BONE STRUCTURE AND TURNOVER IN THE ADULT HUMAN MANDIBLE

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'Than the maxilla and the mandible there are no other bones which undergo greater changes during life' (Humble 1936).
ABSTRACT

This thesis describes some aspects of the bony anatomy of the adult human mandible and the changes that occur with aging and tooth loss. Factors that might influence the resorption phase of remodelling in the edentulous mandible are addressed: including the mineralization density, the effect of the collagen orientation, and the systemic environment.

Bone sections were prepared for scanning electron microscopy, and to aid the study of their complex 3-D architecture, alternative coating and embedding techniques were investigated. Video-rate reflection confocal laser scanning microscopical mapping was used in *in vitro* quantitative resorption studies using isolated osteoclasts to investigate the possible influence of the arrangement of extrinsic collagen fibres; the bone origin (dermal or endoskeletal); and the life or death of the contained osteocytes.

The results showed that extrinsic collagen fibre orientation, the origin of the bone and its vitality all affected the degree to which it could be resorbed by osteoclasts *in vitro*.

To compare changes in other skeletal sites with those in the mandible, the apparent density and the mineralization density of bones from cranial (parietal bone and mandible) and postcranial origins (lumbar vertebra, iliac crest, femoral neck) were compared using quantitative digital backscattered electron imaging. For all bones studied, no relationship was found between the apparent and the mineralization densities. This implies that bone quantity is independent of bone quality. In addition, it was found that bones from postcranial sites possess more similar features to each other than to bones of the head, supporting the view of their having different behaviours related to their embryological and evolutionary origins.

The findings have implications in grafting, the placement of implants, and the interpretation of future resorption experiments, as well as clinical relevance for the aging, edentulous population.
OVERVIEW OF THESIS

Following tooth loss, the mandible shows such an extensive loss of bone in some individuals as to pose a significant management problem. Dental implantology has provided a means by which these otherwise untreatable cases can be handled, but often needs to be combined with grafting procedures increasing the complexity as well as the biological and financial costs of the treatment. To be able to predict which individuals are susceptible to this exaggerated bone loss prior to tooth extraction would be useful so that any appropriate therapy might be instituted promptly. However, as yet, the causes of the differences that occur between individuals have not been fully elucidated.

Many factors, both local and systemic, are likely to influence the structure and turnover of mandibular bone, as they do for bone in general, but studies correlating mandibular with systemic bone status have been inconclusive. This is largely because most clinical studies have used indirect methods of investigating the bone status (such as imaging techniques or blood biochemistry) without a detailed understanding of the anatomical and structural changes that occur in the mandible with aging or upon becoming edentulous. In addition, the multifactorial nature of mandibular residual ridge resorption has made well-controlled, relevant studies difficult to engineer.

This thesis investigates the changes that occur in the mandible with aging and seeks to determine how the bone structure and turnover of the mandible may reflect those occurring at other sites in the skeleton. In addition, it examines how certain anatomical features of the mandible may influence its subsequent resorption.

The literature is reviewed in chapter 1 and covers the morphological changes that occur in the mandible after tooth loss, and the factors that might influence these changes. In particular there is an attempt to analyse the reasons for the conflicting reports found in the literature on the effects that systemic influences may have on mandibular bone, as well as to highlight some of the difficulties in current methods of determining bone density.

Chapter 2 describes attempts to improve upon current bone coating and embedding procedures for scanning electron microscopy.

Chapters 3 and 4 cover the morphological, anatomical and densitometric findings in mandibular bone, how they relate to each other and how they relate to the particular
characteristics found in the fourth lumbar vertebra, the iliac crest of the pelvis and the neck of the femur.

Chapters 5, 6 and 7 examine the effects that the origin of bone, its extrinsic fibre arrangement and its vitality may have upon its resorption in vitro. This has relevance to the mandible which is of a different embryological origin to postcranial bone, contains significant proportions of extrinsic fibres in the bone around the teeth, and may exhibit extensive areas of dead bone in elderly individuals.

The final chapter gives a brief overall discussion of the main findings of this thesis.

Aims of the thesis

The main aims of this thesis are therefore:

- to study the anatomical effects of aging on mandibular structure and density;
- to determine if there is a relationship between mandibular height and bone quality;
- to study the correlation between mandibular and other bone structure and mineralization density; and
- to investigate how some features of mandibular anatomy may influence its resorption postextraction.

This will be done by studying the level of mineralization density as a variation with age, gender and dental status; the apparent bone density as a variation with age, gender and dental status; whether the mineralization and apparent densities are correlated with each other; whether the mineralization and apparent densities of different sites of the mandible are correlated with other bones in the skeleton; how the resorption of cranial bone may differ from that of postcranial bone; and whether the collagen fibre arrangement, or the status of the contained osteocytes may influence the resorbability of bone.
ACKNOWLEDGEMENTS

I would like to thank the many people who have helped me during the period of my PhD. From the Hard Tissue Research Unit at UCL: Professor Sheila Jones for ideas, guidance and encouragement, and without whose advice I would still not have finished; Mo Arora, who tirelessly seeded many of my bone slices with osteoclasts; Roy Radcliffe for his excellent technical assistance; Dr Colin Gray for endless computer instruction and general advice; Dr Peter Howell for imparting his statistical knowledge and for unceasing computing assistance; Dr Leonora Wolfe for allowing me access to the computer.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>1</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>3</td>
</tr>
<tr>
<td>OVERVIEW OF THESIS</td>
<td>4</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>6</td>
</tr>
<tr>
<td>LISTS OF FIGURES AND TABLES</td>
<td>8</td>
</tr>
<tr>
<td>CHAPTER 1 Review of literature and statement of the problem</td>
<td>15</td>
</tr>
<tr>
<td>CHAPTER 2 Investigation of conductive coating and embedding techniques for scanning electron microscopy</td>
<td>35</td>
</tr>
<tr>
<td>Part I - Secondary electron imaging</td>
<td>35</td>
</tr>
<tr>
<td>Part II - Backscattered electron analysis</td>
<td>41</td>
</tr>
<tr>
<td>CHAPTER 3 Mandibular apparent density variation with sex, age, dental and skeletal status</td>
<td>47</td>
</tr>
<tr>
<td>CHAPTER 4 Mandibular mineralization density and its variation with site, sex, age, dental and skeletal status</td>
<td>79</td>
</tr>
<tr>
<td>CHAPTER 5 The effect of bone origin upon osteoclastic resorption</td>
<td>139</td>
</tr>
<tr>
<td>CHAPTER 6 The effect of substrate collagen fibre orientation upon the shape of osteoclastic resorption pits made by chick osteoclasts in vitro</td>
<td>153</td>
</tr>
<tr>
<td>CHAPTER 7 The resorption of vital and devitalized bone in vitro: significance for bone grafts</td>
<td>175</td>
</tr>
<tr>
<td>CHAPTER 8 Discussion</td>
<td>189</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>195</td>
</tr>
<tr>
<td>APPENDIX 1 Lists of specimens</td>
<td>223</td>
</tr>
<tr>
<td>APPENDIX 2 Publications arising from this thesis</td>
<td>227</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 2.1 Photograph of mandibular bone showing the extent of penetration possible using the electroless coating method described in the text........38

Figure 2.2 Secondary emission scanning electron micrograph of horse radius slab coated by the electroless method..........................................................39

Figure 2.3 Same specimen as figure 2.1 at a higher magnification..........................39

Figure 3.1 Diagram showing measurements made from radiographic projections.....50

Figure 3.2 Bone apparent density against age for the mental foramen site of the mandible ..................................................................................52

Figure 3.3a Apparent density against cross sectional area - mental foramen site .....54

Figure 3.3b Apparent density against cortical thickness - mental foramen site.......54

Figure 3.4 Apparent density against age - mandibular midline..............................56

Figure 3.5 Apparent density - mandibular midline against mental foramen site .....56

Figure 3.6 Area against apparent density for mandibular and postcranial sites.......58

Figure 3.7 Photographs of 2 mm thick mandibular sections from mental foramen region ..................................................................................60

Figure 3.8 Photographs of 2 mm thick mandibular sections from the mandibular midline ..................................................................................64

Figure 3.9 Midline mandibular slice from 72 year old male.................................63

Figure 3.10 Near midline slice from 35 year old male.............................................66

Figure 3.11 Midline slice from 48 year old male....................................................67

Figure 3.12 Mandibular midline slices from 70 year old female.........................68
Figure 3.13  Bone slices from mandible, lumbar vertebra, iliac crest and femoral neck of three females ....................................................... 69

Figure 3.14  Bone slices from mandible, lumbar vertebra, iliac crest and femoral neck of three males ............................................................................71

Figure 4.1  Raft of micromilled blocks of PMMA-embedded cranial specimens ......82

Figure 4.2  Variation in percentage occupation of mineralization density fractions with age - lingual split site only ....................................................87

Figure 4.3  Summary diagrams showing sites of highest and lowest mineralization densities in the mandible ......................................................94

Figure 4.4  Four mineralization density fraction histograms for different ages - parietal bone ........................................................ 96

Figure 4.5a  Mean greyscale values for different skeletal sites of each individual (whole bone cross sections) ..............................................................98

Figure 4.5b  Mean greyscale values for different skeletal sites (cont).........................99

