorption, and required a more acidic medium to generate an equivalent effect to chick cells (Jones et al. 1986). Osteoclast volume does not, therefore, correlate with resorptive capability in vitro except within one cell population.

The high correlations between the numbers of nuclei and the cell volume substantiates the use of the number of nuclei as an indiector of cell size within one population. Basile et al. (1988) also found a good correlation between the diameters of human osteoclasts, isolated and cytocentrifuged on to slides, and their number of nuclei.

Effect of APD.

This is the first time that the effect of APD on osteoclast cell volume, independent of the presence of a calcified tissue, has been demonstrated. It is not known how bisphosphonates act to suppress resorption, but it has recently been shown that bisphosphonates can be metabolised by Dictyostelium discoideum, a cellular slime mould (Rogers et al. 1992), and are potent inhibitors of squalene synthase, an enzyme required for cholesterol biosynthesis (Amin et al. 1992).
The reduction in both the volume, volume/nucleus and volume:area ratios clearly suggests that the bisphosphonates act directly on osteoclasts, as indicated by other studies (Boonekamp et al. 1986). Bisphosphonates have also been shown to affect marrow-derived macrophages (Cecchini et al. 1987) as well as osteoblasts after only 5 minutes (Sahni et al. 1993), although no change in cytosolic Ca\(^{2+}\) could be detected in osteoblasts (Antic et al. 1994). In mixed bone cell cultures such as this one, it is impossible to say whether the change in cell size is solely a direct effect on the osteoclast itself or whether it also is due in part from signals from other cells. In order to minimise this, bone cells were seeded sparsely to ensure that the osteoclasts measured were not in contact with any other cell.

There was no change in the tonicity of the culture fluid with the addition of 10^{-6} M APD: therefore this did not influence cell size. The significant difference in cell volume and volume per nucleus following the APD treatment might be explained by a depolarisation of the cell membrane with loss of intracellular K\(^+\) and Cl\(^-\), leading to net water loss and cell shrinkage. An alteration in membrane conductance has been suggested to cause shrinkage in other types of cells exposed to secretagogues (Susuki et al. 1991, Walters et al. 1992), an effect maintained as long as the substance was present. Inwardly rectifying K\(^+\) currents, however, have been described in rat osteoclasts and depolarisation would assist H\(^+\) transport out of the cell and stimulate resorption (Sims et al. 1989). Zimolo et al. (1994) found that alendronate altered membrane fluxes of NH\(_4^+\) and H\(^+\). Most substances that inhibit resorption appear to involve a cyclic AMP-dependant pathway (Ransjö et al. 1988, Allam et al. 1993) and cause a reduction in cell area (Chambers et al. 1986). As there was no significant reduction in plan-areas of the APD-treated osteoclasts it is unlikely that cAMP activation and subsequent rise of intracellular Ca\(^{2+}\) accounts for the change in volume. Although a cAMP-
activation of K\(^+\) and Cl\(^-\) channels is not ruled out, there is no evidence to suggest that APD affects the cAMP pathway in osteoclasts.

The reduction in the volume of the osteoclast supports the hypothesis that the bisphosphonates may be acting as metabolic inhibitors to reduce protein synthesis (Carano et al. 1990). The bisphosphonate alendronate besides altering membrane fluxes of NH\(_4^+\) and H\(^+\) disrupts the ruffled border complex, and stops the proton pump, which may even be pinocytosed back into the osteoclast (Zimolo et al. 1994). These effects may contribute in some part to a reduction in osteoclast volume in the presence of a bisphosphonate, as well as a decrease in the sizes and numbers of the pits that the cells make (Sahni et al. 1994, chapter 2).

The reduction in volume and volume per nucleus of the rat osteoclasts cultured in the presence of 10\(^{-6}\)M APD is less than the reduction in the volume of tissue resorbed by chick osteoclasts cultured under similar conditions (chapter 2). This might be due to a species difference in response to APD, or might indicate that resorbing cells are more affected by the bisphosphonate (Flanagan et al. 1991). However, it does conflict with the idea that only resorbing cells are affected by the drug.

Endo et al. (1993) reported that osteoclasts in the tibiae of mice treated with bisphosphonates became enlarged and the cells contained more nuclei. This assessment was based on observing histological sections. As they did not say whether the change was due solely to an increase in cell fusion, it is not possible to know whether the results of the in vivo study are in conflict with my in vitro data.

Further studies on cell volume investigating the dose-response and time-response to APD, and changes in cell mass, are needed.
Measurement of cell volume.

Measurement of cell volume is not easy. Ideally, wet cell volume should be obtained and the nuclear:cytoplasmic ratio determined. In practice, this is rarely achieved.

Coulter size distribution analysers have been used for estimating volumes of T-lymphoblasts (Bagnara et al. 1992) and volume changes of B-lymphocytes (Morikawa et al. 1993) but are not suitable for an impure population of cells with a wide size range and a tendency to fragment. Most volume estimates of live cells rely on light microscopical images recorded at timed intervals which are analysed for total area, mean height and mean width with an image analysis system; the volume is estimated by assuming a model shape and applying the respective geometric formula (e.g. Walters et al. 1992). Nuclear:cytoplasmic ratios of a number of cell types have been obtained by scrape-loading cells with a mixture of two fluorescent dextrans, one of which diffuses into the nuclear envelope, then calculating the total cellular fluorescence of both probes and the ratio of the two probes in the cytoplasm (Swanson et al. 1991). The volume regulation of astroglial cells in culture has been demonstrated by loading the cells with fluorescent intracellular probes, exciting these at their isosbestic point where the signal emitted depends on the variation in concentration induced by a change in cell volume, and measuring the signal with microspectro-fluorometric equipment (Eriksson et al. 1992). A very different approach for live cells has been employed by Dunn et al. (1987) and Brown et al. (1989) who used microinterferometry to study the movement of dry mass in fibroblasts. This method allows evaluation of the dry mass per projected area but not per unit volume.

Most current estimates of cell and nuclear volumes are based on stereological techniques. For example, neuronal cell areas in monkeys have been measured from fixed, frozen sections by immunolabelling the cells and
microcomputer image analysis (Albanese et al. 1993). Point counting on fixed, stained, embedded sections is commonly used to achieve the same aim (Ghosh et al. 1992, Parr et al. 1993, Hurst et al. 1993), and nuclear volume calculated by the same method (Parr et al. 1993), or from tracing serial sections with a camera lucida (Ghosh et al. 1992). Kirk et al. (1993) used SEM, stereology and geometric formulas to calculate cell volume, whilst Holtrop et al.'s classic study (1979) of changes in the ultrastructure of osteoclasts measured projected areas from photographic negatives of TEM sections. Many groups have used methods of digitising the cell outlines from fixed and stained tissues which were either mechanically or optically sectioned to determine cell area and volume (Adamson 1993, Chu et al. 1993, Stockley et al. 1993, Tipoes et al. 1992).

The method that I have used to measure the volume of osteoclasts by reflection confocal microscopy (CLSM) has proved to be extremely useful, although it falls short of the ideal because the cells are both dead and dry. However, the combination of CLSM with freeze-drying provides the fastest and most accurate method for directly determining the volumes and areas of whole polymorphous cells. The speed achieved by the operator compared to other methods for measuring spread area (Alam et al. 1992, Ransjö et al. 1988) permits larger sample sizes to be measured, an essential requirement when evaluating size changes of cells which have an innately wide range of sizes. Unlike the commonly employed methods reviewed above, geometric formulae for volume estimations are not needed, edge detection of fixed and stained sections is not a problem, and time-consuming, accurate tracing is not required. One circumvents the problems associated with fluorescence methods which involve extreme care when loading cells with fluorescent dyes to ensure that the dye fills the whole compartment to be measured and that the method of detection is sensitive enough to measure the emissions accurately. This new method for measuring cell volumes
and plan areas of fixed osteoclasts may prove to be equally valuable in other fields of cell biology.
Chapter 4. Red deer antler osteoclasts.
Introduction.

The number of nuclei present in osteoclasts varies considerably within one animal at different sites, and at the same site, and at present there is little information on what functional significance this may have in different species.

The frequency distribution of nuclei in osteoclasts of many different animals, mainly mammalian, but also avian and Xenopus, has been under investigation now for some years (Table 4.1).

The methods of obtaining the osteoclasts have varied from: in-situ methods, to smear specimens, cultures, and autoradiographs. There are advantages and disadvantages with each method for ensuring a representative sample of osteoclasts in which to count their nuclei. Whole cell smears have the advantage that the osteoclasts are well separated and flattened so that superimposition of the nuclei is rare, making a more accurate count of the nuclear number possible. However, the smears may not be representative of the whole population. In situ staining of osteoclasts has the benefit that the population of cells will be more representative but it is more difficult to count the nuclei owing to the superimposition of nuclei. In the study carried out by Ries et al. (1987), the mean number of nuclei was lower in the in situ versus the smear specimen (Table 4.1). Autoradiographs of stained osteoclast nuclei smears (Ries et al. 1987), or sections (Miller et al. 1981) have the problem that not all of the osteoclast nuclei may have taken up the marker used, and so one may not get a representative distribution of the nuclear frequency. Cultures of osteoclasts from bones or bone marrow have the disadvantage that the cells that attach and spread during the culture period may not be a true sample, and the distribution of nuclei within the osteoclast population may have changed during the culture period.

Despite limitations in each method some very interesting phenomenological work has been carried out (Table 4.1).
Some pathological conditions, such as Paget's disease, show a change in frequency of cells with higher numbers of nuclei (Basle et al. 1988) and an increase in the numbers of nuclei per cell occurs following treatment with parathyroid hormone (Addison 1978).

Whether there is an optimal size for osteoclasts is unknown. In my in vitro studies of chick osteoclasts (chapter 1), the addition of new nuclei did not appear to confer an advantage on the cell by increasing its productivity (per nucleus). Larger cells were not more effective per nucleus in destroying dentine although the largest cells destroyed more dentine than the smaller cells, but cell fusion may prolong the activity of the cells.

Deer antler is unique in being one of the fastest growing mammalian structures, increasing in length by up to 1.8cm per day (Goss et al. 1983). Therefore the antler is a very active site of both modelling and remodelling of mineralised tissue in a normal, healthy adult mammal. Despite the recent interest in androgen-stimulated bone growth and in the development of the deer antler (Morris et al. 1983), no-one has addressed the frequency distribution of deer osteoclasts of known nuclear number or their resorptive capability in vitro.

The aim of this study was to describe the osteoclasts at a site of endochondral ossification undergoing rapid physiological remodelling, and to measure the rate at which these cells resorb dentine in vitro.
Materials and Methods.

Culture of deer osteoclasts.

Three sets of antlers were obtained from two-year old red deer in the Spring of 1992 during the growing season. The deer were being culled for venison. The antlers were removed at the time of death in the field with a saw. They were then brought back to the laboratory, within one hour. The skin was dissected away from the bony tissues, and the bone cells were obtained from the antler by chopping sections of the antler in phosphate-buffered saline (PBS). The antler was sampled in the area indicated in Fig 4.1.

The cell suspension was then seeded on to tissue culture dishes or sperm whale dentine slices measuring 5mm by 5mm with one corner clipped to aid later identification and allowed to settle at 37°C for 45 minutes. The dishes and dentine slices were gently washed in PBS to remove non-adherent cells, and the cultures incubated in Eagle's Minimum Essential Medium (MEM) with added 10% FCS, 2mM L-glutamine, 0.25pg/ml fungizone, 0.2U/ml penicillin and 0.2pg/ml streptomycin, in 5% CO2 at 37°C for 24 hours.

Counting the nuclei of the deer osteoclasts and identifying the cells with their pits.

The procedure used was similar to the one described in Chapter 1. Briefly, at the end of the culture period, the dentine slices were gently washed in warmed PBS and the cells fixed in 2.5% glutaraldehyde in 0.15M cacodylate buffer at pH 7.4 for 5 minutes. The dentine slices were then washed in distilled water and air dried for 10 minutes. The deer antler-derived cells were stained for the presence of tartrate-resistant acid phosphatase (TRAP: Sigma kit 386) and the specimens were stored in 0.2M cacodylate buffer or 2.5% sodium azide in PBS until recordings of the nuclei counts and pit positions were made. Cultures on plastic culture dishes were fixed and stored in the same way.
The location of the pits and the nuclear counts for the associated osteoclasts and each of the numbered dentine slices were made in exactly the same way as described in Chapter 1.

The nuclei of all the multinucleate cells that were TRAP-positive were counted in the cultures on plastic and parallel cultures on dentine. In addition, the mononuclear TRAP-positive cells in each culture were counted. Nuclear counts were made of 514 putative osteoclasts on plastic and 167 cells on dentine. Therefore, the number of nuclei in each of 681 TRAP-positive cells was recorded in this study.

Measurement of the pits.

The measurement of the pits was carried out as described for the avian osteoclast study in Chapter 1, using the video-rate line-confocal laser scanning light microscope (CLSM) manufactured by the Lasertec Corporation, Japan. The depth recorded was again the maximum depth of the pit. The mean depth was determined from computing volume/area.
Fig. 4.1. Structure of forming antler, growing tips of proliferating undifferentiated mesenchyme (pm) beneath a epidermis (ed), subepidermis (sed) and fibrous perichondrium (fp) covering a central core of forming cartilage (fc). This becomes hypertrophic (hc), calcifies (c) and is remodelled into primary (ps) and secondary spongiosa(ss), which is enveloped by a collar of subperiosteal bone (sb). The antler was sampled between the red lines. Taken from Gray et al. (1992.)
Results.

The total osteoclast population.

Cells harvested from the deer antler were characterised for their nuclear number. 681 osteoclasts and mononuclear stained cells were counted in all. There was a high number of TRAP-positive mononuclear cells, 22% comprising this group. This data is illustrated in Fig 4.2.

When only TRAP-positive multinucleate cells were analysed there was a significantly high proportion of oligonuclear osteoclasts; of the 531 cells: 56% were in the 2-5 nuclei group, 35% in the 6-10 nuclei group, 8% in the 11-15 nuclei group and only 1% in the 16-20 nuclei group (Fig. 4.3).

The results were compared with the previous data on osteoclasts obtained from prehatch chick long bones cultured under similar experimental conditions (see Chapter 1). The purpose of the comparison was to evaluate whether populations of osteoclasts from deer antler and chick long bones, both actively growing and remodelling locations, were similar. The results are illustrated in Figs. 4.4 and 4.5.

The deer osteoclasts had a higher frequency of small cells. The cumulative frequency graph is clearly shifted to the left for the deer cells indicating a higher frequency of smaller osteoclasts (as measured by nuclear number). There were no deer osteoclasts with more than 20 nuclei.

The sizes of the pits and the nuclei per cell

It was possible to measure only 34 combinations of pits and osteoclasts because the shallow excavations present under the small osteoclasts are extremely hard to identify using a light microscope, and even the larger osteoclasts from the deer antler made small pits compared with those produced by the avian osteoclasts. The deer pits proved hard to identify underneath the osteoclast on the
dentine slice. There also was a greater tendency for the pits not to be associated with an osteoclast, as though the osteoclasts started resorption and then migrated away and began again or detached from the surface.

The mean and median results for all the measured parameters are shown in Table 4.2. The mean volume resorbed per nucleus per cell was 507\(\mu\text{m}^3\). The correlation for the 34 pits are shown in Table 4.3.

Not surprisingly, with a sample of only 34 pits the correlations tend to be low. However, the correlation between area and volume is high.

As the pit numbers are so low, the results have been grouped according to number of nuclei in the osteoclast. Two groups were chosen in order to give an approximately even number in both groups: cells with 3-5 nuclei (16 pits), and those with 6-10 nuclei (18) pits. The means, SEMs, and median values for the depths, areas and volumes of the pits are given in Table 4.4. There was a tendency for the depths, areas and volumes to increase with increasing nuclear number, (Figs. 4.6-4.8).

The only significant increase \(p=0.006\) was between the mean volume resorbed between the two groups (twosample t-test). The means, SEMs, and median values for the depths, areas and volumes per nucleus of the pits are given in Table 4.5 and Figs. 4.6-4.8. There was no significant change in the depth, area and volume resorbed per nucleus with increasing nuclear number (Table 4.5). Depths given are the mean of the maximum pit depths.
Table 4.1. The frequency distribution of nuclei from osteoclasts and odontoclasts.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Species</th>
<th>Site</th>
<th>Method</th>
<th>Mean number of Nuclei</th>
<th>Author</th>
<th>Reference</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoclast</td>
<td>Kitten</td>
<td>Femoral metaphyses</td>
<td>Whole cell smears</td>
<td>5.5</td>
<td>Addison</td>
<td>J. Anat. (1980) 130:479</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Insitu 4.56</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Mature 7.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoclast</td>
<td>Cat</td>
<td>Femoral</td>
<td>Bone marrow culture (1 week)</td>
<td>6.0</td>
<td>Suda et al.</td>
<td>Calc. Tiss. Int. (1983) 35:82</td>
</tr>
</tbody>
</table>

143
Table 4.2. The mean value of the sizes of the 34 pits and related deer cells.

<table>
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<tr>
<th></th>
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<th>Median</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td>Nuclei/cell</td>
<td>5.85</td>
<td>6</td>
<td>0.313</td>
</tr>
<tr>
<td>Max depth μm</td>
<td>4.65</td>
<td>3.68</td>
<td>0.411</td>
</tr>
<tr>
<td>Area μm²</td>
<td>1091</td>
<td>844</td>
<td>131</td>
</tr>
<tr>
<td>Volume μm³</td>
<td>3050</td>
<td>2174</td>
<td>411</td>
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</table>
Table 4.3. The correlation matrix

<table>
<thead>
<tr>
<th></th>
<th>Nuclei/cell</th>
<th>Depth</th>
<th>Area</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth</td>
<td>0.281</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area</td>
<td>0.433</td>
<td>0.305</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>0.486</td>
<td>0.670</td>
<td>0.750</td>
<td></td>
</tr>
<tr>
<td>Mean depth</td>
<td>0.109</td>
<td>0.380</td>
<td>0.083</td>
<td>0.464</td>
</tr>
</tbody>
</table>
Table 4.4. Pit size, grouped according to nuclear number.