Figure 4.6  Four mineralization density fraction histograms for all skeletal sites.....102

Figure 4.7  BSE-SEM micrographs showing normal appearances of mandible......104

Figure 4.8  BSE-SEM micrographs of mandibular cement lines .................................106

Figure 4.9  BSE-SEM micrographs of mineralized osteocyte lacunae in mandible..108

Figure 4.10  BSE-SEM micrographs of infilled mineralized Haversian canals .........110

Figure 4.11  BSE-SEM micrographs of bone with low levels of mineralization ......112

Figure 4.12  BSE-SEM micrographs of Sharpey's fibres in the mandible...............114

Figure 4.13  BSE-SEM micrographs of fine bone structure in the mandible............116
Figure 4.14 BSE-SEM micrographs fine bone structure II ...........................................118

Figure 4.15 BSE-SEM micrographs fine bone structure III ......................................120

Figure 4.16 BSE-SEM micrographs of highly calcified tissue in postcranial bones . 122

Figure 4.17 BSE-SEM micrographs of regions of low mineralization ......................124

Figure 4.18 BSE-SEM micrographs of highly mineralized cement lines .....................126

Figure 4.19 BSE-SEM micrographs of highly mineralized cement lines II .................128

Figure 5.1 Confocal micrographs of resorption pits in equine bone slices showing pit measurement techniques using the Lasertec 1LM2W .........142

Figure 5.2 BSE-SEM micrographs of osteoclastic resorption pits in equine frontal and phalangeal bone........................................................................144

Figure 5.3 Histogram showing sizes of pits in cranial and postcranial bone .............145

Figure 5.4 Pseudo-coloured quantitative BSE-SEM micrograph of frontal and phalangeal equine bone slices ....................................................................146

Figure 5.5a BSE-SEM image of equine frontal bone showing many bright reversal lines indicative of previous turnover .................................................. 148

Figure 5.5b BSE-SEM image of equine phalangeal bone, with new bone showing up against the more homogeneous background ..................................148

Figure 6.1 BSE-SEM of block of sperm whale cementum resorption substrate ....156

Figure 6.2 BSE-SEM image of sperm whale cementum with the extrinsic fibre boundary enhanced using an edge finding filter ........................................156

Figure 6.3 Cumulative mean pit areas for each experiment .......................................160

Figure 6.4 Cumulative mean volumes for each experiment .......................................161
Figure 6.5 Cumulative mean depths for each experiment ...........................................162
Figure 6.6 Cumulative mean values for pooled data - randomised order ....................163
Figure 6.7 Volume against area plots for all experiments ........................................164
Figure 6.8 BSE-SEM of embedded sperm whale cementum slice viewed sideways on, showing the profile of a resorption pit ........................................167
Figure 6.9 BSE-SEM showing profile of sperm whale cementum surface ..................167
Figure 6.10 Confocal micrograph showing exposed fibre in base of pit ...................168
Figure 6.11 Confocal micrograph showing how the unmineralized extrinsic fibre core often remained proud relative to the mineralized fibre periphery in the base of the pits on the group I substrate .........................................169
Figure 6.12 BSE-SEM of part demineralized sperm whale cementum slice showing that the extent of demineralization of the slice surface ..................170
Figure 7.1 Histogram showing sizes of pits in vital and nonvital bone ......................181
Figure 7.2a Confocal micrograph of DASPMI-stained live cultured cells ...............183
Figure 7.2b Confocal micrograph of dead cells stained with ethidium bromide ....183
Figure 7.2c Confocal micrograph of autofluorescence of cultured osteoblasts killed by heating .................................................................183
Figure 7.3 Confocal fluorescence image of DASPMI-stained osteocytes in live human mandibular bone .................................................................185
LIST OF TABLES

Table 1.1  Local and systemic factors that may influence residual ridge resorption. 19
Table 3.1  Number, age and sex distribution of MF specimens ..........................51
Table 3.2  Apparent densities of adult MF specimens (g/cm³).............................53
Table 3.3  Mean results from antero-posterior radiographic measurements (mm) ..53
Table 3.4  Number, age & sex distribution of MD mandibular specimens ..........55
Table 3.5  Apparent densities of MD specimens (g/cm³)..................................55
Table 3.6  Mean area and apparent density values for postcranial sites ..........57
Table 3.7  Correlations of apparent densities between skeletal sites.................59
Table 3.8  Correlations between density and area of each skeletal site ............59
Table 4.1  Number, age and sex distribution of 'Is' mandibular specimens ..........85
Table 4.2  Means and sem of the mean mineralization densities of the groups ....86
Table 4.3  Mean and sem of the mean mineralization densities of the groups ....88
Table 4.4  Student's paired t-tests of LS slices.............................................88
Table 4.5  Pearson's linear correlation coefficient of LS slices .......................88
Table 4.6  Mean and sem of the mean mineralization densities of the groups ....89
Table 4.7  Mean and sem of the mean mineralization densities of the groups ....90
Table 4.8  Student's paired t-tests of MD slices.............................................90
Table 4.9  Pearson's linear correlation coefficient of MD slices.......................90
| Table 4.10 | Student's paired t-tests and Pearson's linear correlation coefficients between MD, MF and LS sites of the same mandible. | 92 |
| Table 4.11 | Mean and sem greylevel values for dentate, partially dentate and edentate mandibles | 93 |
| Table 4.12 | Number, age and sex distribution of calvarial specimens | 95 |
| Table 4.13 | Mean mineralization densities for cortical and trabecular zones of postcranial bones | 95 |
| Table 4.14 | Mean mineralization densities for cortical and trabecular zones in L4 - four bin data | 97 |
| Table 4.15 | Mean mineralization densities of whole bone cross sections | 100 |
| Table 4.16 | Significance of differences between sites - paired t-tests | 100 |
| Table 4.17 | Pearson's linear correlation coefficient between sites | 100 |
| Table 5.1 | Results for all pits <10 000 μm³ | 143 |
| Table 5.2 | Roughness measurements of slices | 147 |
| Table 6.1 | Area (A), volume (V) and mean depth (V/A) data - all pits | 159 |
| Table 6.2 | Three dimensional form factor (3DFF) | 189 |
| Table 6.3 | Roughness | 159 |
| Table 7.1 | Factors that may influence bone graft healing | 175 |
| Table 7.2 | Effect of bone cell vitality upon resorption: median pit sizes | 180 |
CHAPTER 1

REVIEW OF LITERATURE AND STATEMENT OF THE PROBLEM

The adult human mandible is a bone which exhibits a large degree of variability (Humble 1936, Kuznetsova et al 1972, Tallgren 1972, Enlow et al 1976, von Wowern & Stoltze 1977, Jacobsen & Krol 1983, Poirot et al 1986, Carter et al 1991). This variation occurs not only between subjects or as a result of aging, but also may exist between the right and left sides in any given individual. After growth has ceased, the single most important factor governing the gross morphological shape of the bone is related to the presence or the absence of the teeth. After tooth extraction, there often follows a considerable phase of remodelling which may result in an extensive loss in the height of the jaws. Historically this phenomenon is of importance since it was the observation of the 'waste of the sockets of the teeth' by John Hunter in the 1750s that prompted him to consider bone as a material capable of remodelling, rather than the immutable and permanent structure it had previously been thought to be (in Cohen 1993).

What happens postextraction
Both internal and external changes occur in the mandible after the teeth are lost (Walkhoff 1900, Neufeld 1958, Atwood 1963). During the initial healing phase, the sockets are filled with blood clot in which woven bone develops, later to be replaced by cancellous bone. At the same time, new bone formation is seen deep to and some distance from the socket surrounding the inferior dental canal (Boyne 1982), the crest of the ridge narrows and the sharp edges of the alveolar processes are reduced (Atwood 1963, Pietrokovski & Massler 1967, Enlow et al 1976). As the bone is reduced in height by periosteal osteoclastic resorption, there is an accompanying endosteal apposition (Pudwill & Wentz 1975), but at no time is new bone formation seen on the periosteal surface of the ridge which remains porous, never developing a complete cortical layer (Neufeld 1958, Atwood 1963, Pudwill & Wentz 1975). Further internal remodelling results in a loss of organisation and a thinning of the trabeculae (Neufeld 1958) as well as disruption in the arrangement of the lamellar and Haversian systems as determined by the split line technique (Seipel 1948) and an increase in the diameter of the Haversian canals in the cortex.

Pattern of bone loss
Most longitudinal studies of the changes in the external form of the bone have been carried out using measurements either from serial study casts (Pietrokovski & Massler
These studies have shown that the loss in vertical height is greatest anteriorly (Lönberg 1951, Carlsson & Persson 1967). Little change is thought to occur in the region of the superior genial tubercles, the mylohyoid and external oblique ridges which become increasingly prominent (Neufeld 1958, Osborne 1963), in extreme cases requiring surgical reduction for the provision of dentures. This has been attributed to these regions being composed of cortical bone (Devlin & Ferguson 1991), though functional factors may have a greater influence. In the horizontal plane, the majority of the bone loss occurs from the buccal aspect in the upper jaw resulting in the palate reducing in width and length, as well as in height (Fish 1947, Likeman & Watt 1974). The situation is more complex in the mandible with the majority of loss occurring from the labial aspect anteriorly and from the lingual aspect posteriorly (Fish 1947, Watt & MacGregor 1986), although some resorption is also seen buccally (Pietrokovski & Massler 1967, Pietrokovski 1975, Enlow et al 1976, Wang 1989).

In a cross sectional histological study of 15 mandibles exhibiting various stages of tooth loss, Enlow et al (1976) described variations in the areas of surface resorption and apposition in the different mandibles, concluding that the resorptive and depository areas are similar to those present during growth. This agrees with the findings of a longitudinal radiographic study spanning a period of 25 years (Tallgren 1972) which showed that the intraindividual pattern of bone loss remains fairly constant, yet that there is interindividual variation in both the pattern and rate of loss. In the subjects studied, between 20 and 120 mm² of bone were lost from the lateral projection of the mandibular midline over a 13.5 year period of denture wear.

Rate and duration of loss
Most of the bone loss occurs in the first year after extraction, with the highest rate being in the first few months (Atwood 1957, Tallgren 1966, Carlsson & Persson 1967, Likeman & Watt 1974). However, continued bone loss from the mandible can still be detected up to 25 years postextraction, reducing much sooner (or even ceasing in some individuals) in the maxilla (Brehm & Abadi 1980), with the latter showing on average one quarter of the reduction of the mandible after a period of seven years (Tallgren 1966, Kalk & de Baat 1989).
Devlin & Ferguson (1991) suggested that the reduction in rate of bone loss was due to the resorbing ridge losing contact with the base of the denture, which would therefore no longer be subjected to unfavourable loading. This is rather unlikely; not only did Tallgren (1992) show this pattern of loss in patients with well maintained dentures, but even poorly fitting dentures must make contact with the underlying tissues in some regions. In addition, if the dentures were thought to be the cause of the resorption, then the resorbed ridge presumably would conform to the shape of the fitting surface of the denture and hence they would not lose contact with each other.

**Extent of loss**

Many texts loosely describe the bone that is lost after extraction as being that which formerly belonged to the alveolar processes (Roth & Calmes 1981), but since the alveolar bone is arbitrarily taken as ending at the root apices (Berkovitz et al 1992) its delineation is lost once the teeth are removed.

In radiographic studies the division between alveolar and basal bone is usually taken as lying at the level of the mental foramen, being the most readily visible landmark (Wical & Swoope 1974a, Ward et al 1977, Kribbs et al 1983, Packota et al 1988, Ortman et al 1989, Benson et al 1991, Hirai et al 1993, Klemetti et al 1994a, Taguchi et al 1995), but the mental foramen has no direct relation to the teeth, and in radiographic projections may lie inferior or superior to the root apices. In addition, identifying the mental foramen and inferior dental canal becomes increasingly difficult in resorbed mandibles (Ulm 1989) especially as in advanced cases, the canal may be exposed by the resorptive process (Gabriel 1958, Gershenson et al 1986).

Inke (1972) claimed that it is possible to see the limit between alveolar and basal bone in the form of 'baseoalveolar sulci'. He even went to the extent of separating bones along these lines to show how the basal parts resemble the shape of a remodelled edentulous mandible and maxilla. Unfortunately the communication only shows exaggerated diagrams of the sulci, which probably otherwise need a strong eye of faith to see. Yet, the sulci may relate to positions of muscle insertions or to the limits of the attached gingiva, both of which have been taken as a functional-anatomical division between alveolar from basal bone (van der Klaauw 1952, Moss 1972, Brown unpublished, Kalk & de Baat 1989). Of the two, the latter accounts better for the different levels of loss that are seen around the mouth; the former is complicated by the fact that the muscle attachments are not fixed landmarks, with clear changes having been noted for the mentalis and buccinator muscles as the ridge recedes (Lammie 1956, Osborne 1963).
This area has not been simplified by the confusion of terms used in connection with the bone that surrounds the teeth. The *alveolar bone proper* (the very thin cribiform plate which immediately abuts the periodontal ligament and gives rise to the radiographic appearance of the lamina dura), which completely disappears shortly after tooth extraction, is not always distinguished clearly from the rest of the bone of the alveolar process (*alveolar bone*) (Becks & Grimm 1945). To help overcome the problems in terminology Edwards (1954) introduced the use of the phrase 'residual ridge resorption' to encompass all the changes that accompany bone loss after tooth extraction.

In 1928 Brash stated that *there is .... no essential difference between alveolar bone and the bone of the base, because the former becomes progressively transformed into the latter*, yet several points remain unexplained: why do some parts of the mandible undergo such profound reduction after tooth loss whilst neighbouring regions show little change? why does the mandible exhibit greater loss than the maxilla? and why do some individuals lose more bone volume than others?

Many factors, both local and systemic, have been put forward as having a potential influence on the postextraction resorption of the mandible (table 1.1). The potential functional, anatomical, physiological, inflammatory and metabolic causes will be discussed in turn, together with how they may be modified by the presence of dentures.

*Functional factors affecting mandibular bone loss*

Since the beginning of the century, many investigators have attributed the changes in the edentulous mandible to the changing function of the bone (Walkhoff 1900, Levin 1913, Seipel 1948, Ortman 1962) as did John Hunter in the 18th century, but it is only recently that the relationship of mechanical function to bone quantity and quality is becoming better understood (Rubin & Lanyon 1984, Skerry & Lanyon 1993). Neufeld (1958) described the changes that occur in the remodelling of the edentulous mandible as mimicking the effects of disuse osteoporosis and some features, such as the increase in cortical porosity and the trabecular thinning, are common to both. Disuse osteoporosis, however, is usually accompanied by endosteal resorption with little in the way of change in the external diameter of the bone (von Wwern et al 1979, Takahashi 1987, Ruff & Hayes 1988) and ceases when the new equilibrium has been established (Devlin & Ferguson 1991), whereas a resorbing ridge shows bone apposition on the endosteal surface, an overall decrease in size, and is continuous. In addition, no relation between cortical porosity and the presence or absence of the teeth has been found (Atkinson & Woodhead 1968).
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<th></th>
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<tr>
<td>lack of mechanical stress</td>
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<tr>
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<td>Harrison 1972</td>
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<td>Atwood 1963</td>
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<tr>
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<tr>
<td>proportion of extrinsic fibres</td>
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</tr>
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It is obvious that many functional changes occur upon the loss of the teeth, both in the source and the magnitude (Sobolik 1960) of the applied strains, as well as in the way they are transmitted to the bone (Ortman 1962). Bite forces reduce considerably with a reduction in the number of teeth (Helkimo et al 1976), and patients with dentures can only apply one eighth of the force of patients with natural teeth (Sobolik 1960, Ortman 1962). The wearing of dentures has been cited as both the cause (de Van 1935, Carlsson & Persson 1967) and the means of prevention (Mauley & Stuart 1937, de Aguiar et al 1968) of residual ridge resorption, although bone loss is observed whether dentures are provided or not (Campbell 1960).

In the past, tension and shearing loads produced by poorly balanced dentures were thought to have the most detrimental effect upon the underlying residual ridge, with the design being modified to reduce lateral loading by the use of teeth with flatter cuspal inclines and by discouraging incision (de Van 1935, Sobolik 1960). However, studies have shown the style of denture teeth to have little effect upon subsequent resorption (Brehm & Abadi 1980), and one five year study (Winter et al 1974) showed the opposite effect to that anticipated, with those in which more shearing loads would be expected showing less resorption. Atwood (1979) attributed this paradox to the great variation that occurs between individuals, but it is known from studies of long bones that the applied loads that are most osteogenetic are those that are applied at high strain rates, are of short duration and are unusual in their distributions (Lanyon 1992) - loading characteristics which are possibly minimised in the flatter type of occlusal design.

The nature of the application of loading is of importance in the maintenance of bone mass. In 1958, Applegate described an exercise appliance for the edentulous ridges of partially dentate patients. The appliance consisted of a close fitting, well extended resin saddle that covers the edentulous areas. The patients placed their fingers on the distal extension saddle prior to biting repeatedly for a few minutes each day (the use of the fingers was advocated so that the patients would avoid biting too hard). After an initial decline, which lasted a couple of weeks, it was shown that this procedure increased the radiographic density of the bone in the area, reaching a maximum at around 12 weeks of stimulation (Smith & Applegate 1961). No statistical analysis was described, but a particularly favourable response was reported for young patients and for those who had experienced only a short period of edentulousness. The differences that may exist between younger and older individuals may be due to age changes in the quality of the overlying soft tissues, the bone and the vasculature as discussed below.
Of the many factors studied by Tallgren (1972), only one was found to correlate with the magnitude of bone loss. She noted that patients with a more acute gonial angle and a greater angle from the mandibular base to the condyle measured from the anterior midline (as may be more frequently associated with patients with a small lower face height), showed a significantly greater amount of resorption compared with long faced individuals. Individuals with different face heights have different shaped musculatures (Mosolov 1972) with the former being capable of generating significantly higher bite forces than people with longer faces (Møller 1966). (Likewise, subjects with advanced tooth wear have been found to have smaller lower face heights than controls, even though the overall face height is the same (Crothers & Sandham 1993), which may be the cause (rather than the effect) of the tooth wear.) Klemetti et al (1994d) found the size of the masseter to correlate with the bone density of the basal parts of the mandible (as determined by quantitative computerised tomography) as well as to the bone mineral content of the femoral neck and lumbar spine.