<table>
<thead>
<tr>
<th>Nuc/No of cell</th>
<th>Depth μm</th>
<th>Area μm²</th>
<th>Volume μm³</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Med.</td>
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<tr>
<td>3-5</td>
<td>3.996</td>
<td>0.498</td>
<td>3.420</td>
</tr>
<tr>
<td>6-10</td>
<td>5.184</td>
<td>0.621</td>
<td>4.685</td>
</tr>
</tbody>
</table>
Table 4.5. Pit size per nucleus, grouped according to nuclear number.

<table>
<thead>
<tr>
<th>Nuc/ cell</th>
<th>No of cells</th>
<th>Depth/nuc Mean</th>
<th>SEM</th>
<th>Med.</th>
<th>Area/nuc Mean</th>
<th>SEM</th>
<th>Med.</th>
<th>Volume/nuc Mean</th>
<th>SEM</th>
<th>Med.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-5</td>
<td>16</td>
<td>0.946</td>
<td>0.125</td>
<td>0.769</td>
<td>194.1</td>
<td>41.9</td>
<td>171.2</td>
<td>441.6</td>
<td>85.7</td>
<td>355.7</td>
</tr>
<tr>
<td>6-10</td>
<td>18</td>
<td>0.729</td>
<td>0.087</td>
<td>0.654</td>
<td>181.4</td>
<td>24.6</td>
<td>170.4</td>
<td>565.6</td>
<td>85.9</td>
<td>464.8</td>
</tr>
</tbody>
</table>
Fig. 4.2. Relative frequencies of the 681 red deer antler TRAP-positive cells with different numbers of nuclei.
Fig. 4.3. Relative frequency of nuclear number of the 531 multinucleate cells.
Fig. 4.4. The relative frequencies of the 730 chick osteoclasts and the 531 deer osteoclasts of each size (nuclear number).
Fig. 4.5 The cumulative frequency histograms of cells with different numbers of nuclei in the samples of 730 chick osteoclasts and 531 deer osteoclasts.
Fig. 4.6. The relationship between the volume of the pit resorbed and the number of nuclei in the osteoclast, and the volume of tissue resorbed per nucleus and the number of nuclei of the osteoclast. The mean volume resorbed is 3050 cubic microns.
Fig. 4.7. The relationship between the plan area of the pit and the number of nuclei in the osteoclast, and the plan area of tissue resorbed per nucleus and the number of nuclei of the osteoclast. The mean area resorbed is 1091 square microns.
Fig. 4.8. The relationship between the mean of the maximum depth of the pit and the number of nuclei of the osteoclast, and the maximum depth of the pit per nucleus and the number of nuclei of the osteoclast. The mean depth resorbed is 4.65 microns.
Discussion.

The nuclear number distribution.

The high proportion of oligonuclear osteoclasts in the cells harvested from the deer antler and mononuclear TRAP-positive cells may indicate a highly active site of resorption, such as would be expected in a mineralised tissue with such rapid growth rates. It could be argued that the cells counted were not a representative sample, either the cells seeded, or the cells spreading and settling not representing the whole population. However, I took samples from three different deer and all showed a similar frequency distribution of nuclei. The settling time allowed and the 24 hour culture period prior to fixation should have favoured a fair representation of all the cell sizes.

Recent work on rat osteoclasts (Zheng et al. 1991) demonstrated that oligonuclear osteoclasts had the highest rates of RNA synthesis per nucleus, and hence may be more active. Their work showed that with increasing nuclear number there was a decrease in basal RNA synthesis per nucleus although total RNA was greater and they suggested that beyond a certain size the osteoclasts became 'aged'.

It is possible that the distribution of nuclei in a population of osteoclasts indicates the relative state of activity of resorption at that site. Early in the resorption cycle there would be a tendency for more mononuclear and oligonuclear, highly active osteoclasts; later in the mid period a more steady state would be reached with more multinucleate cells formed from the fusion of the mononuclear and oligonuclear osteoclasts and hence fewer cells in these two groups. In the late stage there would be few highly active mononuclear cells contributing fresh nuclei to the osteoclast pool, fewer large multinucleate cells as nuclei leaving the larger cells are not replaced, and more oligonuclear cells, some derived from the fission of the multinucleate group. The oligonuclear cells in this
last group would contain older, less active nuclei than those in the first. It might therefore be possible to monitor the stage in a cycle of resorptive activity of certain clinical bone pathologies by monitoring the relative nuclear number distribution of the osteoclasts.

Sizes of the resorption pits.

Deer osteoclasts containing more nuclei made larger pits. This finding was similar to the results obtained for avian cells (see Chapter 1). However, there was not a significant decrease in the volume resorbed per nucleus equivalent to that found with the chick osteoclasts cultured under similar conditions. The mean volume resorbed per nucleus of osteoclasts with 3 nuclei, for example, was 1050\(\mu\text{m}^3\) for chick cells, but only 180\(\mu\text{m}^3\) for deer cells. However, it is possible that the chick cells started resorbing earlier and continued for longer, rather than resorbing tissue at a greater rate.

The finding that the rate of work per deer osteoclast nucleus remained reasonably constant, independent of size, might have been due to the small range of multinuclearity in the deer cells associated with pits (3-10 nuclei), compared with the larger range for the chick osteoclasts (2-27 nuclei). A difference in resorptive efficiency may only be demonstrable with large samples of osteoclasts containing cells at either end of the size spectrum, that is, small, highly active osteoclasts and large 'aged' osteoclasts. Alternatively, deer antler osteoclasts might not be constrained by size but stay as functionally active with both low and high numbers of nuclei. The rate of fusion of new nuclei at very active sites of resorption, such as deer antlers, may be so rapid that none of the nuclei could be termed 'aged'. In a study on the kinetics of nuclei in rat osteoclasts, Ries et al. (1987) demonstrated that the peak level of labelling with [3H]TdR in mononucleate osteoclast-like cells coincided with the increased presence of bi- and tri-nucleate osteoclasts. Possibly then in the deer antler there is an extremely
rapid rate of fusion of new nuclei predominately from the mononuclear cells so that the large osteoclasts at such sites may have all young nuclei, and therefore show no deterioration in their ability to resorb commensurate with their size.

This work needs to be repeated with a larger sample size, but the restricted availability of the material in terms of time and cost made this difficult. It would be valuable to compare the osteoclast population of the antler with that of some other cranial or post-cranial site of the same animal.
Chapter 5. Morphological classification of osteoclasts: changes in shape and numbers of nuclei with age.
Introduction.

Little is known about the life history of cohorts of osteoclasts. This is partly because methods of getting samples of osteoclasts freed from bone may affect the profile of nuclear number, as well as favouring one morphological type rather than another. Although it is now widely accepted that the osteoclasts are derived from a hematopoietic stem cell population (Ash et al. 1980), the life span and fate from mononuclear osteoclast to post-osteoclast are still debated.

Many research groups have demonstrated that mononuclear osteoclasts are functionally active. TRAP-positive mononuclear osteoclasts from chicks (Prallet et al. 1992), mice (Domon et al. 1991), and rats (Baron et al. 1986) are capable of adhering to and resorbing bone. Most of these cells described will be newly differentiated osteoclasts. Whilst the fate of the post-osteoclast, defined as a mononuclear, or oligonuclear, non-resorbing cell derived from an osteoclast has not been elucidated. A dynamic osteoclast turnover with fusion of mononuclear cells, fission of multinuclear cells, and a subsequent recycling of post-osteoclasts has been suggested by some authors (Baron et al. 1984), but others have postulated degeneration and death of these cells (Liu et al. 1982, Klaushoffer et al. 1989). The changes in multinuclearity and shape during the life history of human osteoclasts are still not fully described, nor their importance determined in humans or other mammals.

Size, shape and function of osteoclasts may also be influenced by bone matrix age (Groessner-Schreiber et al. 1991), site (Chappard et al. 1991), and the age of the osteoclasts themselves (Takahashi et al. 1987).

The relationship between the shape of osteoclasts and their functional status is controversial. Hatta et al. (1992) have described two distinct forms, smooth and stellate, and suggested that the stellate form is the active state. However, others believe that the stellate form is not associated with resorption
Osteoclast kinetics have been studied traditionally using $[^3\text{H}]$-thymidine labelling (Young et al. 1961, Fischman et al. 1962, Jaworski et al. 1981) but a major drawback of this technique is the long period required for adequate exposure of photographic emulsions used for autoradiography. Recently a new method has been described using another thymidine analogue, 5-bromodeoxyuridine, and a monoclonal antibody against it for determining osteoclast kinetics (Marshall et al. 1991, 1993). This method has the advantage that it is much quicker to use.

Cell shape and movements are generated by the cytoskeleton. The cytoskeleton comprises a cohesive network of filaments and accessory proteins which link the filaments to one another and are essential for their controlled assembly. The cytoskeleton is regulated by the same molecules and enzymes that act as signalling molecules in the cytoplasm. The function of the cytoskeleton is therefore integrated with other aspects of cell physiology (Bray 1992). Changes in osteoclast activity between migration and resorption phases are associated with changes in the expression of cytoskeletal proteins (Turksen et al. 1988, Lakkakorpi et al. 1991, Marchisio et al. 1984). Calcitonin, for example, which alters osteoclast function, also produces changes in both cell shape and the cytoskeleton (Hunter et al. 1989).

The aims of this study were, firstly, to describe the shapes and nucleation of rat and mouse osteoclasts in vivo at different developmental stages and, secondly, to see if the application of marking nuclei with 5-bromodeoxyuridine and staining cytoskeletal proteins could determine whether mouse stellate osteoclasts were functionally active cells or non-resorptive post-osteoclasts.
Materials and Methods.

Light microscopy.

Calvaria were excised from fourteen Sprague-Dawley rats, two in each age group of 2, 7, 14, 21, 28, 56, 98 days respectively, and from four adult Westray mice and one 14 day old Black-Tan mouse. No other Westray mice were available as after the adult mice were obtained the colony was exterminated for economic reasons. The animals were killed by cervical dislocation and the calvaria removed and stripped of membranes in warmed (37°C) phosphate-buffered saline (PBS) under a dissecting microscope. The membrane-free calvaria were then divided into the two parietal bones and the occipital bone to aid later microscopy. They were fixed at 37°C in 2.5% glutaraldehyde in 0.15M cacodylate buffer at pH 7.4 for 5 minutes.

Following fixation, the calvaria were washed in distilled water and stained for tartrate-resistant acid phosphatase (TRAP). After staining the calvaria were washed again in distilled water and placed in a petri dish in water for microscopy.

The TRAP-positive osteoclasts on the endosteal surface of the calvaria were characterised for both nuclear number, and morphological Types I, II or III (as described by Abe et al. 1990). These morphologies are illustrated in Figures 5.1-5.3. A High Definition Stereo Light Microscope (Edge Scientific Corporation, Los Angeles CA 90066), a new 3-D light microscope described in principle by Greenberg and Boyde (1993), was used for this assessment, with a 20/0.4 or 40/0.55 water immersion lens.

Marking the uptake of nuclei with 5-Bromodeoxyuridine.

Five adult and five 10 day old Black-Tan mice were given intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU) plus 5-fluoro-2'-deoxyuridine (FdU) to give a dose of 60mg BrdU and 6mg FdU per kg body weight. After
making up the solution in PBS this amounted to 10μl per gm body weight. Ten day old mice were chosen, as opposed to the 4 day old mice used by Marshall et al. (1991), because pilot studies had shown that the peritoneal cavity is so taut on the 4 day old mice that the injection fluid tended to leak out. Repeating the injection meant that the dose was imprecise, and the resulting increased handling substantially heightened the chance of the mouse being eaten by its mother.

The mice were killed by cervical dislocation 24 hours after injection. Their cranial bones were dissected out and then stripped of membranes in warmed (37°C) PBS under a dissecting microscope. The cranium was then divided into the parietal and occipital bones, and fixed in 95% ethanol in 5% glacial acetic acid for 10 minutes. Following fixation, the bones were washed briefly with 1mg ml⁻¹ bovine serum albumin in PBS (PBS-BSA). The bones were then incubated for 10 minutes with Sigma kit 386 to reveal TRAP-positive osteoclasts. Following a wash with PBS-BSA, the bones were re-fixed in 12.5% glutaraldehyde in 1M HCL for 5 minutes and then placed in 1M HCL for 30 minutes in order to fix the endogenous mouse IgG, as the second antibody was raised against mouse IgG. The bones were washed 3 times in PBS-BSA and a 1:100 dilution of non-immune sheep serum was then added for 30 minutes. Subsequently the bones were again washed 3 times in PBS-BSA and incubated overnight at 4°C with mouse monoclonal anti-BrdU at a 1:2 dilution. The bones were then washed four times in PBS-BSA and sheep anti-mouse Ig peroxidase conjugate at 1:100 dilution added for 1 hour at 37°C. The bones were again washed and peroxidase activity detected by incubation for 15 minutes at 37°C with 0.5mg ml⁻¹ diaminobenzidine, 0.006% hydrogen peroxide, 1mM nickel chloride and 1mM cobalt chloride in PBS. Afterwards, excess stain was washed off and the bones placed in a petri dish with water ready for microscopy. Control bones received no first antibody.
Actin staining.

Four adult Black-Tan mice and four 10 day old Black-Tan mice were used in this experiment. The mice were killed by cervical dislocation and the cranial bones dissected out and stripped of membranes in warmed (37°C) PBS under a dissecting microscope. The cranium was then divided into the parietal and occipital bones, and the bones fixed in warmed (37°C) 3% formaldehyde in PBS with 2% sucrose for 5 minutes. After a brief wash in PBS, they were permabilized in 1% Triton 1000 in PBS at 4°C and again washed in PBS. The bones were then incubated for 60 minutes at room temperature with TRITC-phalloidin at a concentration of 10μl/ml. Subsequently the TRITC-phalloidin was withdrawn and stored at 0°C for re-use. The bones were stored in water in a container wrapped in tin foil to prevent any light exposure of the stain. Control bones received no antibody to check for any innate autofluorescence. The microscope used was the one described previously.
Fig 5.1. Type I osteoclasts on the inner aspect of the parietal bone of adult Black-Tan mouse. Magnification x20.

Fig 5.2. Type II osteoclasts on the inner aspect of the parietal bone of adult Black-Tan mouse. Magnification x20.
Fig. 5.3. Type III osteoclasts on the inner aspect of the parietal bone of adult Black-Tan mouse. Magnification x20.

Fig. 5.4. Mixed population of all 3 types of osteoclasts on the inner aspect of the parietal bone of adult Black-Tan mouse. Magnification x20.
Results.

Light microscopy.

The numbers of nuclei of 1106 TRAP-positive rat cells were counted and their morphology recorded. TRAP-positive cells were present on the inner aspect of the calvaria of rats aged 2, 7, 14, 21, 56, 98 days, but no osteoclasts were detected at 28 days of age. The data is presented in Table 5.1.

No Type III osteoclasts such as those described in the mouse by Abe et al. (1990) were seen in the rat calvaria at any of the ages examined. The proportion of Type I (circumscribed) and Type II (extended) osteoclasts varied with age. The peak incidence of Type II cells, 12% of total cells, occurred at 14 days, and the lowest incidence at 56 days with no Type II cells being detected. The incidence at other ages as a percentage of total cells was: 2 days 8.5%, 7 days 8.44%, 21 days 2.32 %, and 98 days 6%. This data is illustrated in Figures 5.5-5.10.

The data for the numbers of nuclei in the osteoclasts and the morphological types were for the four adult Westray and one 14 day Black-Tan mice are presented in Tables 5.2 and 5.3.

In the adult mice, 48 % of uninuclear osteoclasts were classified as Type III. Type II was the most common type of cell overall, comprising 44.4 % of the total. Only 7.4% of the type III cells had more than 2 nuclei, and of the 2.6 % of cells with more than 4 nuclei none was a Type III cell. In contrast, the 14 day mouse had very few Type III cells (3% of the osteoclasts), and these had 1 or 2 nuclei only. Most (68%) of the uninuclear TRAP-positive cells were Type I, with Type I being the most abundant cell morphology. 17 % of the cells had more than 4 nuclei. This data is illustrated in Figures 5.11-.5.14.
5-Bromodeoxyuridine.