Functional loads and the way they are applied play a great role in the reshaping of the mandible after tooth loss, and many factors other than the nature and pattern of loading may affect or interfere with the response of bone to functional strain. However, alterations in functional loading are unlikely to be the sole explanation for the reduction of residual ridges, otherwise bone changes would not occur at the insertions of muscles of facial expression (Lammie 1956), which might be expected to undergo little reduction in function upon becoming edentulous. The other factors that may have an influence upon resorption of the residual ridge or the perception and response to functional strain are discussed below.

**Anatomical factors**

**Bone size**

The original size of the mandible and the depth of the extraction sockets have been suggested as having an influence on the subsequent amount of resorption (Atwood 1979), yet no association has been found in longitudinal studies (Carlsson & Persson 1967). Finite element analysis of the distributions of stresses and strains on different shaped mandibles have shown that reduced stresses are found along the lower borders of mandibles with increased corpus height (Korioth et al 1992). This would imply, therefore, that as mandibles become smaller in cross section they must either be experiencing lower stresses so that the added depth of bone is no longer required, or be compensating for the less favourable mechanical characteristics by raising rigidity by another means, such as by increasing the amount of cortical bone, or by altering its mechanical properties. This will be investigated further in chapters 3 and 4.
Bone type
Cortical and cancellous bone are thought to respond differently to local and systemic influences (de Van 1935, Becks & Grimm 1945, Baxter 1987, Buchanan et al 1988, Lanyon 1992). The number, arrangement and distribution of trabeculae is very variable between edentulous mandibles (Becks & Grimm 1945, Parfitt 1962), but the ridge crest never develops a complete cortex postextraction (Neufeld 1958, Atwood 1963, Pudwill & Wentz 1975), and may therefore be more prone to age related or metabolic loss (Baxter 1987). In addition, the concentration of insulin like growth factor differs between cortical and cancellous bone, which may affect the rate of turnover of these tissues (Canalis & Agnusdei 1996).

Bone site
Bone tissue at different locations in the body varies in its rate of remodelling (Jee et al 1991) as well as in its response to mechanical strain. Calvarial bone experiences strains much lower than those required for the maintenance of bone mass and structure in long bones, and yet is preserved well (Rubin & Lanyon 1984, Hylander et al 1991). The mandible is of similar origin to calvarial bone, but is subjected to strains more typical of the postcranial skeleton (Dechow et al 1995) (further aspects of this are discussed in chapters 4 and 5). In addition, bones from different sites in the body vary in their stored growth factors (Baylink et al 1993), with human mandibular osteoblasts reported as producing more fibroblast growth factor and insulin like growth factor, but less transforming growth factor beta, than human iliac cells (Kasperk et al 1995).

Bone composition
Alveolar bone is described as being less brittle than other bone, which has been attributed to its higher glycosaminoglycan content (Waddington et al 1989), and alterations in occlusal loading have been shown to alter the proteoglycan distribution and content in rat mandibles (Shore et al 1996) which may be involved in the strain-memory responses of bone (Davidovitch 1991). Not much, however, is known about the effect that the material properties have upon resorption, although the more highly mineralized a substrate, the longer it takes to resorb (Jones et al 1995). The alveolar crest has been found to have a significantly lower mineral content and fewer extrinsic (Sharpey's) fibres than the adjacent bone (Landini 1991), both of which may favour its removal. These issues are investigated in chapters 4 and 6.

Functional loading has been shown to have an effect upon the intrinsic and extrinsic fibres of the alveolus surrounding teeth subjected to periods of non-, hypo- and hyperfunction (Short & Johnson 1990). Both non- and hypofunctional groups show a
reduction in the level of mineralization of the fibres, which become larger and sparser, compared with the fibres in bone around teeth with normal function. Collagen mass may reduce by as much as 10% at sites of disuse (Akeson et al 1987).

Bone cells
Atwood (1979) suggested that the aging of bone cells may contribute to their defective function, but did not discuss osteocytes which, of the three major bone cells, must be the hardest to replace, and are well known to exhibit age changes (Tonna 1976), in some cases becoming mineralized (Frost 1960). The embryological origin of the bone cells may also influence the turnover of the tissue and its response to mechanical strain (Recker 1992). The resorption of bones of differing embryological origins is tested in chapter 5.

Physiological factors
Blood supply
Experiments undertaken on animals with continuously erupting teeth, have shown that the blood supply to the alveolus is considerably greater than to other bone (Indresano & Lundell 1981). Care has to be exercised in extrapolating this to the human situation, but in Man alveolar bone does seem to have a high metabolic activity (Waddington et al 1989), and changes here can be the first sign of a metabolic disturbance (Bays & Weinstein 1982, Baxter et al 1987).

The pattern of the blood supply in the mandible changes from one that is largely centrifugal in the young dentate individual, to one that is primarily centripetal, with the supply being increasingly dependent upon periosteal and submuscular sources by the time old age is reached (Castelli 1963, Wallenius & Heyden 1972, Bradley 1975, 1981, Poirot et al 1986). This is likely to be of immense importance if the mucosa is used to support a denture (Weinmann & Sicher 1955, Ortman 1962), since prolonged pressure could occlude the fine periosteal plexus of vessels. This could stimulate osteoclastic resorption by altering the local oxygen tension and reducing the pH (Arnett & Dempster 1990). Pressures of long duration could be expected to have a greater effect, especially as the oral mucosa is visco-elastic (Picton & Wills 1978) and recovers more slowly in the elderly (Lytle 1957, Kydd & Daly 1982). Feik et al (1989) in an experimental study which used spring appliances to move rat caudal vertebrae through the surrounding soft tissues, noted that bone resorption occurred wherever the tissues were compressed against the bone, and formation occurred on the trailing edge, demonstrating the influence that the status of the overlying tissues can have on bone remodelling.
In a study of 34 patients, Carlsson & Persson (1967) found the daily period of denture wear to relate to the rate of residual ridge resorption, with those patients wearing their dentures at night as well as during the daytime experiencing significantly more loss (Carlsson & Persson 1967). The effects of pressure could also account for the differences seen between the resorption of maxillary compared with mandibular bone, the latter having nearly twice the area available for support (Woelfel et al 1976). However, subsequent studies have been unable to find any relationship between the extent of resorption and the frequency of denture use (Bergman et al 1971, Harrison 1972).

Atkinson and Hallsworth (1983) suggested that bone turnover in basal parts of the mandible could cut off existing Haversian systems, which would further compromise the alveolar blood supply.

**Bone turnover**

It is a complex interplay between the osteoblasts, osteoclasts, osteocytes and many other factors that is responsible for the regulation of bone turnover. Osteocytes are thought by some to be the cells responsible for detecting and initiating the cellular responses to changes in bone function, but large numbers die following tooth extraction, with many cell-free areas of bone being visible in the initial stages of socket healing (Carlsson et al 1967). These dead cells may account in part for the initial rapid phase of remodelling that is seen after tooth loss, since live osteocytes may release factors that inhibit osteoclastosis (Maejima et al 1995). The resorption of vital and nonvital bone is investigated in chapter 7.

In aging bone, viable osteocytes may become fewer in number to such an extent that the presence of 'overaged' bone becomes an increasingly frequent finding in the mandible (Pudwill and Wentz 1975). Patent canaliculi become reduced in number and diameter, and there is a reduction in the number of lacunae per unit volume of bone (Atkinson & Hallsworth 1983, Mullender et al 1996). Some areas become completely devoid of canalaricul and cellular spaces, probably due to mineralization of the lacunae and their contents (Frost 1960) with, overall, basal bone showing fewer patent lacunae per unit volume than alveolar bone, albeit more evenly distributed (Atkinson & Hallsworth 1983). This may or may not be of importance under conditions of normal usage, but it is likely that older bone will be less able to respond to changes in function if required. This could account for the younger patients benefitting more from the exercise therapy prescribed by Smith & Applegate (1961).
Inflammatory factors

Pre-existing periodontal health
In the western world, teeth are most commonly lost as a direct or indirect (through root caries) result of chronic inflammatory periodontal disease. One of the features of this is a reduction in the bony support around the teeth which results from microbial factors acting either directly, or indirectly via the host's inflammatory responses (Jeffcoat 1993, Henderson & Wilson 1996). Some have suggested that microbial endotoxins from denture plaque and residual bone resorbing factors may cause localised resorption of the alveolar ridge in the same way (Humble 1936, Atwood 1979, Penhall 1980), yet Carlsson & Persson (1967) found no association between either the pre-extraction bony support or the cross sectional size of the mandible and the amount of subsequent bone loss. The latter study based its periodontal assessment on measurements made from lateral cephalograms, which cannot be as reliable an index of periodontal status as the more conventional technique of periodontal probing. On the other hand, the microbial and host interactions that are so crucial to the development of periodontal disease hinge around the fact that the junctional epithelium which links the gingiva with the teeth is highly permeable, a pathway which disappears upon tooth removal. However, pre-existing periodontal disease could affect the bone structure through the altered strain patterns and fibre content that may exist around a tooth with a reduced level of attachment.

Trauma
The amount of bone damage that occurs during the removal of the teeth may affect the subsequent remodelling of the bone (Sobolik 1960). Mucosal inflammation caused by pressure areas under a denture could cause bone resorption via the generation of prostaglandins or interleukins. This is particularly likely to occur in elderly patients with thin, friable and tender soft tissues (Massler 1956), which will also serve indirectly to reduce the mechanical stimulation of the ridge. In one histological study, all individuals studied showed signs of chronic inflammation of the denture bearing mucosa (Pudwill & Wentz 1975).
Hormonal factors

For over half a century it has been recognised that generalised constitutional and/or systemic factors may influence mandibular bone (Becks & Grimm 1945, Massler 1956, Sobolik 1960, Ortman 1962), and this topic has recently been receiving renewed interest. One conference summary devoted to the subject of 'oral' and 'nonoral' bone loss stated that little is still known of the nature of the relationship between mandibular and postcranial bone (Redford & McGowan 1993). This may be for two main reasons: a) as has been discussed, mandibular residual ridge resorption is multifactorial in nature, which severely complicates the construction and interpretation of studies, and b) clinical studies have to rely on indirect methods of investigating the status of the bone, often giving little regard to the underlying anatomical and structural features of mandibular bone and the changes that occur with aging.

Osteoporosis is the commonest systemic bone disease and is characterised by a loss in bone mass with structural changes predisposing to fractures, particularly of the wrist, the lumbar spine and the femoral neck (Christiansen 1993). In the dental literature, the term has been used more freely where it has variously been used to describe a local increase in the diameter of the central canals of cortical Haversian systems (Atkinson & Woodhead 1968); a reduction in the thickness of the mandibular cortex (Bras et al 1982) or; a sparseness of trabeculae as seen on radiographic examination (Horner & Devlin 1992). Spontaneous fractures of the mandible are not a feature of generalised osteoporosis, so there is no convenient endpoint that confirms the diagnosis of osteoporosis in the mandible. In addition, since the pathological effect of skeletal osteoporosis in the mandible is unknown, studies have used a variety of mandibular features as indicative of systemic disease. These include: altered trabecular patterns, cortical porosity, cortical thickness, the height of the residual ridge, the number of teeth and the status of the periodontal tissues.

Trabecular patterns

At first sight, a study of the trabecular architecture of the mandible would seem sensible since in other bones, such as the lumbar vertebrae, this is where most alteration is seen in osteoporotic states (Jayasinghe et al 1994). However, the mandible shows great variation in its trabecular architecture between individuals, and varies markedly with aging and upon tooth loss (Humble 1936, Becks & Grimm 1945, Parfitt 1962). Also the trabecular contribution to structural strength is thought to be negligible in the mandible (Biknevicius & Ruff 1992), which causes one to question the function of trabeculae in this site (it has been suggested that their only real function in the mandible is as a calcium reserve (Roberts 1993), but they may help to hold the marrow elements in place, a role
that may diminish as the marrow becomes increasingly fibrous with age (Klingsberg & Butcher 1960). Moreover, accurate and reliable radiographic interpretation of trabecular architecture is probably not possible since each radiograph can only give a two dimensional representation of the complex three dimensional structure, and usually no account is made either for the varying bone and cortical thicknesses, which will influence the medullary volume available for the trabeculae (de Aguiar et al 1968), or the inhomogeneity of the tissue. Nevertheless, radiography seems to be the most frequent technique used for mandibular trabecular assessment, sometimes being combined with advanced image processing techniques (Ruttiman et al 1992). One study (Horner & Devlin 1992) has claimed that bone density measured from panoramic radiographs could be used as an indication of mandibular osteoporosis. However, not only was the allocation of the radiographs into each category (osteoporotic or not) entirely subjective, but the so-called density measurements were based upon similar criteria so that one would expect there to be a correlation. It has since been shown that panoramic radiographs cannot be used for the individual assessment of skeletal osteoporosis (Klemetti et al 1994a) which is not surprising when one considers the influence that the hard and soft tissue shadows from the out of focus layers over the different regions of the bone may have.

Histological studies in small animal models have shown that aging, ovariectomy and disuse all reduce the mandibular total bone area fraction (trabecular and cortical bone) (Elovic et al 1995), and that the systemic administration of oestrogen causes increased mandibular bone deposition (Stahl et al 1950), but even animals show an increased variation in tissue morphology with age (Klingsberg and Butcher 1960). One would still have to exercise extreme caution in drawing conclusions from human mandibular trabecular architecture in the absence of longitudinal data.

Cortical thickness

Cortical thickness measurements have been used to assess the effect of osteoporosis on many bones, but even proponents of this method say that it should not be applied to the mandible (Garn et al 1966). Nevertheless, several workers have shown an age related decrease in mandibular cortical thickness (von Wawern & Stoltze 1979, Bras et al 1982, Kribbs 1990), but no association with skeletal bone mass as measured by dual photon absorptiometry of the lumbar spine or femoral neck (Mohajery & Brooks 1992). In a study of over 350 patients, Klemetti et al (1994a) found that there is an association between the height and radiographic quality of the cortex with varying severities of osteoporosis, but that the sensitivity was so low that no indication of osteoporosis risk could be obtained on an individual basis.
Bone density

The term 'density' has been used to refer to a number of aspects of bone quality and quantity, other than just the mass per unit volume of the tissue. The Oxford English Dictionary (Little et al 1959) defines density as: 'the quality or condition of being dense; thickness; closeness of consistence ... the degree of consistence of a substance, measured by the quantity of matter in a unit of bulk' and 'dense' as: 'having its constituent particles closely compacted together'. Therefore, just as the term 'bone' can be used to describe the organ or the tissue, so can 'bone density' refer to the amount of tissue present in a given volume, or to its constitution at a microstructural level. The use of 'bone mineral content', 'bone mass' and 'bone density' may superficially help to clarify the situation, but when one considers the techniques used for their determination the division between them is not so clear. This review uses the term inexactly, as has been done in the literature, but the distinction between them and their interrelationships will be made clearer in chapters 3 and 4.


The relationship between density and quality is even less well defined for the mandible than it is for the rest of the skeleton. Treatment modalities for osteoporosis have traditionally aimed at increasing bone density, but this does not always result in the anticipated reduction in fracture incidence (Riggs et al 1982, Burckhardt & Burnand 1993). Bone quality is used to describe the material, architectural and mechanical characteristics of the tissue (Sherman & Hadley 1993) and must be considered together with bone density.

It is only since the recent upsurge in the use of dental osseointegrated implants that attempts have been made to define mandibular bone quality. The most frequently used classification for the mandible is a descriptive one based upon the intraoperative (or radiographic) arrangement of the bone in which four types are detailed (Lekholm & Zarb 1985). Quality 1 - nearly all compact bone; quality 2 - thick layer of compact bone surrounding dense trabecular bone; quality 3 - thin layer of compact bone surrounding
dense trabecular bone; quality 4 - thin layer of cortical bone with low density trabecular bone. Qualities 2 and 3 are thought to be the best types for implantation and failure of osseointegration is most likely in quality 4 bone (Bass & Triplett 1991).

One can see why this area of research may have caused difficulty before there was an agreed consensus as to what constitutes good quality bone, since the effects of disease and the aims of treatment could not be quantified. One example is that Sobolik (1960) thought that a radiographically dense appearance of alveolar bone was the most desirable, whilst Smith & Applegate (1961) argued that less dense bone was to be preferred because it was more 'organic' and hence more conducive to a normal rate of bone maintenance since its turnover would be facilitated. Even so the present classification is more mechanical than biological with no allowance being made for the cellular status, the blood supply, the age of the individual or the resilience of the bone tissue.

Periodontal disease
Several workers have found a weak, sometimes indirect, correlation between skeletal bone status and periodontal disease (Phillips & Ashley 1973, Manson 1976, von Wogram et al 1994), with teeth with large amounts of attachment loss being more likely to be retained in patients with higher skeletal bone densities (Klemetti et al 1994b). Some other studies have found a relationship between tooth number and skeletal bone density as measured at the radius (Krall & Dawson-Hughes 1993, Taguchi et al 1995) or other sites (Kribbs 1990), although attempts to use tooth number as a predictor for the existence of systemic osteoporosis fall woefully short of being useful (Taguchi et al 1995).

Ridge height
The majority of studies have found no relationship between the height of the mandible and the skeletal parameters tested, even when a good correlation of the bone density between the sites exists (Ward et al 1977, Dyer & Ball 1980, Kribbs et al 1983, Ortman et al 1989, von Wogram & Kollerup 1992, Taguchi et al 1995). However, Habets and co-workers (1988a,b) found that nearly all iliac crest biopsies from patients with severely resorbed mandibles showed disturbances in mineralization, and that over half had a secondary hyperfunction of the parathyroid glands. Unfortunately, no information was given on the time of year of examination, which can affect the incidence of mineral disturbances seen histologically in femoral and mandibular bone (Aaron et al 1974, Stutzmann et al 1981).
In a study of 10 patients, Bays & Weinstein (1982) found that patients with advanced mandibular resorption had low bone densities in the radius as measured by absorptiometry. In only one paper has the height of the residual ridge been positively correlated with the degree of severity of osteoporosis as determined from vertebral radiographs (Hirai et al 1993).