Neither in the 10 day old mice nor in the adult mice could any uptake of the 5-BrdU be detected. It was evident that the stain was working, as endothelial cells stained positively (Fig 5.15).

Actin staining.

The 10 day old mice showed both generalised actin staining as described in non-resorbing osteoclasts and also a regular broad F-actin ring like structures, or localised intense staining found in osteoclast engaged in resorption. There was a large proportion of the cells showing a broad F-actin ring (Figures 5.16-5.18). In contrast, the adult mice showed only broad F-actin rings, or localised intense staining in the Types I and II osteoclasts, whereas in the Type III osteoclasts a more generalised staining was detected (Figures 5.19-5.21).
Table 5.1. Nuclear distribution and morphology of rat osteoclasts.

<table>
<thead>
<tr>
<th></th>
<th>2d</th>
<th>7d</th>
<th>14d</th>
<th>21d</th>
<th>28d</th>
<th>56d</th>
<th>98d</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>0</td>
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</tr>
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<td>70</td>
<td>107</td>
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<td>93</td>
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<td>65</td>
<td>103</td>
<td>0</td>
<td>23</td>
<td>59</td>
</tr>
<tr>
<td>Type II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>2-5</td>
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<td>30</td>
<td>16</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>8</td>
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<tr>
<td>6+</td>
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<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 5.2 Nuclear distribution and morphology of the adult Westray mice osteoclasts.

<table>
<thead>
<tr>
<th>No of nuclei</th>
<th>No. osteoclasts</th>
<th>% of total osteoclasts</th>
<th>Classification of osteoclasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Type I</td>
</tr>
<tr>
<td>1</td>
<td>158</td>
<td>46.2</td>
<td>32</td>
</tr>
<tr>
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<td>96</td>
<td>28.1</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
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<td>9</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>11.1</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>1.2</td>
<td>2</td>
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</tbody>
</table>
Table 5.3. Nuclear distribution and morphology of the 14 day Black-Tan mouse.

<table>
<thead>
<tr>
<th>No. of nuclei</th>
<th>No. osteoclasts</th>
<th>% of total osteoclasts</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>5</td>
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</tr>
<tr>
<td>2</td>
<td>30</td>
<td>31.2</td>
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<td>9</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>10.4</td>
<td>7</td>
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<td>0</td>
</tr>
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<td>4</td>
<td>18</td>
<td>18.8</td>
<td>12</td>
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<tr>
<td>5</td>
<td>7</td>
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<td>7</td>
<td>0</td>
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</tr>
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<td>3</td>
<td>3.1</td>
<td>3</td>
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</table>
Fig. 5.5. The distribution of nuclei and morphology of osteoclasts on the inner aspect of 2 day old rat calvarial bones.
Fig. 5.6 The distribution of nuclei and morphology of osteoclasts on the inner aspect of 7 day old rat calvarial bones.
Fig. 5.7 The distribution of nuclei and morphology of osteoclasts on the inner aspect of 14 day old rat calvarial bones.
Fig. 5.8 The distribution of nuclei and morphology of osteoclasts on the inner aspect of 21 day old rat calvarial bones.
Fig. 5.9 The distribution of nuclei and morphology of osteoclasts on the inner aspect of 56 day old rat calvarial bones.
The distribution of nuclei and morphology of osteoclasts on the inner aspect of 98 day old rat calvarial bones.

**Fig 5.10**
Fig. 5.11 The distribution of nuclei in osteoclasts on the inner aspect of 14 day old mouse calvarial bones.
Fig. 5.12 The distribution of nuclei and morphology of osteoclasts on the inner aspect of 14 day old mouse calvarial bones.
Fig. 5.13 The distribution of nuclei in osteoclasts on the inner aspect of adult mice calvarial bones.
Fig. 5.14 The distribution of nuclei and morphology of osteoclasts on the inner aspect of adult mice calvarial bones.
Fig 5.15. Positive staining of endosteal cells with BrdU. Magnification x20

Fig 5.16. Fluorescence image of broad F-actin rings present on the inner surface of a 10 day old mouse calvaria. Magnification x20.
Fig 5.17. Fluorescence image of localised intense actin staining in an osteoclast present on the inner surface of a 10 day old mouse calvaria. Magnification x20.

Fig 5.18. Corresponding resorption lacunae. Magnification x20.
Fig 5.19. Fluorescence image of localised intense actin staining in Type I and II osteoclasts present on the inner surface of adult mouse calvaria. Magnification x20.

Fig 5.20. Fluorescence image of generalised diffuse actin staining in Type III osteoclasts present on the inner surface of adult mouse calvaria. Magnification x20.
Fig 5.21. Fluorescence image of generalised diffuse actin staining in Type III osteoclasts present on the inner surface of adult mouse calvaria. Magnification x20.
Discussion

Types and sizes of osteoclasts

This study of rat osteoclasts gives support to the hypothesis proposed in Chapter 4 of a resorption cycle with a waxing and waning of the numbers of nuclei and activity in osteoclasts. At 2 days, most cells were oligonuclear (2-5 nuclei) osteoclasts, suggesting a high rate of bone modelling as oligonuclear osteoclasts have been shown to have the highest rates of basal RNA (Zheng et al. 1991) and are the most functionally active resorbers in 19 day old chicks (chapter 1). At 14 days, a steady state in the resorption cycle seems to have been reached, with nearly equal numbers of oligonuclear osteoclasts and osteoclasts with more than 6 nuclei and also the highest proportion of Type II cells. A similar pattern emerges at 21 days. At 28 days no resorption was taking place as no osteoclasts were detectable. Later, at 56 days, a new period of modelling or of remodelling seems to be occurring, with no Type II cells being present and again a high proportion of oligonuclear osteoclasts. At 98 days, there again appears to be a slowing down with the appearance of Type II cells. Abe et al. (1983, 1990) described a similar 3 week cycle of osteoclasts in mice, with numerous Type I osteoclasts at 1-2 weeks of age, at 4 weeks of age a very scanty distribution of osteoclasts and in the adult mice a predominance of Type II and III osteoclasts. However, no Type III cells as described by Abe et al. (1990) were found in the rats. Although one can not rule out that either they were present at some other date than the ones that I examined, or that they occur in much smaller numbers in the rat and went undetected, it is possible that there is a true species difference and that they do not exist in the rat.

The mice osteoclasts demonstrate further a change in morphological type within resorption cycles. In the young mouse Type I cells predominated, being essentially uni or bi-nucleate. The morphology of the osteoclasts observed in this
study confirmed the work of Abe et al. (1990), and in addition demonstrated a change in the frequency of osteoclasts with differing numbers of nuclei. The patterns of resorption pits and osteoclasts in situ on the inner aspect of the adult Westray mice parietal bones were also studied, using scanning electron microscopy (Jones et al. 1994). The adult mice also had large numbers of uni and bi-nucleate osteoclasts but these were predominately Type III osteoclasts which Abe (1990) thought were weaker resorbers than the Type I cells, with Type II cells being an intermediary form. The predominance of Type III cells in the adult mice tends to suggest that they may be at the end of their life cycle, and the result of fission of other osteoclasts. This conclusion is compatible with observations made by Groessner-Schreiber et al. (1991) who noticed that chick osteoclasts formed in response to matrix from older rats (16 weeks) had a greater periphery, and Marshall et al. (1991) thought that the stellate mice osteoclasts were non-resorbing cells. In addition recent studies on isolated neonate rabbit osteoclasts have showed that osteoclasts with a more rounded morphology expressed higher levels of carbonic anhydrase II mRNA (CA-II mRNA) than spread osteoclasts with similar numbers of nuclei (Asotra et al. 1994).

These preliminary results should be extended in further work with more time points and animals so that a clearer pattern is obtained.

5-Bromodeoxyuridine.

The aim of the experiment labelling the acquisition of new nuclei by osteoclasts was to show whether Type III osteoclasts were a form of post-osteoclast or were newly differentiated osteoclasts prior to fusion with another cell. However, I observed no evidence of positive staining for BrdU in my osteoclasts either in the ten day old or adult mice. Marshall et al. (1991) reported that in their 4 day old mice only 7.9% of osteoclast nuclei stained positive for BrdU one day after injection, and in any of the labelled osteoclasts only one to
two nuclei were stained. Work carried out by Scheven et al. (1986) using $[^3\text{H}]$-thymidine to label the uptake of new nuclei found that in young mice uptake of $[^3\text{H}]$-thymidine could be detected, but in the older mice new nuclei did not show this pattern. They postulated that this was because the osteoclasts in the older mice were progressively more derived from post-mitotic unlabelled precursors. A study carried out by Tanaka et al. (1993) using a co-culture system of mouse cells and labelling with $[^3\text{H}]$-thymidine recorded that the labelling index of $[^3\text{H}]$-thymidine in the nuclei of TRAP-positive multinucleate cells was 51% if added on day 3 but dropped off rapidly to 7% if added on day 5.

The reasons that I may not have seen any staining in the osteoclasts may have been that the uptake of stain within the slightly older cell population was so low that it went undetected or that in the older mice and even in the ten day old mice the nuclei were being derived from post-mitotic precursors or other osteoclasts. It was not due to a failure in the staining technique, because other cell types showed positive staining. However, the results do not conflict with the concept that the Type III osteoclasts do not have recently acquired nuclei.

**Actin Staining.**

The pattern of actin staining seen in the mice corresponds well with previous studies carried out by other groups (Turksen et al. 1989, Lakkakorpi et al. 1991). Generalised diffuse cytoplasmic staining is associated with non-resorbing osteoclasts and a pattern of intense F-actin localised over a resorption lacunae (Turksen et al. 1989), or a broad regular F-actin ring, is associated with resorptive osteoclasts (Lakkakorpi et al. 1991). In the ten day old mice the observed pattern of diffuse cytoplasmic staining and broad F-actin rings is one that would be expected with an active resorption cycle. However, in the adult mouse broad F-actin rings were only seen in the Types I or II osteoclasts. Only diffuse staining was present in the Type III osteoclasts, suggesting that these cells may
well be a non-resorptive stage of the osteoclast life cycle and possibly a form of post-osteoclast.

This work clearly needs to be extended in order to prove whether the Type III osteoclasts are a form of post-osteoclast or not, and whether they have ruffled borders. In addition, studies on the morphological categories in human or primate osteoclasts should be conducted.
Chapter 6. A preliminary study in the use of phase-shifting interference microscopy applied to rat bone cells.
Introduction.

The measurement of osteoclast volume and spread area with relation to nuclear number was discussed in chapter 3. To overcome the disadvantage of fixing and drying the cells, an approach measuring the dry mass and the areas of live osteoclasts using interference microscopy was explored.

Phase-shifting interference microscopy is not new and has been used extensively in the study of cultured fibroblasts (Brown et al. 1989, Dunn and Zicha 1993) but not for the measurement of osteoclasts. The image obtained is a quantitative representation of the distribution of non-aqueous cellular material, and the cell periphery is easily determinable, unlike the case associated with visualising cell peripheries using phase-contrast video images (Boyde et al. 1994).

As phase-shifting interference microscopy allows images of live cells to be obtained, the changing areal distribution of mass can be followed over a period of time. and the movement, growth or shrinkage of the cell can be established.

Osteoclasts garnered from the bones of animals are part of a mixed population of other cell types and tissue debris. Phase-shifting interference microscopy has been used in the past predominately for the study of cells that have been grown on from plated cells because cells which touch one another and cell debris interfere with the measurements.

The aims of this study were to establish whether phase-shifting interference microscopy was a practical method for studying osteoclasts, to establish the correlations between mass and nuclear number and to compare the results from the same cells measured first by phase-shifting interference microscopy and then after fixation and freeze-drying using reflection confocal microscopy.
Materials and Methods.

Cell culture.

Osteoclasts were obtained from the long bones of 4 day old Sprague-Dawley rat pups in a similar manner to that described in chapter 1. Briefly, the long bones were excised, and their shafts, freed of periosteum, were chopped in 199 medium with Hanks salts supplemented with 10% heat-inactivated foetal bovine serum, 100 units ml$^{-1}$ Penicillin and 100 $\mu$ml$^{-1}$ Streptomycin, and 1mM glutamine. The change to the use of medium 199 was required because the cells were later placed in sealed chambers under the microscope, where the buffering system used by 199 medium was found to be the most successful in ensuring cell viability. The resultant cell suspension was seeded on to sterile 22mm square No. 2 glass coverslips which were placed in sterile plastic 35mm petri dishes. The cells were allowed to settle, and overlaid with the above medium after 15 minutes and cultured in 2.5% CO$_2$ at 37°C for 4 hours till the cells were well adherent and spread sufficiently to allow easy counting of the osteoclast nuclei. The coverslips were then washed gently in 199 medium to remove non-adherent cells and fragments of tissue debris which interfere with both the microinterferometry and later data analysis. Each coverslip was mounted onto a Helber Bacteria Counting Chamber (Z3 special unruled, Weber Scientific International Ltd) and sealed down using equal parts of beeswax, soft paraffin and paraffin wax, leaving a small bubble of air trapped in the medium to ensure efficient buffering.

Phase stepping interference microscopy and image recording.

The method used is described more fully by Dunn and Zicha (1994) and Zicha and Dunn (1994). Briefly, the culture temperature remained constant at 37°C because
the interference microscopy was carried out in a temperature-controlled room. The chamber and attached coverslip were placed under the 20x twin objective of the Horn type Transmitted Light-Interference Microscope (Leitz, Wetzlar, Germany) which was coupled to a TM-765 monochrome CCD camera (Pulnix Europe Ltd, Bassingstoke, UK). Phase-stepping was carried out using a stepper motor controlled by the host computer, and four digital images were acquired rapidly whilst the optical path of the microscope's reference beam was altered in quarter wavelength steps. Microscopic images were then digitised using a Magic frame grabber (Matrox, Swindon, UK). These images were recorded at 15 second intervals for 10 minutes and stored on hard disk. Fields of view containing osteoclasts not touching other cells were selected, and an area free from cells also had to be in the field of view, as the cell-free regions of the image act as a reference plane having a uniform dry mass density of zero.

Data analysis.

The initial data analysis was automatic and calculated the dry mass, spread area (im) and (x,y) co-ordinates of the mass centroid for each cell or cell cluster within each phase-stepped image of a sequence. The images of the bone cells were displayed on a computer screen as a movie sequence whilst I identified each osteoclast and any cell that collided with it during the recording. This was done by tracking, firstly, each osteoclast or stromal cell, then any colliding cells with a cursor placed within the cell boundary using a mouse control. The computer then constructed a table linking the objects that it had detected and measured to the identified cells. The final result was a list of all the cells, cell clusters and cell fragments in all the frames together with their time, mass, area and centroid data. The analysis of the data was the carried out using Mathematica software (Wolfram
Research Inc., Champaign, Illinois, USA). Data from cell clusters and fragments is rejected so that a cell colliding with an osteoclast at known points in time will not have its dry mass included in the dry mass of the osteoclast. This results in gaps in the graphs at the corresponding time (Fig. 6.1). Further, mean mass and mean area were computed using Mathematica. In all, data was available for 23 osteoclasts and 79 stromal (osteoblastic) cells.

Fixation and drying of the cells.

After the osteoclasts on the coverslips had been measured the wax seal was removed and the cells fixed for 24 hours in 2.5% glutaraldehyde in 0.15M cacodylate buffer (initially pre-warmed to 37°C). The cells were then freeze-dried and vacuum coated with gold as described in chapter 3.

Identifying the osteoclasts and measuring their cell volume.

Black and white images taken from the last frame of each 10 minute sequence were laser printed using Correldraw. This enabled subsequent identification of the freeze-dried and gold-coated osteoclasts (Fig. 6.2). Osteoclast volumes and areas (rcm) were measured in the same way as described in chapter 3.
Fig. 6.1. Graph obtained from Mathematica showing dry mass and area measurement.

Fig. 6.2. Picture from the last frame using Correldraw.
Results

Interferometry measurements.

The mean dry mass of the 23 living osteoclasts derived from 4 day old rats was 372pg; the mean number of nuclei per cell was 2.83. The mean mass of the 79 stromal cells in the same preparations was 66pg and the mean area 224μm². The means, medians and SEM.s for the measured parameters for the 23 osteoclasts are given in Table 6.1 and for the 79 stromal cells in Table 6.2. The correlation coefficients for the areas, dry mass and the numbers of nuclei were all high. The strongest correlation was between dry cell mass and area.

Mean data for the cells grouped according to nuclear number is given in Table 6.3. Larger cells had larger areas, volumes and mass. The mean dry mass per nucleus for the osteoclasts was 109.9pg. The scatterplot of mean mass per nucleus for cells with 2-7 nuclei suggests a possible trend for larger cells to have a disproportionately larger mass per nucleus (Fig 6.4) but there is not enough data as yet to confirm this. Mean and SD values for the groups of cells of 2 and 3 nuclei and the significant differences between the two groups are given in Table 6.4. There was a significant difference between the two groups with respect to mass, area(im), volume and area(rcm), but no significant difference between the groups with respect to mass, area, volume per nucleus.

Scatterplots and correlation coefficients are shown in Fig 6.4-6.6 for the 23 osteoclasts and in Fig 6.14 for the 79 stromal cells.

Freeze-dried measurements.

The means, medians and SEM.s for the same osteoclasts measured after freeze-drying are also given in Table 6.1. The correlation coefficients for the areas,
volumes and the numbers of nuclei were still high but were slightly weaker than the interferometry measurement. The strongest correlation was between cell volume and area. Scatterplots and correlation coefficients are shown in Fig 6.7-6.9.

Comparison of the two methods.

The mean and median values for osteoclast area measured using interferometry (square microns) were greater than those for area measured using the confocal microscope after freeze-drying the cells (square microns) Table 6.1. Although not directly comparable, the mean and median values for cell volume (cubic microns) were greater than those for mean dry mass (pg).

Regression lines comparing the two methods for area, dry mass and volume and the area:dry mass ratio and area:volume ratio are shown in Fig. 6.10-6.12. There was a significant difference between ratios of dry mass:area and volume:area p<0.001. There was no significant difference between the two different methods of measuring area. There was a good correlation between cell mass and cell volume and the scatterplots and correlation coefficient are shown in Fig. 6.13.
### Table 6.1. The sizes of the rat osteoclasts.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/ cell</td>
<td>2.83</td>
<td>2</td>
<td>0.279</td>
</tr>
<tr>
<td>Dry mass</td>
<td>372</td>
<td>223</td>
<td>101</td>
</tr>
<tr>
<td>Area (im)</td>
<td>1268</td>
<td>920</td>
<td>248</td>
</tr>
<tr>
<td>Volume</td>
<td>720</td>
<td>576</td>
<td>143</td>
</tr>
<tr>
<td>Area (rcm)</td>
<td>1171</td>
<td>888</td>
<td>228</td>
</tr>
</tbody>
</table>

Areas in μm², volumes in μm³, dry mass in pg.

im= phase-shifting interference microscopy.

rcm= reflection confocal microscopy.

### Table 6.2. The sizes of the rat stromal cells.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry mass</td>
<td>66.48</td>
<td>55.16</td>
<td>4.34</td>
</tr>
<tr>
<td>Area (im)</td>
<td>223.9</td>
<td>201.6</td>
<td>10.5</td>
</tr>
</tbody>
</table>
Table 6.3. Mean values grouped by nuclear number.

<table>
<thead>
<tr>
<th>NN</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>7</th>
</tr>
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<tbody>
<tr>
<td>n=</td>
<td>13</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mass</td>
<td>175.1</td>
<td>342</td>
<td>330.3</td>
<td>1288.9</td>
<td>2281.0</td>
</tr>
<tr>
<td>Area (im)</td>
<td>644.4</td>
<td>1353</td>
<td>1809</td>
<td>3350.8</td>
<td>5509.4</td>
</tr>
<tr>
<td>Volume</td>
<td>413.3</td>
<td>786.8</td>
<td>928</td>
<td>1018</td>
<td>3602</td>
</tr>
<tr>
<td>Area (rcm)</td>
<td>611.5</td>
<td>1222</td>
<td>1729</td>
<td>2999</td>
<td>5191</td>
</tr>
</tbody>
</table>
Table 6.4. Mean values of osteoclasts with 2 or 3 nuclei.

<table>
<thead>
<tr>
<th></th>
<th>nn=2</th>
<th>nn=3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mass</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>175.1</td>
<td>342</td>
</tr>
<tr>
<td>SD</td>
<td>71.51</td>
<td>139.91</td>
</tr>
<tr>
<td>Sig.</td>
<td>p=0.003</td>
<td>p=ns</td>
</tr>
<tr>
<td>Mass/nuc</td>
<td>87.53</td>
<td>114.0</td>
</tr>
<tr>
<td>SD</td>
<td>35.8</td>
<td>46.64</td>
</tr>
<tr>
<td>Sig.</td>
<td>p=ns</td>
<td>p=ns</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Area (im)</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>nn=2</td>
<td>644.4</td>
<td>1353.2</td>
</tr>
<tr>
<td>Area/nuc</td>
<td>322.2</td>
<td>451.1</td>
</tr>
<tr>
<td>SD</td>
<td>297.9</td>
<td>397.4</td>
</tr>
<tr>
<td>Sig.</td>
<td>p=0.000</td>
<td>p=ns</td>
</tr>
<tr>
<td>nn=3</td>
<td>1353.2</td>
<td>451.1</td>
</tr>
<tr>
<td>Vol</td>
<td>413.3</td>
<td>786.8</td>
</tr>
<tr>
<td>Vol/nuc</td>
<td>206.7</td>
<td>262.3</td>
</tr>
<tr>
<td>SD</td>
<td>170.9</td>
<td>230.3</td>
</tr>
<tr>
<td>Sig.</td>
<td>p=0.001</td>
<td>p=ns</td>
</tr>
<tr>
<td>nn=3</td>
<td>786.8</td>
<td>262.3</td>
</tr>
<tr>
<td>Area (rcm)</td>
<td>611.5</td>
<td>1222.0</td>
</tr>
<tr>
<td>Area/nuc</td>
<td>305.8</td>
<td>407.3</td>
</tr>
<tr>
<td>SD</td>
<td>299.2</td>
<td>308.4</td>
</tr>
<tr>
<td>Sig.</td>
<td>p=0.001</td>
<td>p=ns</td>
</tr>
<tr>
<td>nn=3</td>
<td>1222.0</td>
<td>407.3</td>
</tr>
<tr>
<td>Vol</td>
<td>85.5</td>
<td>76.8</td>
</tr>
<tr>
<td>Vol/nuc</td>
<td>5.7</td>
<td>76.8</td>
</tr>
<tr>
<td>SD</td>
<td>85.5</td>
<td>76.8</td>
</tr>
<tr>
<td>Sig.</td>
<td>p=ns</td>
<td>p=ns</td>
</tr>
<tr>
<td>13 cells in nn=2, and 6 cells in nn=3 groups.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 6.3 Mean mass per nucleus.
Fig. 6.4. Scattergram and correlation coefficient for the relationship between nuclear number and dry mass for the rat osteoclasts.
Fig. 6.5. Scattergram and correlation coefficient for the relationship between nuclear number and area (im) for the rat osteoclasts.
Fig. 6.6. Scattergram and correlation coefficient for the relationship between dry mass and area (μm) of the rat osteoclasts.
Fig. 6.7. Scattergram and correlation coefficient for the relationship between nuclear number and volume for the rat osteoclasts.
Fig. 6.8. Scattergram and correlation coefficient for the relationship between nuclear number and area (rcm) for the rat osteoclasts.
Fig. 6.9. Scattergram and correlation coefficient for the relationship between cell volume and area (rcm) of the rat osteoclasts.
Fig. 6.10. A comparison of the regression lines for nuclear number and volume or dry mass for the same rat osteoclasts.
Fig. 6.11. A comparison of the regression lines for nuclear number and area for the same osteoclasts.
Fig. 6.12. A comparison of the regression lines for the relationship of cell volume or dry mass and area for the same rat osteoclasts.
Fig. 6.13. Scattergram and correlation coefficient for the relationship between cell volume and dry mass of the rat osteoclasts.
Fig. 6.14 Scattergram and correlation coefficient for the relationship between dry mass and area (im) of the rat stromal cells.
Discussion.

The benefits of measuring a live cell, where fixation has not caused shrinkage or swelling of the cell, are obvious. Interference microscopy is well suited to measuring 'plated cells' such as fibroblasts or osteoblasts where culture conditions can be kept constant for weeks, and cells are 'clean' and not affected by debris. However, osteoclasts obtained from bones are in different stages of their life cycle, which may affect dry mass. In addition, cultures onto glass coverslips are contaminated both with other cells and tissue debris which interfere with the dry mass measurements. The small chamber area of the Helber slide reduces the probability of finding osteoclasts which have been seeded onto the coverslip because this has to be fixed down to the chamber. The number of available osteoclasts to measure is, therefore, low once one has ruled out any cells that have debris lying on top of them. Once the cells are obtained, the actual measurement is relatively easy although a lot of data processing is required.

The sizes of the rat osteoclasts measured in this study were small as compared to a similar population described in chapter 3. This may be because larger cells were selectively removed during the more vigorous washing required to dislodge as much debris as possible, or because the probability of encountering larger osteoclasts in a small chamber area was low.

The significant difference between the groups with 2 and 3 nuclei in all the measured parameters confirms my earlier observations that larger osteoclasts have larger areas, volumes and mass. The trend for an increase in mass per nucleus with increasing nuclear number may indicate an advantage of larger size, although the data sets at present are too small to confirm this. This advantage might result from the acquisition of 'younger' cytoplasm containing cytoplasmic regulators that delay
programmed cell death, with the nuclear number merely acting as a marker of cell fusion (Jacobson et al. 1994).

This is the first time to my knowledge that anyone has measured non-aqueous material in living osteoclasts and correlated the dry mass to nuclear number. The correlation between nuclear number and dry mass was very high, as was the relationship with cell area. Dry mass:area analysis likewise produced very high correlations, similar to those described previously for volume:area. Osteoclasts when compared to stromal cells in culture had a greater dry mass and area when the nuclear number of the osteoclasts was greater than 2.

There was no significant difference between the areas of the same osteoclasts live or fixed and freeze-dried, supporting my earlier contention that cell area does not change as much as volume on drying, due to the adhesion of the cell to the underlying substratum. The observation that freeze-dried osteoclasts often fracture through the centre of the cell, leaving the periphery unaltered, lends additional support to this observation. There was a significant difference between the dry mass and volume although both were closely correlated. One would expect that if dry mass measured all the non-aqueous mass and freeze-drying had removed all the water, that these values would tend to be closer together. It is possible that the difference between the two groups resulted from the dry mass measurements being marginally inaccurate for the very low mass areas of the cell (e.g. at the periphery) or the freeze-dried cells being slightly rehydrated prior to measuring the volume, or both.

This method of analysis was excellent for showing the patterns of movement of the osteoclasts, and illustrating the way in which the dry mass distribution changed as the cell outline altered. It confirmed the relative stability of the perinuclear zone in osteoclasts (Jones et al. 1995).
Conclusions
This work has shown for the first time that the volume of calcified tissue that an osteoclast resorbs in vitro is directly proportional to its size, measured as the number of nuclei it possesses, its cell volume or its dry cell mass. The coefficients of correlation between these three parameters are all high for one cell population. Larger osteoclasts resorb more calcified tissue than smaller ones but may be less efficient resorbers with increasing nuclear number. Rat osteoclasts isolated from bones were demonstrably larger than chick osteoclasts of comparable number of nuclei and had a mean dry mass per nucleus of 109.9 pg. There was a trend in the small sample studied for mass to increase disproportionately and this might confer a functional advantage to larger osteoclasts.

There was no evidence to suggest that 3-amino-1-hydroxypropylidene-1,1-bisphosphonate affected one cell size differently from another in vitro. However, the presence of the bisphosphonate in the medium diminished the sizes of resorption pits in a dose-dependent manner, and decreased freeze-dried cell volume, but not cell area, suggesting that the 3-amino-1-hydroxypropylidene-1,1-bisphosphonate may act directly on the osteoclast.

My studies indicated that the resorption cycle of mice and rat osteoclasts spans 3-4 weeks, with a changing population of both numbers of nuclei and morphology of osteoclasts. Rapidly growing deer antler was shown to have a high proportion of oligonuclear osteoclasts, consistent with active osteoclastogenesis.
Discussion
The primary aim of the work embodied in this thesis was to examine whether the ability of multinucleate osteoclasts to resorb bone was altered by the acquisition of more nuclei. Prior to these studies, although it had always been thought that larger osteoclasts must be able to destroy more tissue, the only evidence for this assumption was circumstantial. Routine histological sections of bone and other calcified tissues provide ample examples of large osteoclasts residing in large resorption lacunae, and small ones abutting small pits. Empirically then, it was thought that the larger cells which result from the fusion of smaller ones could remove bone more rapidly. However, the rate of resorption had not been investigated, and until in vitro assays for the resorptive function of individual osteoclasts were devised in the early 1980s, this was not feasible. Moreover, whether the rate of resorption increased or decreased disproportionally when osteoclasts attained greater size was generally disregarded, probably because it was impossible to measure.

The first chapter in this thesis examines the importance of multinuclearity in osteoclasts from one cell population, avian osteoclasts derived from chick long bones. It demonstrates that whilst larger osteoclasts resorb more calcified tissue than smaller ones they are less efficient per nucleus in doing so. This finding correlates well with the work of Zheng et al. (1991) who showed that oligonuclear osteoclasts had higher rates of mRNA synthesis and were thus functionally likely to be more active.

However this finding was not demonstrable in further experiments where avian osteoclasts were untreated or treated with APD, or in experiments investigating the function of deer osteoclasts in vitro. In both these experimental sets the numbers of nuclei were more closely grouped, there being fewer large osteoclasts. Potentially the effect is only demonstrable in a 24 hour culture period if there are large numbers of cells at either end of the size scale. In the original experiments the range was 2-27 nuclei per osteoclast, and the larger cells may
have contained more older and perhaps effete nuclei. Further work looking specifically at this aspect would be required to establish whether the initial finding is true for all osteoclast populations. Nevertheless, it was never the case that more nuclei increased the rate of resorption per nucleus. And, in the experiments using APD, there was no evidence that larger osteoclasts were more (or less) resistant to the effects of the drug than smaller ones.

Of course, larger osteoclasts may have a functional advantage by virtue of their increased size enabling them to have a greater sphere of influence (Jones et al. 1994) or by having a disproportionately increased cell mass. For this reason, I attempted to measure the dry cell mass of living rat osteoclasts in which the numbers of nuclei were known. The discovery of a trend for the larger cells (those with more nuclei) to have a proportionately greater mass is most intriguing. However, the technique of phase-shifting interference microscopy is new for osteoclasts and the technical problems encountered seemed to select preferentially for osteoclasts with smaller numbers of nuclei. It is important that this work is repeated with further experimental sets in order to increase the numbers of osteoclasts measured and the range of sizes, and to investigate the cells of other species. If verified in further short term cultures, a larger cell mass might indicate a more productive enzyme synthesis or greater resorptive capacity or indicate an increase in cytoplasmic factors which delay programmed cell death (Jacobson et al. 1994).

Chapter 2 demonstrated that the volume of dentine resorbed by cultured chick osteoclasts decreased with increasing concentrations of the bisphosphonate APD in the medium and that pre-treatment of the dentine slice with APD produced the most profound effect, possibly because of a degenerative effect on the osteoclasts as postulated by Marshall et al. (1990) and Flanagan et al. (1991). This pattern has also be shown with other types of bisphosphonate (Selander et al. 1994).
There has been dispute in the literature as to whether the action of the bisphosphonates is mediated solely through the calcified tissue to which it becomes adsorbed, and from which it is released when the osteoclasts resorb the tissue, or whether it can act directly on bone cells. One approach to this problem is to determine whether a reduction occurs in the cell volumes and volume:area ratios of osteoclasts cultured on glass and exposed to APD at a concentration known to affect their resorption of bony tissues. Therefore, freeze-dried osteoclasts, initially cultured on glass in the presence or absence of APD, were measured using a video-rate, line-confocal laser scanning microscope. The results obtained indicate that part of the effect of bisphosphonates may be directly on the osteoclast, since there was a reduction in cell volume per nucleus. This evidence supports the hypothesis of Carano et al. (1990) that bisphosphonates may be acting as metabolic inhibitors to reduce protein synthesis.

However, the reduction in the volume of rat osteoclasts cultured on glass in the presence of the APD was found to be less than the reduction in the volume of tissue resorbed by chick osteoclasts cultures under similar conditions. This of course may be merely a species difference. Freeze-dried rat osteoclasts were shown to be larger than chick osteoclasts containing the same number of nuclei although previous studies have shown that within the same culture period and under identical culture conditions they make smaller pits (Jones et al. 1986), perhaps partly owing to a later onset of resorption. These findings, however, necessitate a comparable resorption study of the affects of APD in the medium to be carried out using rat osteoclasts.

The population of deer osteoclasts obtained from growing antler was found to contain a very high proportion of both mononuclear and oligonuclear (2-5 nuclei) osteoclasts. This finding, in the light of the work from chapter 1 and the work carried out by Zheng et al. (1991), suggests that these osteoclasts are from a very active site of resorption. A hypothesis is proposed that the distribution of
nuclei in a population of osteoclasts indicates the relative state of activity of resorption at that site. Further work looking at the distribution of osteoclasts from different ages of developing antler and other bones is needed to determine the population dynamics. This should be correlated with mRNA levels in the same population of osteoclasts.