The reasons that most workers have found no correlation and that only a few have could depend upon two main factors. Habets and co-workers (1988a,b) and Bays & Weinstein (1982) selected their patients on account of their severely resorbed mandibular ridges. This might preselect the patients who are more likely to have systemic disease than a random selection of edentulous patients, especially as the ridges are more likely to be severely resorbed in more severe cases of osteoporosis (Hirai et al 1993).

The greatest problem in this area, however, stems from the use of the fact, given by Wical & Swoope (1974a), that the total height of the mandible is 2.9 times greater than the distance from the lower border of the mandible to the mental foramen. It is acceptable to use such a ratio in longitudinal studies (Packota et al 1988), where it eliminates differences according to size, but some have relied upon this ratio to calculate the pre-extraction height of the mandible, and hence calculate the loss that has occurred (Wical & Swoope 1974b, Ward et al 1977, Taguchi et al 1995). Such studies find no association between mandibular height and the skeletal variable tested. In the original study, the individuals used for calculating the ratio were only selected if certain criteria were met, such as having a full complement of teeth in a good arrangement, and a clearly visible mental foramen. No mention is made of the age range of the individuals that did fulfill the requirements and so it is not known whether this ratio can be applied reliably to the aging population, which may experience continued apposition (Whittaker et al 1990) or loss (Enlow et al 1986, Ulm 1989) on the lower border of the mandible throughout life. If this is true, one would anticipate the ratio of alveolar to basal bone to change with age, even without any changes occurring at the alveolar crest. Another important point is that great variability has been reported in the position of the mental foramen (Tebo & Telford 1950) which may be located between 9.0 mm and 18.5 mm from the lower border of the mandible. This would imply that the original height of the mandibles would range from 26.1 mm to 53.7 mm (a height perhaps more frequently attained by a great ape).

**Gender**

In general, it seems that the sex of the individual has some influence on mandibular bone. In edentulous individuals, mandibular height is greater in males than females although
there is no difference whilst the teeth are still present (Engstrom et al 1985, Ortman 1989). Devlin et al (1994) supported this view by reporting that alveolar crestal resorption lacunae are deeper in women than in men. However, Winter et al (1974) found males to show more bone loss than females in the posterior mandible which they attributed to the males having a greater bite force, and Bergman et al (1971) found no differences between the sexes. The porosity of the cortical bone in the femoral neck only correlates to mandibular cortical porosity in females (Dyer & Ball 1980), and von Wowern (1988) showed that the bone mineral content of the mandible in young dentate women is lower than in young dentate men.

Nutritional factors

Diet can have both a local and a systemic effect on the mandible. In rats, the consistency of the food has been shown to alter mandibular structure and low calcium diets result in resorption of mandibular bone (Sones et al 1986). Several human studies have suggested that a secondary hyperparathyroidism caused by lack of dietary calcium may affect mandibular structure (Syrğänen & Lampainen 1983, Habets et al 1988 a&b), and dietary calcium and vitamin supplements may help to maintain ridge size and mass at one year postextraction (Wical & Swoope 1974b, Kribbs 1992). However, Corten et al (1993) found absorptiometry techniques to be one third as sensitive for the mandible as for the lumbar spine and suggest that it would take around two years to detect reliably bone loss in the mandible at the rate that it usually occurs postmenopausally in the rest of the skeleton.

In a study of dental panoramic radiographs from 18 patients with secondary hyperparathyroidism, no correlation was found between serum calcium, inorganic phosphate, alkaline phosphatase or parathyroid hormone with the radiographic parameters tested, although the patients were judged to have fewer trabeculae compared with controls (Syrğänen & Lampainen 1983). With such a small number of patients, and the large subjective bias that panoramic radiographic assessment has (Klemetti et al 1994a), no firm conclusions can be drawn, yet it is interesting to note that no association has been found between primary hyperparathyroidism and residual ridge reduction (Lekkas 1989).

Midgett et al (1981) investigated the effect of raising parathyroid hormone levels by decreasing the dietary calcium:phosphate ratio on orthodontic movement of beagle teeth. Tooth movement occurred much more quickly in the hyperparathyroid dogs, which also showed less than one quarter of the amount of trabecular bone.
Age

Clinically it is known that age changes in the mandible may increase the difficulty of orthodontic tooth movement, extractions, or other surgery (Grant & Bernick 1972), but exactly what feature of aging causes these differences is unknown. The aging of the osteocytes has already been mentioned, and it is possible that their death could slow the rate of response during tooth movement by failing to aid in the response to strain; in addition, if the osteocytes do mineralize, they may contribute to changes in the mineral content of the tissue. The contained levels of insulin like growth factor are also known to change with age (Hammerman 1987, Nicolas et al 1994), which might be recognised as changing the rate of turnover of bone as it ages. These issues are addressed in chapters 4 and 7.

In some sites in the skeleton, bone may increase in mineral density with age (Boyde et al 1995b). The changing mineral density with age and location in the mandible is unknown. But the bone may become increasingly taken up by compact cortical bone (Humble 1936), even though radiographic and anatomical cortical thickness measurements have usually shown a decrease with aging as already stated (von Wowern & Stoltze 1979, Bras et al 1982, Kribbs 1990). The cortex also becomes increasingly porous with age (Atkinson & Woodhead 1968) but tends to increase initially in early adulthood prior to reducing in older age (Manson and Lucas 1962). Chapters 3 and 4 investigate the changes in mandibular density with age.
CHAPTER 2

INVESTIGATION OF CONDUCTIVE COATING AND EMBEDDING TECHNIQUES FOR SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) is a versatile technique providing a range of modes of study. In anatomical investigations the two most frequently used modes detect either the high energy reflected (backscattered) electrons or the low energy (secondary) electrons leaving the surface of the specimen. When an electron beam is incident upon a nonconducting material the latter becomes charged. This build up of electrons not only has a repellant effect upon the beam, but it may also discharge; both phenomena have a detrimental effect upon the image obtained, producing either lines or artefactually bright areas. For this reason, nonconductive specimens usually have a conducting coating applied in the form of sputtered or evaporated metal or evaporated carbon. Part I of this chapter describes a coating technique for use with secondary electron imaging; Part II describes (unsuccessful) attempts at producing alternative embedding media for backscattered electron analysis.

PART I

Secondary electron imaging

Secondary electron (SE) analysis is of use in studying complex three dimensional structures, and is thus of immense value in studying the trabecular architecture of bone. Problems are encountered, however, in trying to coat successfully such a complex and porous structure; sputtering coats the superficial trabeculae of the cut surface, but these often remain insulated from the rest of the structure. Even though this problem can be partly overcome by coating the specimen from different angles and using thinner sections, it is often not possible to coat all aspects of the deeper trabeculae. Coating techniques which rely upon immersion of the specimen have the advantage that it should be possible to place a continuous layer upon all aspects of the bone. The dipping of bone into an organic antistatic agent (Duron) has been described (Boyde 1984), but immersion in metal coating solutions for SEM has not been detailed. Electroless plating (so called because it does not require the use of electrodes) is a process by which nonconductive specimens can be plated with metal (Canning 1953, Reidel 1991). Since the specimen is completely immersed, all aspects of the sample can be coated simultaneously. The use of this technique for the preparation of bone specimens for SEM is investigated.
Method

Electroless coating with nickel

The nickel coating procedure is based on a technique described by Reidel (1991). The prepared bone specimens (which had been prepared by a combination of mechanical (waterpik) and biochemical means) were sonicated in solution A (see below) for 5-10 seconds then immediately washed in distilled water. After removal of excess moisture, the specimens were then suspended in solution B which had been heated to 40°C in a waterbath. Small amounts (a few ml) of solution C were added slowly until effervescence occurred: the samples were removed after various time intervals between five seconds and thirty minutes and sonicated in distilled water. Specimens which had been selected for complete embedding in nickel were left for four hours (during which time the solutions were refreshed to maintain effervescence) and were then coated electrolytically in solution D with a current density of 0.33 mA/mm² at approximately 50° (a current density greater than this was found to cause a dendritic deposition).

Solution composition

A - an acidified solution of palladium chloride (Aldrich Chemical Co Ltd, Gillingham, Dorset, SP8 4JL) (containing 10-50mg palladium chloride dissolved in 100ml 0.1M hydrochloric acid).

B - an aqueous solution containing 0.23M nickel (II) chloride (Aldrich) and 0.28M citric acid (Aldrich), adjusted to a pH of 7 with ammonia.

C - 0.84M solution of dimethyl aminoborane (Candorchemie Gmbh, Prinz-Regent Str 48, D-4630 Bochum, Germany) dissolved in ethanol.

D - a mixture of 0.92M nickel (II) sulphate (Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire), 0.13M nickel (II) chloride and 0.49M boric acid (Fisons).

Copper coating

A similar procedure was used as above, but, after palladium chloride treatment, the bones were suspended in an aqueous solution containing 0.15M copper (II) sulphate (BDH Laboratory Supplies, Poole, BH15 1TD), 0.66M potassium sodium tartrate (Aldrich) and 2.07M sodium hydroxide at room temperature; the reductant in this instance was 38% (w/v) formaldehyde (Canning 1953).