One site which can be monitored relatively easily with its population of osteoclasts maintained \textit{in situ as in vivo} is the endocranial aspect of the parietal or frontal bone. A study was therefore conducted to determine the sizes and shapes of osteoclasts present on the endocranial surface of known ages of rats and mice. Rat osteoclasts appeared to have a 3 week cycle with two distinct morphological types of osteoclast present—the types I and II osteoclast as described by Abe et al. (1983, 1990). At 28 days no osteoclasts were seen in either of the rats studied and this finding merits further investigation with larger numbers of animals at each time point. Mouse osteoclasts also exhibited a similar pattern but in addition Type III osteoclasts were noted predominately on the endocranial surfaces from older animals. These cells are postulated to be a form of post-osteoclast. Experiments using stains labelling for the presence of actin in the osteoclasts on the endocranial surfaces of the mice cranium showed patterns of actin staining in the type III osteoclasts consistent with that expected for non resorbing cells. This work clearly needs to be extended in order to prove whether these cells are post-osteoclasts or not, methods for determining their mRNA levels would be most useful in this situation.

Thus the work encompassed in this thesis has addressed some aspects of osteoclast physiology for the first time and suggested several avenues of further investigation that should elucidate the rationale for multinuclearity in osteoclasts.
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Appendix 1.

Publications and abstracts arising from the work presented in this thesis.

Abstracts.

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Publications.

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Piper K, Boyde A, Jones SJ (1992)  

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Volumes of chick and rat osteoclasts cultured on glass. Calcif Tiss Int (accepted Nov 1994)
The relationship between the number of nuclei of an osteoclast and its resorptive capability in vitro

Kim Piper, Alan Boyde, and Sheila J. Jones

Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, U.K.

Accepted June 15, 1992

Summary. This study examined the relationship between the number of nuclei in an osteoclast and its resorptive efficiency, as demonstrated by the size of the pit it can make in a mineralized tissue in 24 h in vitro. Osteoclasts released mechanically from prehatch chick long bones were cultured on dentine slices or on plastic dishes for periods of 6 or 24 h. The frequency distribution of the multinucleate tartrate-resistant acid phosphatase (TRAP)-positive cells with different numbers of nuclei was determined: the mean number of nuclei per cell was 6.92, with a mode of 4.47% had 5 or fewer nuclei and only 11% more than 10 nuclei. The pits associated with 292 osteoclasts with known numbers of nuclei were measured using a confocal laser light microscope (Lasertec) and dedicated image analysis system, and depths, plan areas and volumes determined. There was a positive correlation between the number of nuclei per osteoclast and the volume of the pit made, but a trend for the volume resorbed per nucleus to decrease with increase in the number of nuclei per osteoclast.

Key words: Osteoclast – Nuclei – Resorption – Pit size – In vitro

Introduction

The volume of an osteoclast varies throughout its life history, and may both increase and decrease. There are no published data giving values for this crucial parameter although one would expect intuitively that there must be some functional advantage gained by the cell by virtue of added size. At the very least, cell size may give some indication of resorptive fervour at a particular site or time, or in response to local or humoral factors (Holtrop et al. 1979), including matrix components (Groessner-Schreiber et al. 1991). This is recognised by investigators’ descriptive comments on the sizes of osteoclasts seen on the bone surface in various pathological (Kukita et al. 1990) or experimental conditions (Katoh et al. 1991), although a negative relationship may also be present (Shapiro et al. 1988).

Large cells are associated with large or small pits and small cells with small pits. Of course, large pits may develop from small ones by maintaining the same form but growing in proportion; or grow by changing form radically, beginning with an extensive plan area and deepening to a greater relative amount; or they may vacillate between different patterns of growth (Jones and Boyde 1988). As osteoclasts are motile cells, and can move whilst resorbing, the sculpting of the surface by an osteoclast is a resultant of its lateral translation across the mineralised tissue and its ability to secrete protons (Baron et al. 1985) and degradative enzymes into the ruffled border zone: movement during formation therefore changes the form of pits. Motile but non-resorptive phases may intervene; and when resorption starts, or restarts, round pits are initially favoured. The best geometric measure of the work done by an osteoclast must, therefore, be the volume of tissue it has destroyed (Boyde and Jones 1987, 1991, 1992).

An osteoclast is a cell that may be uninuclear but is most commonly recognised at the bone or tooth surface in a multinuclear state. Increase in the size of an osteoclast, beyond a certain but unknown amount, is a consequence of cell fusion, and there is a turnover in the nuclei (Kembrer 1960; Young 1962; Zambonin Zallone and Teti 1981; Ries et al. 1987). The number of nuclei that are present in an osteoclast is often taken as an indication of total cell size. However, it is not known whether multinucletarity or the act of cell fusion confers some special ability on the cell, such as is reported for multinucleated macrophages (Vignery et al. 1991), in addition to the effect of increase in size alone; nor, if this were so, whether the benefit accrues with each fusion or in relation to the numbers of nuclei present. An increase in nuclear number could also be detrimental to resorptive efficiency, perhaps beyond a critical cell size or youth of component nuclei, or as a result
of a reduction in RNA synthesis by some nuclei (Zheng et al. 1991).

An indirect measure of resorptive ability might be the ratio of the total volume of the cytoplasm to that of the nuclei, or the relation between the enzyme content of the cell and the numbers of nuclei. These cell features are, however, promissory, and could be misleading (Umita et al. 1991); proof of the pudding is in the eating.

The purpose of this study was to measure the volume of the pits made by osteoclasts of known nuclear number under standardized conditions in vitro in order to discover whether cell fusion resulted in increased overall resorptive activity. For example, is an osteoclast with seven nuclei more, or less, efficient at the removal of a calcified tissue than two cells with a combined total of seven nuclei?

Materials and methods

Culture of chick osteoclasts. Long bones were removed from 19-day prehatch chicks, the cartilaginous epiphyses cut off, and the bones chopped in phosphate-buffered saline (PBS) with added 10% fetal calf serum (FCS). The release of osteoclasts from the bones was aided by flushing the fragments up and down a plastic pipette. The milky cell suspension was then seeded on to rectangular slices of sperm whale dentine, which measured 5 mm x 5 mm, and had one clipped corner in order to make relocation of the pits easier.

The cells were allowed to settle for 45 minutes at 37°C. Each chick provided four aliquots for seeding, and a total of 21 chicks were used in two experimental sets for measuring the resorption pits.

The nuclei of all the multinucleate cells that were TRAP-positive were counted in the 6- and 24-h cultures on plastic, and parallel cultures on plastic or dentine. The nuclei of osteoclasts with different numbers of nuclei, in 6 h (on plastic) and 24 h (on plastic or dentine) were included in the study. The plan shape of the osteoclast and its pit were drawn to assist in the identification of the pit after the cells had been removed. This set included 292 osteoclasts and pits on 75 dentine slices.

The nuclei of the multinucleate cells that were TRAP-positive were counted in the 6- and 24-h cultures on plastic, and parallel 24-h cultures on dentine. Nuclear counts were made of 227 osteoclasts at 6 h (on plastic) and 211 cells at 24 h (on plastic or dentine) in this set. Including the 292 osteoclasts above, the nuclei of a total of 730 osteoclasts were counted in this study.

The cells were then removed from the dentine by washing with a mild detergent, 1% chlorohexidine gluconate, pH 7, followed by immersion for 30 min in 30% H2O2, and the 292 pits identified again using the graticule and chart.

Measurement of the pits. The method which we used (demonstrated at the Davos Bone and Cartilage Cells and Cytokines meeting, Jan. 1992) is based upon a video rate, line confocal scanning light microscope (CSLM) manufactured by Lasertec Corporation, Japan. The ILM 11 instrument was developed for the semiconductor industry market. A He-Ne laser beam (λ = 633 nm) is broadened to a line using a cylindrical lens, which is scanned down the specimen field at TV frame rate. The signal corresponding to each point in the line is directed to a single detection element in a linear diode array. Thus images are acquired at full standard TV rate. The microscope and its special Z-axis controller provide a hard-wired solution to the problem of acquiring the max and the map images during multiple plane acquisition. Images derived from 256 planes are acquired in approximately 10 secs: an improvement in time and acquisition that allows us to achieve a better confocal Z resolution – a practical match to the SEM photogrammetric method at ten times the speed.

Volume data from the map image was acquired by tracing (with a mouse and a screen cursor) a line (one pixel wide) around the pit to be measured. Software, written by SIS Münster-West, then found the volume enclosed within that area, deriving the height at which the surrounding, reference surface was found from the values under the trace binary. The area of the pit was derived from a second screen cursor trace placed close to the edge of the pit. The depth recorded was the maximum depth of the pit. The mean depth was determined from computing volume/area. Analysis of the data was performed using Minitab statistical software (Minitab, 1989).

Results

The total osteoclast population

The numbers of nuclei present in the osteoclasts harvested by the standard procedure from the chick long bones and seeded on to plastic culture dishes or on to dentine for periods of 6 and 24 h are shown in the histograms (Fig. 1). All the osteoclasts in these cultures were

<table>
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<tr>
<th>Nuclei/chick osteoclast</th>
<th>Frequency %</th>
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Fig 1. Histogram showing the relative frequencies of the chick osteoclasts with different numbers of nuclei, in 6 h (on plastic) and 24 h cultures (on dentine or plastic), excluding those dentine samples used for pit measurements.
Table 1. Number of nuclei in chick osteoclasts

<table>
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<tr>
<th>Culture Substratum</th>
<th>Mean</th>
<th>SD</th>
<th>Max. nuclei</th>
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<tbody>
<tr>
<td>6 h Plastic</td>
<td>6.20</td>
<td>3.47</td>
<td>18</td>
</tr>
<tr>
<td>24 h Plastic</td>
<td>7.29</td>
<td>5.03</td>
<td>24</td>
</tr>
<tr>
<td>24 h Dentine</td>
<td>6.11</td>
<td>3.69</td>
<td>20</td>
</tr>
<tr>
<td>24 h Plastic/dentine</td>
<td>6.88</td>
<td>4.64</td>
<td>24</td>
</tr>
<tr>
<td>24 h Dentine</td>
<td>7.51</td>
<td>4.91</td>
<td>27</td>
</tr>
<tr>
<td>24 h Plastic/dentine</td>
<td>6.92</td>
<td>4.54</td>
<td>27</td>
</tr>
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</table>

pooled. Figure 2 is a histogram of the distribution of nuclei for all the osteoclasts in the study. The frequency % and cumulative frequency histograms for the total number of osteoclasts (730) are shown in Fig. 3. Only 11% of the cells had 10 or more nuclei and 47% had five or fewer nuclei.

There was a significant difference (P = 0.0004) between the numbers of nuclei/osteoclast in the 6-h cultures on plastic and the 292 cells associated with a pit at 24 h. This was because there were more very large cells in the second group, and a slightly lower frequency of cells with very low numbers of nuclei with a pit. However, the type of substratum did not appear to influence the frequency of attachment of cells of different nuclear number.

**The nuclei/cell in the population of the osteoclasts with measured pits**

Figure 4 shows the distribution of the cells according to the number of their nuclei for the 292 osteoclasts related to a measured pit. The number of nuclei per cell ranged from 2 to 27, with a mean of 7.514 and a median of 6. 44% of the multinucleate osteoclasts had 5 or fewer nuclei, and 81% had 10 or fewer nuclei. Only 11% had 15 or more nuclei.

**The sizes of the pits and the nuclei per cell**

The depths, areas and volumes of the pits measured are shown in the histograms of Fig. 4, and the means and medians of the maximum depths, mean depths, areas, and volumes in Table 2.

The correlation matrix for nuclei/cell, pit depths, areas and volumes is shown in Table 3. Whether considered as the total measured or in the smaller groups of the two individual experiments, the correlation between the number of nuclei/cell and the area of the matching pit was always greater than that between nuclei and volume, although both showed a positive correlation. The correlation between the number of nuclei per cell and...
the depth of its pit was always weakest, even when the 14 most dubious associations were excluded from the analysis (Table 3). As we have found previously, the correlation between the volume and the area of a pit formed under standard conditions and over short culture periods is high. As the size of the cells (as reckoned from their number of nuclei) increased, the correlation between size and volume within the group increased. For example, grouping the cells into three sets of those with 2–5 nuclei, 6–10 nuclei, or more than 10 nuclei, gave correlations of number of nuclei/cell with volume of associated pit, within the group, of 0.008, 0.049 and 0.347. This reflected the much greater diversity of size in the third group, comprising 19% of the total number.

Six random samples of 25 cell-pits from the total of 292 gave correlations between nuclei/cell and pit volume with a range of −0.108 to 0.751, and for nuclei/cell and pit area with a range of 0.060 to 0.847, showing the necessity for a large sample size. Only the correlations between pit area and volume were consistently high ($r = 0.599$ to 0.916).

When the cells were grouped with regard for their size (number of nuclei) and the pits for their size (volume), the trend was obvious. Most small pits were made by small osteoclasts and larger osteoclasts were more frequently associated with larger pits (Fig. 5). The mean volume resorbed/nucleus/cell was 692 μm$^3$. There were weak negative correlations between the number of nuclei in a cell and the volume or plan area resorbed per nucleus, but a significant decrease in the depth of resorption per nucleus. Thus an increase in the number of nuclei did not appear to increase an osteoclast’s resorptive activity per nucleus but to decrease it.
Table 3

<table>
<thead>
<tr>
<th>Nuclei/cell</th>
<th>Volume (cu.microns)</th>
<th>Area (cu.microns/1000)</th>
<th>Max. depth (microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation matrix for the 292 cells and pits</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>0.419</td>
<td>0.801</td>
<td>0.324</td>
</tr>
<tr>
<td>Area</td>
<td>0.530</td>
<td>0.538</td>
<td>0.138</td>
</tr>
<tr>
<td>Max. depth</td>
<td>0.227</td>
<td>0.613</td>
<td>0.577</td>
</tr>
<tr>
<td>Correlation matrix for the 175 cells and pits of Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>0.371</td>
<td>0.881</td>
<td>0.391</td>
</tr>
<tr>
<td>Area</td>
<td>0.405</td>
<td>0.571</td>
<td>0.153</td>
</tr>
<tr>
<td>Correlation matrix for the 117 cells and pits of Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>0.482</td>
<td>0.623</td>
<td>0.269</td>
</tr>
<tr>
<td>Area</td>
<td>0.737</td>
<td>0.438</td>
<td>0.808</td>
</tr>
<tr>
<td>Max. depth</td>
<td>0.264</td>
<td>0.502</td>
<td>0.152</td>
</tr>
<tr>
<td>Mean depth</td>
<td>0.107</td>
<td>0.475</td>
<td>0.502</td>
</tr>
</tbody>
</table>

*14 cells, with <10 nuclei but associated with pits of >10000 μm² omitted

Correlation coefficients for the 292 osteoclasts and pit size/nucleus

<table>
<thead>
<tr>
<th>No. of nuclei/osteoclast</th>
<th>Volume/nucleus</th>
<th>Area/nucleus</th>
<th>Max. depth/nucleus</th>
<th>Mean depth/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume/nucleus</td>
<td>-0.119</td>
<td>-0.181</td>
<td>-0.589*</td>
<td>-0.564*</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max. depth/nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean depth/nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P<0.0001

Fig 6. The mean pit volumes and the volume/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the pit volume to increase as the number of nuclei/osteoclast increases, but for the volume of tissue resorbed per nucleus to decrease.

Fig 7. The mean pit areas and the area/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the plan area of the pit to increase as the number of nuclei/osteoclast increases, but for the plan area of tissue resorbed per nucleus to decrease.

Fig 8. The mean pit maximum depth and the maximum depth/nucleus for the cells grouped by the numbers of nuclei they contain. There is a trend for the maximum depth to increase as the number of nuclei/osteoclast increases, but for the depth resorbed per nucleus to decrease.
Efficiency of resorption related to number of nuclei per osteoclast

The depths, areas, and volumes resorbed per cell and per nucleus were assessed further by pooling groups of osteoclasts according to their number of nuclei, first in 27 sets (Figs. 6-9) and then in four larger sets (Figs. 10-13; Table 4). There was a clear trend for the depth, area, and volume of the pit to increase with an increase in the number of nuclei, but for the depth, area and volume resorbed per nucleus to decrease with increasing numbers of nuclei per cell.

When analysed in four sets so that there was an adequate number for statistical analysis in each group, the volume resorbed per nucleus and the area resorbed per nucleus were significantly less when the smallest (less than 5 nu-

Table 4. Work accomplished by the chick osteoclasts, grouped into four sets by their number of nuclei

<table>
<thead>
<tr>
<th>Nuclei</th>
<th>Cells</th>
<th>Mean vol.</th>
<th>SEM</th>
<th>Mean vol./nucleus</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-4</td>
<td>92</td>
<td>2577</td>
<td>378</td>
<td>984</td>
<td>142</td>
</tr>
<tr>
<td>5-7</td>
<td>89</td>
<td>3895</td>
<td>329</td>
<td>657</td>
<td>51</td>
</tr>
<tr>
<td>8-10</td>
<td>55</td>
<td>5375</td>
<td>986</td>
<td>597</td>
<td>117</td>
</tr>
<tr>
<td>11+</td>
<td>56</td>
<td>8849</td>
<td>1250</td>
<td>543</td>
<td>75</td>
</tr>
</tbody>
</table>
nuclei) and largest (more than 10 nuclei) osteoclasts were compared (Figs. 10, 11). All sets were significantly different for the volumes and areas of the pits, except for the difference between the two middle groups. However each of these was significantly different from all other groups. There was no significant difference between the steps for depths (apart from the final group of maximum depths reaching significance), but each step was significantly different from the next as regards the depth/nucleus (Figs. 12, 13). The significance levels for each step are given in the captions to Figs. 10-13.

Discussion

Sizes of osteoclasts

The relative frequencies of osteoclasts with a given number of nuclei have been determined previously for a number of species. Finding the number of nuclei in such an irregular and often large cell efficiently requires it to be kept whole during the count rather than sectioned, especially as many cells need to be counted. Addison (1980), for example, used bone imprinting to obtain whole osteoclasts from kittens, and Jones et al. (1986) counted the nuclei in osteoclasts dispersed from chick, rat and rabbit bones by mechanically agitating bone fragments in fluid until the osteoclasts became dislodged, then allowing them to settle on to a dish or tissue slab. Ries and Gong (1982) studied rat osteoclasts obtained from periosteal smears, and Helfey and Stern (1982) isolated fetal rat osteoclasts by enzymatic digestion. All these methods rely on the released osteoclasts being representative of the whole population.

Remarkably, the relative frequencies of the numbers of nuclei in osteoclasts of different animals are quite similar, and not very different from counts of nuclei from human or kitten odontoclasts (Addison 1978). By counting very large numbers of cells, Addison (1980) was able to find a difference between the cumulative frequencies for nuclei counts in osteoclasts in control animals and those treated for 6 h with parathyroid extract, but the shift in the curve was small. This suggests that the number of nuclei in an osteoclast is normally controlled either by the life span of the constituent nuclei or of the cell for, although osteoclasts may reach a gigantic size, the great majority (81% in our present study; 80% of human odontoclasts in Addison’s) have ten or fewer nuclei. The addition of extra nuclei does appear to parallel activation of the cells by, for example, PTH (Holtrop et al. 1979). This suggests that cell fusion may enhance resorptive activity, and not merely accompany it. The supply of preosteoclasts will affect the renewal rate of nuclei in osteoclasts, and the frequency distribution of the cells with different numbers of nuclei. It may also be that the addition of new nuclei acts as a survival factor, preventing the suicide of the cell (Raff 1992), but that there is a limit to the number of times this normally operates. Pagetic osteoclasts appear to disregard these restraints, whether produced in vivo (Basle et al. 1988) or formed in long-term cultures of Pagetic marrow (Kukita et al. 1990).

We deliberately excluded mononuclear osteoclasts or preosteoclasts (Athanasou et al. 1991) from our analysis of the distribution of nuclei in osteoclasts, although there is a growing body of support for these to be capable of resorption (Hattersley and Chambers 1989). This claim is sometimes based only on the small size of some resorption pits—a hazardous assumption given the tiny pits that may be made by any osteoclasts, and that are always made by binucleate cells at the initial stage of resorption. However some cultures with only mononuclear cells are reported to make pits, and we have found that many tiny pits as well as huge ones are produced in long term bone marrow cultures which are generating new osteoclasts (Jones et al. 1986). Amano and colleagues (1992) considered only two features as distinguishing mature osteoclasts: a ruffled border and a clear zone. We did not see pits below mononuclear cells in this study, but cannot claim to have especially sought them. Vignery and colleagues (1991) have suggested that macrophage multinucleation is accompanied by a change in gene expression and confers a specific function to macrophages. Whether this is true also for osteoclasts is not known.

It is not clear whether in vivo the addition of extra nuclei to a multinucleate cell is only by the fusion with it of mononuclear cells. If this is usually the case, the production of gigantic cells in pathological conditions might result from the unusual fusion of multinucleate cells, or from the rapid production of many new uninuclear osteoclasts and their addition to a multinucleate cell. Indeed, oversize osteoclasts generated in long-term normal marrow cultures, and capable of eroding huge single-scoop lacunae (Jones et al. 1986), are probably witness to the success of the preosteoclast production in vitro. We do not exclude the possibility that fusion of osteoclasts, or preosteoclasts with osteoclasts, may
have occurred during the culture period use in this study – we would expect some fusion. The cumulative frequency curves for the distribution of the nuclei in the osteoclasts after 6 h in culture and after 24 h were very similar, there being some cells at 24 h larger than the largest at 6 h. The smallest osteoclasts, those with two or three nuclei, may also have been slightly under-represented in the pit sample, because a tiny pit, or light etching without cavitation, below an osteoclast went undetected. However, this did not shift the cumulative frequency curves to a significant amount.

**Sizes of resorption pits**

One might expect that the correlation between pit size and osteoclast size which we found would be seen to be higher if the period of time to make each individual resorption pit were known. The choice of 24 h for the culture period was in order to allow time for the majority of the osteoclasts to make pits, but not several pits. Obviously some osteoclasts would have started later than others, and some would have made more than one pit, and not all would have continued resorption until the end of the experiment. Undoubtedly, there were also a few misfits, where an osteoclast was wrongly identified as being responsible for the adjacent pit. Additionally, the rate at which an osteoclast resorbs can vary during the making of one pit (Jones and Boyd 1988), and may even vary at two pits being made simultaneously by one osteoclast (Kanehisa and Heersche 1988). We cannot exclude at present the possibility that oligo-nuclear cells may begin to resorb earlier in vitro than larger osteoclasts.

We were not able to find any enhancement of resorptive voracity in the cells with the addition of extra nuclei beyond that due to increase in nuclear number and hence size. Rather, we found a trend for the volume of mineralized tissue stripped away by an osteoclast per nucleus to decrease as the number of nuclei per cell increased. This result is consistent with the data of Zheng and colleagues (1991). These authors reported that oligo-nuclear rat osteoclasts showed greater nuclear uptake of tritiated uridine than cells with many nuclei and that the nuclei of the latter cells showed greater variation in their nuclear labelling. They concluded that the nuclei of oligo-nuclear osteoclasts have higher rates of RNA synthesis than those of osteoclasts with many nuclei. Our findings that oligo-nuclear cells resorb more dentine per nucleus than cells with higher numbers of nuclei could therefore show a direct result of a reduced synthetic potential for proteases and enzymes involved with proton secretion per nucleus of the larger cells. The plan area of a pit is dictated to a large extent by the size of the cell – its contact area; in contrast the depth of resorption strongly reflects the working efficiency of the cell.

Measuring the work done by an osteoclast is a challenge that has occupied us since 1979 (Boyd and Jones 1979; Boyd et al. 1983). The methods we have used have changed as newer technologies have become available (Boyd and Jones 1992) but the need for a volumetric measurement has not (Boyd and Jones 1991). On the assumption that no proton is wasted, and that we know the composition of the resorbed tissue, it allows us to determine how many protons are produced by an osteoclast in unit time – an indulgence in sub-molecular biology. Although we have again shown that the correlation between the area and the volume of a pit is high under standard culture conditions of short duration, it is a changing relationship with time (Delaisse et al. 1987), and changes under different experimental conditions (Taylor et al. 1990). To think otherwise would be to err.

**Acknowledgements.** This work has been supported by the MRC, SERC and The Wellcome Trust. We are grateful for the skilled assistance of Maureen Arora, and thank Mr. Komiya of Laserte Corporation for kindly lending us the equipment to measure the resorption pits.

**References**


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The Effect of 3-Amino-1-Hydroxypropyldene-1,1-Bisphosphonate (APD) on the Resorptive Function of Osteoclasts of Known Nuclear Number

K. Piper, A. Boyd, S. J. Jones

Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

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Abstract. Bisphosphonates (BP) are known to suppress osteoclastic resorption in vivo and in vitro, but doubt persists as to how, and the effect of BP on the resorptive capability of osteoclasts of known nuclear number is unknown. We aimed to find whether the addition of 3-amino-1-hydroxypropyldene-1,1-bisphosphonate (APD) changed the nuclear profile of an osteoclast population in vitro, and to measure the resorptive efficiency of individually characterized osteoclasts in the presence and absence of the BP. Prehatch chick bone cells were cultured for 24 hours on slices of dentine in medium with or without added APD at 10^{-6} M or 10^{-8} M, or in control medium on dentine presoaked with 10^{-6} M APD for 48 hours. Total pit counts, and pit depths, areas, and volumes for pits made by osteoclasts of known nuclear number, were found using confocal video-rate laser reflection microscopy and 3-D image analysis software. APD in the medium inhibited resorption and reduced the volume, area, and depth resorbed per nucleus per chick osteoclast. The nuclear number distribution did not shift significantly, suggesting that no preferential effect arose from the APD affecting one size of cell more than another. The large reduction found in pit numbers, depths, areas, and volumes in the APD dentine-pretreated group supports previous views that BP released during resorption act as metabolic inhibitors, altering protein synthesis by the cell. Larger cells made larger pits, but resorative efficiency was similar for different cell sizes within the control or APD-treated groups. There was a strong negative correlation between the maximum depth resorbed per nucleus and the number of nuclei in the osteoclast in all groups.

Key words: Osteoclasts — Bisphosphonates — Pits — Nuclei — In vitro

Bisphosphonates suppress osteoclastic resorption in vivo and in vitro [1] and are used in the treatment of Paget's disease [2], osteoporosis [3], and hypercalcemia of malignancy [4]. A possible new use is to prevent bone loss associated with hypokinesia, including microgravity [5].

Controversy persists about the ways in which the bisphosphonates may inhibit resorption in vivo and in vitro [6], and whether or not its effects are confined to osteoclasts and their precursors [7]. Variations of the in vitro 'isolated' osteoclast resorption assay have been used to evaluate osteoclastic function in the presence of bisphosphonates [8-11], but there is no information on the effect of bisphosphonates on the resorative capability of individually identified osteoclasts.

Recent work on RNA synthesis in isolated rat osteoclasts has shown that there may be a decrease in basal RNA synthesis and hence activity with increasing nuclear number [12], and our previous work on resorption with prehatch chick osteoclasts lent support to that theory [13]. We found that the osteoclasts having more nuclei made larger pits in a 24-hour culture period, but that the volume of calcified tissue destroyed per nucleus tended to decrease as the cells increased in size. It has been suggested that bisphosphonates may affect resorption in vivo by altering the size of osteoclasts as well as their number [6, 14]; this would change the nuclear number distribution in the population and, possibly, resorative efficiency of the osteoclasts.

The attachment of osteoclasts to a substrate, and their retention on it, is also affected by the presence of bisphosphonates [9, 15], but whether some cells are more affected than others has not been determined. This study set out to examine whether the addition of 3-amino-1-hydroxypropyldene-1,1-bisphosphonate (APD) changed the nuclear profile of an osteoclast population in vitro, and to measure the resorative efficiency of individually characterized osteoclasts in the presence and absence of the bisphosphonate.

Materials and Methods

Culture of Chick Osteoclasts

Osteoclasts were obtained from 19-day, prehatch, chick long bones in the manner previously described [13]. The bones of one chick were chopped in phosphate-buffered saline (PBS) with added 10% fetal calf serum (FCS). The release of osteoclasts from the bones was aided by repeatedly sucking the fragments up and down a plastic pipette. The milky cell suspension was then seeded on to one of four sperm whale dentine slices measuring 5 mm x 5 mm, cut from centimetre squares. One slice of each set of four was pretreated by soaking for 48 hours in 10^{-8} M APD, the APD solution was sucked off, replaced with fresh PBS three times using 1-ml aliquots. (A further dilution of any residual APD in solution would occur at stages in the culturing process.)

Following seeding, cells were allowed to settle for 45 minutes at 37°C before washing in PBS to remove nonadherent cells. The cells remaining on the dentine were cultured for 24 hours in Eagle's medium (MEM) containing 10% FCS, 2 mM L-glutamine, 0.25 pg/ml
fungizone, 0.2 U/ml penicillin, and 0.2 pg/ml streptomycin in 5% CO₂ at 37°C.

In each experiment, the control and pretreatment slices were cultured in the control medium without added bisphosphonate; the other two slices received medium with added APD at 10⁻⁵ M or 10⁻⁶ M.

In addition, seedings were made on to plastic culture dishes for 24 hours and cultured under identical conditions, receiving either control medium or medium with either 10⁻⁵ M APD or 10⁻⁶ M APD. Both experiments were repeated 45 times.

Counting Nuclei of Osteoclasts, and Identifying the Cells with Their Pits

At the end of the culture period, the dentine slices and tissue culture dishes were gently washed in PBS and the cells were fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.2. The cells were stained for the presence of tartrate-resistant acid phosphatase (TRAP) (Sigma 386 kit). The numbers of nuclei in all TRAP-positive multinucleate cells were counted on tissue culture dishes.

Locating the pit and counting the nuclei of the associated osteoclast were carried out as before [13]: each of the dentine slices was drawn using an Olympus IMT-2 microscope and a graticule that had been lined up at one corner of the slice, and all multinucleate TRAP-positive cells with a pit clearly ascribed to them were included in the study. The plan shape of the osteoclast and its pit were drawn to assist later identification when the cells were removed. The cells were removed from the dentine slices by washing in a mild detergent, 1% chlorhexidine gluconate, pH 7, followed by immersion in 30% H₂O₂, and the pits were reidentified using the graticule and chart. In some cases, osteoclasts were found to have had more than one pit beneath them.

Total pit numbers were counted on all specimens using a Bausch and Lomb reflected light microscope.

Measurement of Pits

Measurement of the pits was carried out using the video-rate, line-confocal light microscope ILM12 (Lasertech, Japan). A He-Ne laser beam is broadened to a line using a cylindrical lens, which is scanned down the specimen field at TV frame rate. The signal corresponding to each point in the line is directed to a single detection element in a linear diode array. Thus, images are acquired at full standard TV rate. The microscope and its special Z-axis controller provide a hard-wired solution to the problem of acquiring the max and the map images during multiple plane acquisition. Using an 80 x objective, images were derived from 256 planes acquired in approximately 10 seconds. Volume and area data were acquired from the map image by tracing (with a mouse and screen cursor) a line around the pit edge, volume data being acquired by the software (SIS D4400, Münster, Germany) deriving a trace binary outside the pit to determine the height of the surrounding reference surface and then deriving the volume beneath the surface. The area was calculated at the same time. The depth recorded was the maximum depth of the pit.

Analysis of the data was performed using Minitab statistical software (Minitab 1989).

Results

Nuclear Number Distribution

The nuclei of 2412 osteoclasts cultured on tissue culture dishes were counted, comprising 828 control cells, 794 cells at APD 10⁻⁵ M, and 790 cells at APD 10⁻⁶ M. After a 24-hour culture period, there was no significant difference between the cells of the treatment groups and the control groups with regard to their size (as indexed by the number of nuclei), as illustrated in Figure 1. There was no evidence that the presence of the bisphosphonate affected the fusion or fission of the osteoclasts or preferentially targeted a particular cohort of cells during the 24-hour culture period, for example, by affecting the adherence or viability of smaller or larger cells in the population.

Number of Pits

The total number of pits on the dentine slices varied among the groups (Table 1). There were fewest pits in the slices that had been pretreated with APD, and fewer in the group cultured in the presence of 10⁻⁶ M APD than in the remaining two culture groups. Only a tenth of the pits in any group were suitable for inclusion in the study linking the sizes of osteoclasts with their resorption sites; this was because it was difficult to find cells, unequivocally on pits that they had made, with nuclei that could be counted accurately. In addition, the number of cells still attached to the dentine in the pretreatment group was small. There was a reduction in the percentage of cells with more than one resorption site with 10⁻⁶ M APD in the medium, and only one cell was associated with more than a single pit in the pretreatment group.

Sizes of Resorption Pits in Dentine

The depth, areas, and volumes of all pits ascribed to a par-

### Table 1. The numbers of pits and osteoclast-pit sites measured after 24-hour culture period

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10⁻⁵ M APD</th>
<th>10⁻⁶ M APD</th>
<th>Pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total pits</td>
<td>543</td>
<td>578</td>
<td>483</td>
<td>178</td>
</tr>
<tr>
<td>Osteoclasts in</td>
<td>57</td>
<td>60</td>
<td>43</td>
<td>14</td>
</tr>
<tr>
<td>measured set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pits measured</td>
<td>81</td>
<td>82</td>
<td>57</td>
<td>17</td>
</tr>
<tr>
<td>% Osteoclasts in</td>
<td>24.6%</td>
<td>23.3%</td>
<td>16.3%</td>
<td>7.1%</td>
</tr>
<tr>
<td>measured set</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with &gt;1 pit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Maximum depth resorbed by osteoclast

<table>
<thead>
<tr>
<th>Depth resorbed/nucleus (microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 2. The means and SEM for the maximum depths of the measured pits, and maximum depths resorbed per nucleus by the osteoclasts, in the control and treatment groups. The depth resorbed per nucleus corrects for variation in cell size (number of nuclei), the cells in the group with $10^{-6}$ M APD having more larger cells.

Area resorbed by osteoclast

<table>
<thead>
<tr>
<th>Area resorbed/nucleus (sq. microns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1400</td>
</tr>
<tr>
<td>1200</td>
</tr>
<tr>
<td>1000</td>
</tr>
<tr>
<td>800</td>
</tr>
<tr>
<td>600</td>
</tr>
<tr>
<td>400</td>
</tr>
<tr>
<td>200</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>50</td>
</tr>
</tbody>
</table>

Fig. 3. The means and SEM for the areas of the measured pits, and areas resorbed per nucleus by the osteoclasts, in the control and treatment groups. The area resorbed per nucleus corrects for variation in cell size (number of nuclei), the cells in the group with $10^{-6}$ M APD having more larger cells.