Upon removal from the plating solutions, the specimens were rinsed in distilled water (or acidified palladium chloride solution) to remove any loose surface debris and dried in an oven or vacuum. The surface resistivity of each specimen was then calculated (using a four probe test apparatus) prior to viewing in a scanning electron microscope.
Results
All specimens, irrespective of treatment time, appeared to have a continuous adherent surface coating which covered all aspects of the bone including fine trabeculae at the centres of the largest samples tested (ca 10 x 20 x 30mm). The coating was black (in those specimens which had been treated for less than 30 minutes) or silver in colour and thus provided good contrast for optical microscopical applications. The depth to which the coating could penetrate a bone with a complex morphology was extremely good (figure 2.1, overleaf)

Specimens treated for less than 30 minutes had surface resistivities between 13 and 23 ohms, which may be compared with a mounting medium ("Leit C Plast", plastic conductive carbon cement, Neubauer Chemikalien, D-4400 Münster, Germany) value of 136 ohms. The specimens coated for four hours or more had surface resistivities of less than 0.1 ohms.

Up to instrumental magnification values of 100x, the microscopical surface texture was not unduly perturbed by the metallic crystalline structure of the applied coating. The appearance was acceptable (figure 2.2, overleaf), and charging effects were dramatically reduced when compared with conventionally coated samples of the same nature.

It was found to be important to rinse the specimen thoroughly upon removal from the plating solution or loosely adherent surface debris remained which was prone to charging. At higher resolution, the metal totally obscured the fine structure of the bone: details such as the collagen fibre arrangement and resorption pits could no longer be readily identified (figure 2.3, overleaf).

The thickness of the metal coating of the heavily coated samples could be measured directly in the SEM after one surface of the bone had been polished to expose the profile of the coating. In general, bones treated for longer than 10 minutes were found to have coating thicknesses in the region of 10-20μm.

During electroplating, approximately 1 g/100 mm² nickel was deposited on a specimen in 24 hours. Once one surface had been polished to expose the bone the surface resistivity of the bone on that side was found, as would be expected, to have increased markedly (from < 0.05 ohms prior to polishing to > 1 x 10⁷ ohms afterwards). The larger pore spaces within these specimens were seen to have been penetrated.
Figure 2.1  Photographs of a segment of the body of the mandible which has been coated by the electroless method described in the text. The mandibular segment was coated whole and has been sectioned in the lower picture, to reveal the depth to which this coating method can penetrate.
Figure 2.2 Secondary emission scanning electron micrograph, 10kV, Zeiss DSM 962 SEM, horse radius slab coated by the electroless method described in the text for 30mins, then rinsed in water. 10kV SE. Scale marker for 2mm in image.

Figure 2.3 Same specimen as above. 30mins treatment (far longer than is necessary to secure a continuous coating). The crystalline structure of the metal obscures the fine detail of the bone. 4kV SE. Scale marker for 20μm in image.
Discussion
This procedure provides a satisfactory solution to coating awkward three dimensional structures such as trabecular bone and may be tested as an alternative to established procedures. For low magnification, wide field, SEM where the fine structure of the sample surface is not important, the procedures outlined here may have special advantages.

Alternative procedures for dealing with intractable charging problems in porous composites include (a) soaking in a solution of an organic antistatic, which may also lead to obscuration of surface detail but has the advantage of optical transparency for cathodoluminescence applications (Boyde 1984): (b) using a low accelerating voltage, e.g. 1-2kV and video-rate scanning and frame averaging, but this will usually be limited to 512 line resolution: (c) the use of backscattered electrons and an applied positive surface voltage to inhibit low energy electron emission (Boyde and Cowham 1980) and (d) the use of uncoated samples, careful selection of the accelerating voltage (in our case to ca 1.24kV) and a multichannel plate detector - such detectors are currently very expensive and require exceptionally careful operation. In particular, the vacuum conditions required (better than 5x10^-6 Torr) are rather stringent.

Sputter coating is obviously very convenient, and if surface detail can be sacrificed, charging problems can be eliminated by applying thick coatings. However, most laboratories will be equipped with coaters with precious metal targets, so that cost will become an important element. In comparison, the time cost of preparing the solutions used here contrasts favourably with the labour involved in cleaning either a sputter or an evaporative coater after heavy use.

Conclusions
Specimens with complicated, intricate three dimensional structures, which are otherwise difficult to coat adequately by conventional means (sputter or evaporative coating) for scanning electron microscopy, can quickly and simply be rendered conductive by electroless plating. However, this technique should only be used when studying specimens at low magnification as fine detail may be lost.
PART II

Backscattered electron analysis

Backscattered electrons (BSE) provide similar information to that obtained from the secondary electrons, but if applied to a surface with minimal topographical relief, they can also provide information upon the variation in the mean atomic number of the substrate. This allows a 'density' map of the tissue to be obtained where the areas with a high signal (white) represent those with high atomic number, and conversely a low signal (black) represents a low atomic number. With bone, the flat surface is best obtained from an embedded sample which has been polished or milled. The most commonly used embedding medium for this application is poly(methylmethacrylate) (PMMA) which is capable of taking a high polish and also has the advantage of having a monomer with very low viscosity. In other respects it is far from ideal: exhibiting a substantial shrinkage upon polymerisation, being insulating in nature and being unstable under exposure to an electron beam. Its insulating properties are generally overcome by using a coating of sputtered carbon. If a specimen is being used for a serial milling procedure to give three dimensional information of the specimen, then this coating must be reapplied each time. It may be that this process would be unnecessary with a conductive embedding medium. For this reason, alternative methods of embedding were investigated including the use of conductive polymers, as described below.

The conductivity of plastics and other insulating materials may be increased in a number of ways, usually involving the incorporation of conducting particles or polymers, which may also reduce the polymerisation shrinkage. When particles are used, the loading has to be sufficiently high for electrical continuity to exist. This level of loading not only alters the mechanical properties of the material but may significantly increase the viscosity of the monomer, impairing its ability to penetrate small, or even moderately sized, pores. Particles (such as graphite) that do not adhere to the matrix have a tendency to settle during the long curing period, and also to rub off the surface of the block.

Inherently conducting polymers (ICPs) have been the subject of much interest over the last two decades, mainly in connection with their potential application in the electronics industry. There are two main groups of ICP: one includes polymers based upon pyrrole, thiophene and indole which all have a heterocyclic structure; and the other includes aniline, azulene and pyrene which are aromatic. The polymers may be produced chemically by oxidation with iron III (Bocchi & Gardini 1986) or copper II salts (Myers 1986), electrochemically or by radiation polymerisation. Chemical polymerisation requires the least sophisticated equipment and since the technique might be suitable for
wider applications, the simplest approach was chosen which was thought unlikely to
damage the bone. Since aniline involves a stage in synthesis that requires a low pH or a
high temperature, pyrrole, thiophene and 3-methylthiophene were selected for further
investigation.

ICPs tend to exist as soft powders with a particle size in the region of 75 nm (Armes and
Vincent 1987). Because of this they are unsuitable for use on their own as embedding
media; however, like particles, they may be introduced into a nonconductive polymer
matrix, and have the advantage that they may form a continuous phase rather than a
dispersion (Bargon 1987), as long as the two polymers are compatible (Hotta et al
1987).

Method
A number of methods of incorporating ICPs into PMMA were studied. These included:

1. imbibition of PMMA with the ICP monomer followed by its polymerisation
This technique relies on the fact that pyrrole can penetrate the surface of a block of
PMMA. PMMA blocks measuring 20 x 20 x 50 mm were placed into pyrrole monomer
for 30 minutes. The impregnated blocks were then placed into a 0.2 M aqueous solution
of ammonium peroxydisulphate and sodium paratoluene sulphonate to oxidise the
monomer and bring about polymerisation, after which they were washed and oven dried.
Additionally, PMMA slabs measuring 2 x 20 x 60 mm were suspended over pyrrole
monomer before oxidising in a saturated aqueous solution of iron III chloride.

2. polymerisation of pyrrole in dissolved PMMA
2 g of pyrrole were polymerised in a solution containing 7.7 g iron III chloride and 3 g of
PMMA dissolved in 25 ml dichloromethane.

3. polymerisation of 3-methylthiophene (3-MeT) in dissolved PMMA
0.19 g nitrosonium tetrafluoroborate was added to a solution containing 0.83 g 3-MeT
and 1 g PMMA dissolved in a 50:50 solution of chloroform and dichloromethane. After
evaporation of the remaining solvent, the mass produced was washed in acetone.

4. polymerisation of both the pyrrole and the MMA
1.12 g pyrrole was introduced into a solution of 1.76 g MMA, 1.2 g iron II sulphate and
0.4 g iron III chloride and left in a refrigerator. When the reaction was complete, the
solution was washed in water and dried.
5. prior manufacture of polypyrrole prior to embedding in PMMA
In this instance, the pyrrole was polymerised first prior to embedding in PMMA.

6. electrochemical formation of ICP in solubilised PMMA; A bone slice which had a very thin coating of polypyrrole applied by dipping the bone in pyrrole and then into a dilute aqueous solution of iron III chloride, was suspended in an electrochemical bath containing a solution of 0.2 M pyrrole, 0.2 M tetrabutylammonium toluene-4-sulphonate and PMMA dissolved in dichloromethane.

Results and Discussion
It was possible to improve to a small extent the conducting properties of PMMA, but most of the techniques could not be applied readily to the embedding of bone.