A particular osteoclast were measured (Figs. 2-4). The mean values for both areas and volumes of pits decreased in a dose-dependent manner, although there was a small but nonsignificant increase in the maximum depths of the pits and numbers of nuclei per cell in the group cultured in the presence of $10^{-6}$ M APD. The most striking reduction in depth, area, and volume of pits was in the group in which the dentine had been pretreated with $10^{-6}$ M APD. The depth, area, and volume resorbed per nucleus also decreased in a dose-dependent fashion. The reduction in the area resorbed per osteoclast was evident at a lower concentration in the medium than the reduction in maximum pit depth.

Median values and significance levels for difference from control values are given in Table 2.
Table 2. The sizes of pits made by osteoclasts of known nuclei number

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 57)</th>
<th>10^-8 M APD (n = 60)</th>
<th>10^-6 M APD (n = 43)</th>
<th>Pretreated (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei/cell</td>
<td>6</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Pit depth</td>
<td>5.94</td>
<td>5.11</td>
<td>6.22</td>
<td>2.71</td>
</tr>
<tr>
<td>Depth/nucleus</td>
<td>1.02</td>
<td>0.82</td>
<td>0.73</td>
<td>0.56</td>
</tr>
<tr>
<td>Pit area</td>
<td>802</td>
<td>563</td>
<td>430</td>
<td>382</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>123</td>
<td>82</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>Pit volume</td>
<td>3425</td>
<td>1895</td>
<td>1542</td>
<td>727</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>552</td>
<td>323b</td>
<td>244b</td>
<td>130b</td>
</tr>
</tbody>
</table>

Median values: depths in μm, areas in μm², volumes in μm³. The median values for the sizes of the pits are given because the data is nonparametric. (The mean values for the numbers of nuclei/cell for the control, 10^-8 M APD, 10^-6 M APD, and pretreatment groups were 7.16, 6.73, 8.21 and 6.21, respectively.) The median values for the numbers of nuclei counted per osteoclast in the groups cultured on plastic (Fig. 1) were 6 for all three groups, however. This data emphasizes the value of correcting for cell size variation.

"P < 0.001; "P < 0.0001 values significantly different from control (Mann-Whitney test)

The results were also analyzed excluding the cells to which more than one pit had been ascribed. The means and medians of the volumes of the pits, and the volumes of dentine resorbed per nucleus showed the same pattern (Table 3).

The proportion of cells with 10 or more nuclei in the 10^-6 M APD group (34.9%) was nearly twice that in the control (15.8%) and 10^-8 M APD groups (15.0%). These values vary from those for the data in Figure 1 (% of cells with 10 or more nuclei: control 27.4%, 10^-8 M APD 28.5%, 10^-6 M APD 23.8%), and this might influence the results if the activity decreased with increased cell size. We therefore analyzed

Table 3. (a) The sizes of the pits made by cells associated with a single pit at end of culture period

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 43)</th>
<th>10^-8 M APD (n = 46)</th>
<th>10^-6 M APD (n = 36)</th>
<th>Pretreatment (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>Mean 4749</td>
<td>4652</td>
<td>2937</td>
<td>1005</td>
</tr>
<tr>
<td>(SEM)</td>
<td>(806)</td>
<td>(1087)</td>
<td>(675)</td>
<td>(263)</td>
</tr>
<tr>
<td>Median</td>
<td>3235</td>
<td>1894</td>
<td>1439</td>
<td>701</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>Mean 610.4</td>
<td>590.7</td>
<td>410.7</td>
<td>144.1</td>
</tr>
<tr>
<td>(SEM)</td>
<td>(57.2)</td>
<td>(93.5)</td>
<td>(96.7)</td>
<td>(26)</td>
</tr>
<tr>
<td>Median</td>
<td>486.0</td>
<td>329.2</td>
<td>215.3</td>
<td>109.3</td>
</tr>
</tbody>
</table>

Mann-Whitney test, values significantly different from controls: "P = 0.03; "P = 0.005; "P = 0.0001; "P = 0.01; "P = 0.005; "P = 0

Table 3. (b) Sizes of 150 unselected pits in each group

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 150)</th>
<th>10^-8 M APD (n = 150)</th>
<th>10^-6 M APD (n = 150)</th>
<th>Pretreatment (n = 150)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>Mean 1586</td>
<td>1749</td>
<td>1198</td>
<td>402.7</td>
</tr>
<tr>
<td>(SEM)</td>
<td>(178)</td>
<td>(265)</td>
<td>(239)</td>
<td>(71.1)</td>
</tr>
<tr>
<td>Median</td>
<td>699</td>
<td>589</td>
<td>463b</td>
<td>161.5b</td>
</tr>
<tr>
<td>Area</td>
<td>Mean 422.8</td>
<td>358.0</td>
<td>333.8</td>
<td>172.9</td>
</tr>
<tr>
<td>(SEM)</td>
<td>(42.8)</td>
<td>(41.8)</td>
<td>(51.2)</td>
<td>(25.7)</td>
</tr>
<tr>
<td>Median</td>
<td>252.5</td>
<td>168b</td>
<td>170b</td>
<td>95b</td>
</tr>
</tbody>
</table>

Mann-Whitney test, difference from control: "P = 0.0005; "P = 0; "P = 0.004; "P = 0.003

The cell-associated pits fall in the upper part of the range of the unselected pits, as would be expected, and the response to the APD is clearer when the size of the osteoclasts is taken into account. Depths in μm, areas in μm², volumes in μm³.
Table 4. Correlations between the number of nuclei of a cell and the size of its resorption pit/s

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>10^{-8} M APD</th>
<th>10^{-6} M APD</th>
<th>Pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth</td>
<td>0.331</td>
<td>0.312</td>
<td>0.189</td>
<td>0.427</td>
</tr>
<tr>
<td>Depth/nucleus</td>
<td>-0.605</td>
<td>-0.516</td>
<td>-0.627</td>
<td>-0.429</td>
</tr>
<tr>
<td>Area</td>
<td>0.810</td>
<td>0.734</td>
<td>0.429</td>
<td>0.671</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>0.286</td>
<td>0.053</td>
<td>0.044</td>
<td>0.231</td>
</tr>
<tr>
<td>Volume</td>
<td>0.774</td>
<td>0.574</td>
<td>0.291</td>
<td>0.694</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>0.237</td>
<td>0.112</td>
<td>-0.098</td>
<td>0.468</td>
</tr>
</tbody>
</table>

predict if no preferential effect arose from the APD affecting one size of cell more than another, given that the short time course of the experiment and the dispersion of the cells after seeding limit the opportunities for cell fusion either between mature osteoclasts or between precursor cells in the bone marrow cell population and osteoclasts. The bisphosphonate may have prevented the attachment of osteoclast precursors present in the bone marrow fraction to the mineralized surface [15, 19], a step suggested to be essential for their differentiation. However, osteoclast recruitment in mice has been shown to increase following APD treatment in vivo [6], with new osteoclast nuclei being added to preexistent osteoclasts, and an increase in the number of osteoclasts [14]. Whether the precursor cells in the mice attached to the bone or not before fusing with the resident osteoclasts is unclear, but fusion was not prevented by the APD. The time courses of these in vivo experiments suggest that no change would be expected to occur within our 24-hour culture period, and that the response of the hemopoietic marrow in vivo is to the change in serum calcium as resorption is inhibited, not to the presence of bisphosphonates per se [6].

Marshall et al. [20] found that the number of osteoclasts in cultured mouse parietal bones was reduced by adding APD, an effect counteracted by the addition of parathyroid hormone to the medium. They found that the osteoclasts had a changed morphology, and concluded that these changes were degenerative, an opinion with which Flanagan and Chambers [11] concurred. The large reduction in pit numbers, depths, areas, and volumes in the pretreated group in our study lends further credence to this view, for the accumulation of APD in the surface layer of the dentine would be maximal in this group and its release as resorption proceeded increasingly detrimental to the cells. Calcitonin, which inhibits resorption, also protects the osteoclasts from the effects of bisphosphonates [10, 11, 19]; it is therefore not surprising that we found the least percentage of cells associated with more than one pit, and the smallest pits, in the pretreatment group.

The correlation coefficients for the different treatments raise a very interesting point. They may be lower in the 10^{-6} M APD group because the cells respond unevenly to the APD, but without regard to their size. In fact, there seems to be a dose-related trend for the drop in size of resorption as size and pit size to lessen with APD in the medium. The question then arises as to why this drop in correlation is not a feature of the pretreatment group, where the reduction in resorption is greatest. A possible explanation would be that in this case the APD does not affect the cells until resorption is well under way, and then does so overwhelmingly, so that resorption ceases abruptly. Thus, the relationship between cell size and resorptive activity is not disrupted in the same way as a more gradually increasing suppression with APD in the medium, where the correlation between pit size and cell size falls as the drug takes effect.

Our results show that there is a progressive loss in efficiency of resorption, measured by the volume of tissue resorbed by an individual cell, or by the volume resorbed per osteoclast per nucleus, with increasing bisphosphonate concentration in the medium, supporting the hypothesis of Capron et al. [7] that the bisphosphonates act as metabolic inhibitors, altering the protein synthesis of the cell. Other workers, using rat bone-derived cells, have reported that pit numbers increased with low concentrations of bisphosphonates in the medium [9]. We also found a slight increase in total pit numbers with 10^{-6} M APD, and this would offset, to some extent, the reduction in the mean volumes of the individual pits made by a population of cells. It emphasizes the
need to measure as many parameters as possible in order to allow a full picture of the resorptive activity to be established [21, 22].

This study has confirmed our earlier finding [13] that larger cells make larger pits, but we did not find a reduction in resorptive efficiency, measured as the volume of dentine destroyed per nucleus, in the larger cells compared with the smaller in either the control or APD-treated groups. However, in all groups, there was again a strong negative correlation between the maximum depth resorbed per nucleus and the number of nuclei in the osteoclast.

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References

Volumes of Chick and Rat Osteoclasts Cultured on Glass

K. Piper, A. Boyde, S. J. Jones

Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

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Abstract. We have examined the relationship between the number of nuclei of an osteoclast and its volume. Chick and rat cells were released from long bones by chopping the shafts and flushing the fragments in Eagle's Minimum Essential Medium with added 10% fetal calf serum. The bone cell suspension was seeded onto glass coverslips. In Experiment 1, rat and chick cells were allowed to settle for 15 minutes, more medium was then added, and the cells were cultured in 5% CO₂ at 37°C for 4 hours. In Experiment 2, only rat cells were used, and the cells were cultured in the presence or absence of 10⁻⁶ M 3-amino-1-hydroxypropyridene-1,1-bisphosphonate (APD) in the medium for 4 or 6 hours. The coverslips were washed in 37°C phosphate-buffered saline and fixed for 24 hours in 2.5% glutaraldehyde in isotonic cacodylate buffer (initially 37°C). The chick cells were critical point dried (CPD) or freeze dried (FD); all rat cells were FD. After drying, cells were coated with gold by vacuum evaporation. The volumes and areas of osteoclasts were measured using a video-rate, line-confocal reflection laser scanning microscope and the number of nuclei in each cell was counted. The volumes and volumes per nucleus of the FD cells were larger than those of the CPD cells but there was no significant difference in plan-areas. Rat osteoclasts were larger than chick cells in all the measured parameters except the mean number of nuclei/cell. The correlation coefficients for the areas, volumes, and the numbers of nuclei for rat and chick cells were all high (r > 0.725). The volumes and volumes per nucleus, but not the areas or areas per nucleus, of the osteoclasts cultured with APD were significantly smaller than control cells. We conclude that FD causes less shrinkage than CPD; chick osteoclasts are about two-thirds the size of rat osteoclasts; and 10⁻⁶ M APD caused a reduction of rat osteoclast volume and volume per nucleus of 21%.

Key words: Osteoclast — Volume — In vitro — Nuclei — Confocal microscopy — Bisphosphonate.

Osteoclasts are multinucleated, polymorphous cells that are able to destroy calcified tissue. The work capacity of each cell is related to its size, for which the best measurement is probably its total volume. Though determining the volume of a complex shape is difficult, especially on a resorbed, rough substrate, the measurement of cell volume on a flat substrate is practical.

Most attempts to monitor changes in osteoclast size have been made by measuring areas of spread cells either by using time-lapse video recordings of live osteoclasts in bone cell cultures, using a system for digitizing the cell outlines and hence calculating the spread area [1-3], or by using fixed and air-dried cells and digitizing the cell outline [4-6]. Holtrop et al. [7] fixed rat osteoclasts in situ and digitized images of transmission electron microscopy (TEM) sections to estimate the change in volume of the cells as a result of treatment with parathyroid hormone (PTH). Domon and Wakita [8] extended this method to cultured cells and calculated the volume of resorbing osteoclasts from serial TEM images.

There are innate problems with fixing cells for measurement. Cells take time to die, and might change shape and size while they do. They also bleed and lose wet cell volume [9]. Drying cells that are adherent to a substratum produces considerable volume shrinkage but not an equivalent reduction in plan area. It is not known whether changes on fixation and drying affect osteoclasts with different numbers of nuclei equally because measurements are lacking for multinucleate cells.

Digitizing outlines of living cells from time-lapse video recordings using phase contrast microscopy is a lengthy procedure. Some difficulty is experienced with visualizing and tracing the cell outline from phase-contrast video images which may lead to inaccuracies, and reports are often based on a very small sample size (e.g., n = 5 [2]). Furthermore, the approach is limited by phase halo effects which provide poor discrimination of the true location of the cell periphery [10]. Perhaps the most accurate and simplest method is to capture images of the interface of the cell with a flat surface, such as a glass coverslip, using confocal reflection microscopy [11]. However, measurement of spread area will not necessarily demonstrate changes in the metabolic activity of osteoclasts in response to different reagents: this necessitates measuring cell volume.

As most resorbing osteoclasts are multinucleate, and the nuclei are not necessarily of equal age or size, obtaining the nuclear:cytoplasmic volume ratio is very difficult and it is perhaps more useful to ascertain the nuclei count:whole cell volume ratio. The aims of this study were to examine the relationship between the number of nuclei of an osteoclast and its volume, to compare the volumes of chick and rat osteoclasts, and to determine whether cell volume changes in the presence of a bisphosphonate (3-amino-1-hydroxypropyridene-1,1-bisphosphonate) (APD).

Materials and Methods

Culture of Osteoclasts

Osteoclasts were obtained either from the long bones of 19-day-old...
prehatch Cobb White chicks or from 4-day-old Sprague-Dawley rat pups in the manner previously described [12]. The long bones were excised and freed of periosteum, and their shafts were then chopped in Eagle's Minimum Essential Medium with added 10% fetal calf serum. The release of the osteoclasts from the bones was aided by repeatedly sucking the fragments up and down a plastic pipette. The resultant cell suspension was seeded onto sterile 18-mm glass coverslips, square to allow a raster search to be carried out.

In Experiment 1 both rat and chick cells were used. The coverslips with the droplets of seeded cells were placed in sterile plastic 35-mm Petri dishes and the cells were allowed to settle for 15 minutes. More medium was then added and the cells were cultured in 5% CO\textsubscript{2}, at 37°C for 4 hours until they were well adherent and spread sufficiently to allow easy counting of the osteoclast nuclei. In Experiment 2 only rat cells were used, and the cells were seeded and cultured in the presence or absence of 10^{-7} M APD in the medium. The coverslips were placed in 35-mm plastic Petri dishes under conditions identical to the previous experiment for either 4 or 6 hours.

Fixation and Drying of Cells

At the end of the culture period, the coverslips were washed gently in warmed (37°C) phosphate-buffered saline (PBS) to remove non-adherent cells, and then fixed for 4 hours in 2.5% glutaraldehyde in 0.15 M cacodylate buffer (initially at 37°C). In Experiment 1, the chick cells were either critical point dried or freeze dried. Rat cells in both experiments were freeze dried.

For critical point drying (CPD), the cells were dehydrated in ethanol and substituted with Freon 113 (CC\textsubscript{3}FCClF\textsubscript{2}) prior to CPD from CO\textsubscript{2}. Before freeze drying (FD), the coverslips were immersed for 1 hour in chloroform water [13]. Immediately before quenching in Freon 12 (CC\textsubscript{3}F\textsubscript{2}) at -155°C, the gross excess moisture was removed by touching the side of each coverslip onto a piece of filter paper. The coverslips were placed in a specimen holder that had been precooled in liquid nitrogen. Freeze drying was conducted in an Edwards Speedivac freeze dryer at -60°C for 24 hours. The freeze dryer was gradually warmed up to room temperature at the end of the 24-hour period to avoid condensation when air was admitted.

All the coverslips from one experiment were dried in the same holder and at the same time. After drying, both the critical point-dried and the freeze-dried cells were coated by vacuum sublimation of gold to provide a reflective surface.

Counting the Nuclei of the Osteoclasts and Measuring their Cell Volume

The coverslips were attached to glass slides with the coated cells uppermost, and the cells were measured using a video-rate, laser confocal laser scanning microscope (IM.Z21, Lasertec Corporation, Japan) [14]. Each osteoclast that was encountered on a raster search was first checked to see whether it was suitable for inclusion in the study. The criteria used were that the nuclei had to be clearly visible so that they could be accurately counted, the cell body free of overlying debris that would influence measurement of cell volume, and the cell adequately distant from other cells or debris to allow a trace binary to be derived to determine a reference surface [15].

Nuclei were counted using phase contrast optics (200/0.20 objective). The cell was then mapped using a 400/0.95 lens with a coverslip thickness correction collar and with a coverslip cemented to the objective. A full description of the method, which employs a 256 focus level pass, is given in Boyde and Jones [15]. Volume and area data were derived from the map image using dedicated software (SIS D4400 Münster, Germany). The cell territory was defined by the operator tracing a screen cursor carefully around the periphery of the cell. The software derived a reference binary encircling the cell to determine the height of the surrounding glass surface; it then derived the volume above the surface and the cell area.

Analysis of the data was performed using Minitab statistical software (Minitab 1989).

Results

Experiment 1

The Difference in Cell Size with Respect to Freeze Drying and Critical Point Drying

The means, medians, and SEMs for the measured parameters for 67 freeze-dried chick osteoclasts and 10 critical point-dried chick osteoclasts are given in Table 1. The volumes of the freeze-dried cells were significantly larger than those of the critical point-dried cells (cell volume \( P = 0.0045 \), volume per nucleus \( P < 0.0001 \); Mann-Whitney test) but there was no significant difference in the cell area.

The difference in volumes as a result of the drying methods is shown in Figure 1. Taking the slope of the line from the equation \( y = c + x \frac{f(x)}{f(0)} \) for the freeze-dried cells \( f = 389 \) and for the critical point-dried cells \( f = 221 \). The volume of each cell may be calculated by determining the nuclear number of the cell and multiplying this value by the f value.

Comparison of Freeze-dried Rat and Chick Osteoclasts

Fifty freeze-dried rat osteoclasts, seeded for 4 hours prior to fixation, were measured in the same way as the 67 chick osteoclasts that had been freeze dried. The data for the sizes of the rat osteoclasts are presented in Table 1.

The rat osteoclasts were significantly larger cells in all the measured parameters except the median number of nuclei per cell where they had the same value as the chick cells. The mean number of nuclei per cell was larger in the chick cells (5.28 ± 0.54 compared with 4.14 ± 0.27 SEM for the rat cells) due to the inclusion of a number of chick osteoclasts with more than 12 nuclei. The significant difference for the cell volume was \( P = 0.0012 \), cell area \( P = 0.0044 \), and for

### Table 1. Experiment 1: The sizes of the chick and rat osteoclasts

<table>
<thead>
<tr>
<th></th>
<th>Chick (CPD)</th>
<th>Chick (FD)</th>
<th>Rat (FD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 67</td>
<td>n = 50</td>
</tr>
<tr>
<td>Nuclei/cell</td>
<td>4.80 (1.14)</td>
<td>5.28 (0.54)</td>
<td>4.14 (0.27)</td>
</tr>
<tr>
<td>Volume</td>
<td>735 (254)</td>
<td>1727 (222)</td>
<td>2354 (245)</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>130 (14.1)</td>
<td>367 (13.4)</td>
<td>539 (32.4)</td>
</tr>
<tr>
<td>Area</td>
<td>2695 (638)</td>
<td>2590 (306)</td>
<td>2929 (249)</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>561 (56.2)</td>
<td>433 (15.4)</td>
<td>708 (40.9)</td>
</tr>
</tbody>
</table>

Significantly different from the Chick FD values (Mann-Whitney test) Areas in \( \mu m^2 \); volumes in \( \mu m^3 \)

* \( P < 0.005 \); ** \( P < 0.0001 \)
both the volume per nucleus and area per nucleus, $P < 0.0001$ (Mann-Whitney test). The correlation coefficients for the areas, volumes, and the numbers of nuclei for rat and chick cells were all high. Scatterplots, linear regression lines, and the correlation coefficients are shown in Figures 2 and 3.

**Experiment 2**

**Effect of APD on Size of Rat Osteoclasts.** Rat osteoclasts were seeded and cultured for either 4 or 6 hours. As there was no significant difference in the sizes of the control or APD-treated osteoclasts in the two different culture periods, the results from the two experiments were pooled. The means, medians, and SEM for 96 control and 96 $10^{-6}$ M APD-treated osteoclasts are given in Table 2.

The volumes, volumes per nucleus, and volume:area ratios of the osteoclasts cultured with APD were significantly smaller than control cells ($P = 0.0023$, $P = 0.0003$, and $P < 0.0001$, respectively; Mann-Whitney test). There was no significant difference in the area and area per nucleus, and the median number of nuclei in both groups remained the same. The mean number of nuclei for the control rat osteoclasts was $4.97 \pm 0.31$ SEM compared with $4.93 \pm 0.32$ SEM for the APD group. The correlation coefficients in both groups remained high. These, regression lines, and scatterplots are illustrated graphically (Figs 4 and 5). The regression lines for the cell areas and cell volumes are compared in Figure 6. The osmolarity of the culture medium did not change detectably with the addition of APD.

The sizes of the control freeze-dried rat cells in Experiment 1 ($n = 50$) and Experiment 2 ($n = 96$) were compared. There was no difference in the numbers of nuclei, volumes, or volumes per nucleus of the two groups, but the areas, areas per nucleus, and volume:area ratios of the cells were significantly different ($P = 0.0009$, $P = 0.0009$, and $P = 0.0000$, respectively, Mann-Whitney test), reflecting different degrees of spreading of the cells on the substrates in the two series.

**Discussion**

**Drying Methods**

The relative shrinkages of the cell volumes in this experiment for freeze-dried and critical point-dried cells are in good agreement with previous data for cellular tissues (see review by King [16]). The freeze-dried volume would be approximately 80% of the wet volume of the cell, and the critical point-dried cells 40% of the total volume of the cell [17, 18]. On air drying, even from nonpolar solvents, cells shrink 80% by volume. Thus, solvent evaporation drying is the least satisfactory method for preparing cells for volume measurement, although it has been used for estimating osteoclast areas [4-6]. The choice of freeze drying in preference to critical point drying is unequivocal. For this reason,
Fig. 3. Scattergrams and correlation coefficients for the relationship between cell size and nuclear number of 50 rat osteoclasts (Experiment 1).

we only measured a small number of CPD cells to confirm the relationship between CPD and FD volumes for osteoclasts.

The ratio of volumes after CPD and FD (FD/CPD 2.35) is relevant to all biological microscopy. These values are in agreement with those reported for bulk test specimens composed of uniform cellular tissues with a low collagen content [16-18]. They indicate that we may place some confidence in an estimate of living cell volume in which we multiply the FD volumes by 1.25.

Wheatley et al. [19] found that the wet weight/dry weight ratio and protein/unit mass of cell remained constant throughout the HeLa cell cycle. There is no reason to believe that the ratio would change with cell fusion in osteoclasts.

The consistency of the results in the two sets of control, freeze-dried, rat osteoclasts with respect to the volume and volume per nucleus values suggests that the nuclear count is a valid index of the size of an osteoclast within similar cell populations. The difference in the areas and area per nucleus values is explained by variation in the extent of spreading in the two experiments: this also would result in a difference in the mean height of the cells. It confirms that volume measurements are more meaningful when comparing between resorption pit experiments, although the correlation between area and volume is always very high within one experimental group.

If we compare the sizes of chick cells, assuming a 20% volume (i.e., 5% linear) shrinkage on drying, with the sizes of the resorption pits made under similar culture conditions in dentine during the first 24 hours after seeding [12, 20], it becomes clear that chick osteoclasts can destroy more than their own volume of dentine per day. We have previously continuously monitored the rate of resorption of a live chick osteoclast in its second day of culture on dentine and found that it destroyed 128% of its own volume in 18 hours [21].

Species Differences

The results show that rat osteoclasts are larger than chick osteoclasts with the same number of nuclei. This is interesting in view of the previous finding that the pits made by rat cells in 1-day cultures are smaller than those made by chick cells [22]. We found that rat cells adhered and spread faster than chick cells but made smaller pits within the 24-hour culture period than either rabbit or chick osteoclasts, and suggest that the rat cells were tardy in commencing resorption. Empirically, we and others have found rat cells to be poorer resorbers in vitro, requiring a more acid medium to generate an equivalent effect to chick cells. Osteoclast volume does not, therefore, equate with resorptive capability in vitro except within one cell population. It is not possible to say whether rat osteoclasts are less efficient resorbers than chick ones in vivo as each will be responding to species-specific environmental influences rather than to artificially similar ones.

The high correlations between the number of nuclei and the cell volume vindicates our use of the number of nuclei as an indicator of cell size within one population in resorption pit assays [12, 20]. Basle et al. [23] also found a good correlation between the diameters of human osteoclasts, isolated and cytocentrifuged onto slides, and their number of nuclei. The different volumes per nucleus for chick and rat cells is intriguing, however, and we do not know whether the nuclei of rat osteoclasts are also larger than chick ones. This property of osteoclasts should be explored for other species, ages, and stages of development.

Effect of APD

The rationale for the APD experiment was to select one concentration of a drug that had profound effects on the resorptive capability of osteoclasts and to determine whether we could detect any volumetric change. The dose of $10^{-6}$ M APD was chosen because we had shown previously [20] that this caused a highly significant reduction in the volumes of pits made in vitro, and in the volume of tissue resorbed/
Table 2. Experiment 2: The sizes of rat osteoclasts cultured in the presence or absence of 10^{-6} M APD

<table>
<thead>
<tr>
<th></th>
<th>Control n = 96</th>
<th>APD n = 96</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Median</td>
</tr>
<tr>
<td>Nuclei/cell</td>
<td>4.97 (0.31)</td>
<td>4.0</td>
</tr>
<tr>
<td>Volume</td>
<td>2799 (196)</td>
<td>2193</td>
</tr>
<tr>
<td>Volume/nucleus</td>
<td>559 (23.9)</td>
<td>521</td>
</tr>
<tr>
<td>Area</td>
<td>4535 (293)</td>
<td>3986</td>
</tr>
<tr>
<td>Area/nucleus</td>
<td>926 (36.8)</td>
<td>875</td>
</tr>
</tbody>
</table>

Significantly different from control (Mann-Whitney test)

Areas in μm^2; volumes in μm^3; freeze-dried osteoclasts

* P < 0.005; ** P < 0.0005

nucleus by chick osteoclasts. We therefore did not do dose- or time-response studies.

This is the first study to report an effect of APD on osteoclast cell volume, independent of the presence of a calcified tissue. It is not known how bisphosphonates act to suppress resorption, but it has recently been shown that bisphosphonates can be metabolized by amebae [24] and are potent inhibitors of squalene synthase, an enzyme necessary for cholesterol biosynthesis [25]. The reduction in osteoclast volume, volume per nucleus, and volume:area ratios suggests that the bisphosphonates act directly on osteoclasts, as indicated by other studies [26]. Bisphosphonates have also been shown to affect marrow-derived macrophages [27], and have an effect on osteoblasts after only 5 minutes [28], although no change in cytosolic Ca^{2+} could be detected in osteoblasts [29]. As our bone cell cultures contain osteoblasts and macrophages, it is impossible to say whether the change in cell size is solely a direct effect on the osteoclast or whether it also is due in part to signals from other cells. To minimize this problem, and aid measurement, we seeded the bone cells sparsely to ensure that the osteoclasts were not in contact with any other cell.

The possibility that a change in the tonicity of the culture fluid with the addition of 10^{-6} M APD resulted in cell shrinkage can be discounted. The 21% lower volume and volume per nucleus of the APD-treated osteoclasts might be explained by a depolarization of the cell membrane with loss of intracellular K^+ and Cl^-, leading to net water loss and cell shrinkage. An alteration in membrane conductance has been suggested to cause shrinkage in other types of secretory cells exposed to secretagogues [30, 31], an effect maintained as long as the substance was present. However, Sims and Dixon [32] have described inwardly rectifying K^+ currents in rat osteoclasts, and depolarization would assist H^+ transport out of the cell and stimulate resorption. Zimolo et al. [33] found that alendronate altered membrane fluxes of NH_4^+ and H^+. Most substances that inhibit osteoclastic resorption appear to involve a cyclic AMP-dependent pathway [3, 4] and cause a reduction in osteoclast area [1]. As we did not find a significant reduction in the plan-areas of the APD-treated osteoclasts, it is unlikely that cAMP activation and subsequent rise of intracellular Ca^{2+} accounts for the change in volume. A cAMP activation of K^+ and Cl^{-} channels cannot be ruled out, although there is no evidence that APD affects the cAMP pathway in osteoclasts.

The reduction in the volume of the osteoclast also supports the hypothesis that the bisphosphonates may be acting as metabolic inhibitors to reduce protein synthesis [34]. The bisphosphonate alendronate, besides altering membrane fluxes of NH_4^+ and H^+, disrupts the ruffled border complex of an osteoclast and stops the proton pump, which may be

![Fig. 4. Scattergrams and correlation coefficients for the relationship between cell size and nuclear number of 96 control rat osteoclasts (Experiment 2).](image-url)
pinocytosed back into the osteoclast [33]. These effects may contribute to a reduction in osteoclastic volume in the presence of a bisphosphonate, as well as a decrease in the sizes and numbers of the pits that the cells make [20, 28].

The 21% reduction in volume and volume per nucleus of the rat osteoclasts in the presence of 10^{-6} M APD is less than the reduction in the volume of tissue resorbed by chick osteoclasts when cultured under similar conditions for 24 hours [20]. In neither case was there any indication that cells of different sizes (numbers of nuclei) were affected differently by the bisphosphonate. There might be a species difference in the response of chick and rat cells to APD, and resorbing cells may be more affected by the bisphosphonate [35].

However, the results of this experiment conflict with the notion that only resorbing cells are affected by the drug.

Endo et al. [36] reported that osteoclasts in the tibiae of mice treated with bisphosphonate enlarged and the cells contained more nuclei. This assessment was made by observing histological sections. As the authors do not say whether the change was due solely to increased cell fusion, it is not possible to know whether the results of the in vivo study are at variance with our in vitro data.

**Measurement of Cell Volume**

Measurement of cell volume is not easy. Ideally, wet cell
volume should be obtained and the nuclear:cytoplasmic ratio determined. In practice, this exemplar is rarely achieved.

Coulter size distribution analyzers have been used for estimating volumes of T-lymphoblasts [37] and volume changes of B lymphocytes [38] but are not suitable for an impure population of cells with a wide size range and a tendency to fragment. Most volume estimates of live cells rely on light microscopical images recorded at timed intervals which are analyzed for total area, mean height, and mean width with an image analysis system; the volume is estimated assuming a simple geometric model shape and applying the respective geometric formula (e.g., [31]).

Nuclear:cytoplasmic ratios of a number of cell types have been obtained by scrape-loading cells with a mixture of two fluorescent dextrans, one of which diffuses into the nuclear envelope, then calculating the total cellular fluorescence of both probes and the ratio of the two probes in the cytoplasm [39]. The volume regulation of astroglial cells in culture has been demonstrated by loading the cells with fluorescent intracellular probes, exciting the two probes in the cytoplasm at their isosbestic point where the signal emitted depends on the variation in concentration induced by a change in cell volume, and measuring the signal with microspectrofluorometric equipment [40]. A very different approach for live cells has been employed by Dunn and Brown [41] and Brown and Dunn [42] who used microinterferometry to study the movement of dry mass in fibroblasts. This method allows evaluation of the dry mass per projected area, but not per unit volume.

Most current estimates of cell and nuclear volumes are based on stereological techniques. For example, neuronal cell areas in monkeys have been measured from fixed, frozen sections and microcomputer image analysis [43]. Point counting on fixed, stained, embedded sections is commonly used to achieve the same aim [44–46] and nuclear volume is calculated by the same method [45] or from tracings of serial sections with a camera lucida [44]. Kirk et al. [47] used SEM, stereology, and geometric formulae to calculate cell volume; Hol trop et al.’s [7] classic study of changes in the ultrastructure of osteoclasts measured projected areas from photographic negatives of TEM sections. Many groups have used methods of digitizing the cell outlines from fixed and stained tissues which were either mechanically or optically sectioned to determine cell area and volume [48–51].

The method we have devised to measure the volume of osteoclasts by reflection confocal microscopy (CSLM) has proved to be extremely useful, although it fails short of the ideal because the cells are both dead and dry. However, the combination of CSLM with freeze drying provides the fastest and most accurate method available for directly determining the volumes and areas of whole, polymorphous cells. Unlike the commonly employed methods reviewed above, geometric formulae for volume estimations are not needed, edge detection of fixed and stained sections is not a problem, and time-consuming, accurate tracing is not required. One circumvents the problems associated with fluorescence methods which involve extreme care when loading cells with fluorescent dyes to ensure that the dye fills the whole compartment to be measured and that the method of detection is sensitive enough to measure the emissions accurately. This new method for measuring the cell volumes and plan areas of fixed osteoclasts may prove to be equally valuable in other fields of cell biology.

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References