1. imbibition of PMMA with the ICP monomer followed by its polymerisation
To ensure adequate penetration of the pyrrole monomer into the PMMA blocks, it was necessary to allow a prolonged soaking period. However, since PMMA is slightly soluble in pyrrole, the block surface was partially dissolved. This meant that after polymerisation the polypyrrole produced a poorly adherent black and sticky surface which would not be suitable to prepare for electron microscopy.

The thin slabs of PMMA that were suspended over pyrrole became only slightly softened in the process. This allowed the formation of a more adherent polypyrrole layer, but the coating produced was too thin and uneven to be conductive.

2. polymerisation of pyrrole in dissolved PMMA
This reaction produced a black gel-like nonconducting mass. The softness was probably due to the presence of impurities, both the unreacted pyrrole and excess FeCl₃. These impurities may have in part accounted for the poor conductivity, but the use of dissolved PMMA (rather than forming PMMA from MMA in situ) probably hinders the complete mixing of the two polymers which may help in insulating the structure.

3. polymerisation of 3-methylthiophene (3-MeT) in dissolved PMMA
This experiment produced a hard, very poorly conducting (1 x 10⁻⁴ S/cm) solid.

4. polymerisation of both the pyrrole and the MMA
This experiment used iron II and iron III salts in the 3:1 ratio as this was found to produce the best conductivity in the chemical polymerisation of pyrrole (Kathirgamanathan 1993). This experiment produced a copolymer of polypyrrole and
PMMA with a reasonable conductivity (0.026 S/cm), but it was in the form of a powder which had to be compressed into a pellet before a contiguous conducting mass was obtained. This could not be used for embedding a delicate structure like trabecular bone.

5. prior manufacture of polypyrrole prior to embedding in PMMA
The chemical polymerisation of pyrrole results in a fluffy, matted material. This can be embedded successfully in PMMA, but it would probably not be possible to incorporate the polypyrrole as a single continuous mat for the embedding of bone.

6. electrochemical formation of ICP in solubilised PMMA
The electrolytic process used in this experiment requires there to be a current density in the order of 1.5 mA/cm². However, the dissolved PMMA impaired the conductivity of the solution so greatly as to only allow the passage of 0.5 mA. The particular bone specimen used in this experiment had a surface area of approximately 4 cm² so the current density was very much reduced. In addition, continued evaporation of the solvent rapidly worsened the situation.

Conclusion
The conductivity of PMMA can be improved by loading with a variety of conducting fillers yet, for anatomical investigations, there is currently no simpler or more effective method than the use of sputtered carbon and conductive carbon paint for the examination of block surfaces for BSE imaging. This method also avoids the problems caused by variation in the composition of the polymer mixture from region to region which could affect the local electron backscattering properties of the medium, making it unsuitable for use with a highly quantitative technique such as that used in chapter 4 of this thesis. However, there are many instances where it may be desired to re-embed or remount a block, and where the enclosing or enshrouding resin might advantageously have excellent conductive properties. The use of the materials studied above might be considered in such applications.
CHAPTER 3

MANDIBULAR APPARENT DENSITY VARIATION WITH SEX, AGE, DENTAL AND SKELETAL STATUS

As mentioned in chapter 1, interpretation of the literature regarding mandibular changes with aging and osteoporosis is complicated by the wide range of techniques which have been employed and the different parameters investigated.

Most often, density is correctly used to refer to the bulk mass per unit volume of a substance as measured in g/cm$^3$. With bone, it is the reduction of this, together with changes in the architecture, that is responsible for the pathological fractures that occur in osteoporosis. Information on this 'apparent' density has been obtained in many ways. Direct methods such as weighing the bone and calculating the volume by water displacement or other equivalent techniques (Atkinson & Woodhead 1968, Dyer & Ball 1980) have the advantage of simplicity and reliability, but cannot, of course, be applied clinically. Histological sections of small samples of bone (such as bone biopsies from the iliac crest) provide a two dimensional picture of the bone:marrow space ratio and can be further analysed by a variety of histomorphometric techniques to provide some measure of the bone architecture, but these have rarely been applied to the mandible (Devlin et al 1994, Elovic et al 1995).

Clinically, bone densitometry (using dual photon, or dual energy x-ray absorptiometry) has become increasingly widely used as a noninvasive and relatively quick method for skeletal assessment, but takes little account of the overall size of the bones and returns a measurement of mass per unit area rather than volume.

Apart from the histological techniques which are only influenced by the amount of bone in the section, most techniques will be influenced by the quantity as well as by the level of mineralization of the bone tissue. In this thesis, the measure of bone density in terms of mass per unit volume will be referred to as 'apparent density' and the measure of how highly mineralized the tissue is as 'mineralization density'. This and the next chapter look at how these two aspects of density of the mandible change with aging and compare with other sites of the skeleton. The relationship between the two is investigated in chapter 4.

Material and Methods

Human mandibular material was collected from oral histopathology laboratories, museums and dissecting rooms. For 14 of the dissecting room cadavers, samples of fourth lumbar vertebra (L4), iliac crest (IC) and femoral neck (FN) were also harvested.
Cleaning
All material was cleaned with an enzyme detergent solution (Terg-A-Zyme®, Alconox Incorporation, 215 Park Street South, New York, NY 1003, using 7.5 g of powder per litre of water at 40°C, Boyde 1984, Reid & Boyde 1987). The soft tissue on the dissecting room material was resistant to digestion so the bones were first sectioned and washed (>100 hours (h) in running tap water - after Nimni (1994) personal communication) before cleaning in frequent changes of Terg-A-Zyme solution for 1-2 years. Despite this prolonged cleaning period, the contents of the inferior dental canal had to be dissected out by hand, care being taken to avoid contacting the bone with metal instruments in order to minimise surface damage. The use of a water pick to remove the marrow contents was also avoided for fear that the more fragile trabeculae within the thin bone slices might be lost.

Sectioning
Mandible
The specimens were clamped in a specially constructed holder and were cut into 2 mm thick vertical slices using a low speed water-cooled diamond saw (Labcut 1010, DR Bennett Limited, Leicester, UK) with minimal weight. The bones were oriented so that the cut took the shortest route across the cortex, which meant that large fragments had to be reclamped several times to allow for the curvature of the bone. In most instances the whole hemi-mandible, or as much as was present, was sectioned. Slices from the mandibular midline (MD specimens) and 2-6 mm posterior to the mental foramen (MF specimens) were selected for apparent density analysis. The midline site corresponds to the site investigated in the longitudinal studies carried out by Lönberg (1951), Atwood (1963), Tallgren (1972) and Carlsson & Persson (1967): the mental foramen site has been used by many previous researchers (Parfitt 1962, Carlsson & Persson 1967, de Aguiar et al 1968, Atkinson and Woodhead 1968, Wical & Swoope 1974a, von Wowern & Stoltze 1977, Kribbs et al 1983, Kribbs et al 1989, Benson et al 1991, Horner & Devlin 1992, Hirai et al 1993, and Taguchi et al 1995) and has become the 'standard site' for anatomical investigations of the mandible (von Wowern & Stoltze 1979).

Postcranial bone
Three slices 4 mm thick were taken from each of the bones using the same water-cooled diamond saw as above. The bodies of the lumbar vertebrae were sectioned in the mid-sagittal plane; the anterior portion of the ilium of the pelvis was sectioned in the horizontal plane so as to include the crest and the site 2 cm posterior and inferior to the anterior iliac spine preferred for iliac crest biopsies; and the femoral neck slices were taken across the maximum constriction of the neck.

All of the sections were cleaned, washed in distilled water, and dehydrated in ethanol.
Radiography

Before sectioning, the wet mandibles from the individuals for which other bone slices were available were radiographed on a custom-built microfocal x-ray unit which has an effective spot size of 6-8 μm (Buckland-Wright 1989, Buckland-Wright & Bradshaw 1989) at a focus-film distance of 1 metre, 50 kV, 150 mA and 0.02 secs. After sectioning, all the slices obtained were radiographed at a set distance both from a lateral projection (60 kV, 3mA and 20 secs) and from a mesio-distal direction (50 kV, 3mA and 20 secs) in an x-ray cabinet fitted with a 0.2 mm aluminium filter (Todd Research Ltd, in the Institute of Archaeology, UCL). All radiographs were taken with the specimens lying directly upon the film thus minimising any magnification effects.

These radiographs were used for measurement of the thickness of the inferior mandibular cortex (Klemetti et al 1994a, Taguchi et al 1995). The radiographs were placed on a light box and landmarks were traced onto tracing paper. Intervals between the landmarks were measured with digital callipers to the nearest 0.5 mm.

Lateral projections

The interval between the highest point on the alveolar crest and the lowest point on the mandibular border was taken as a measure of the overall height of the mandible on a line passing through the mental foramen (Taguchi et al 1995). The centre of the mental foramen was marked, as was the internal extent of inferior mandibular cortex. Due to the indefinite transition between cortical and trabecular bone, the cortex was taken as having its limits where the bone first appeared to be perforated. No attempt was made to correct for body or head size.

No equivalent measurement was made for the midline, because orientation of the curved fragments was difficult, the radiographic exposure required to penetrate the great volume of bone at the base of the midline caused 'burn out' at the alveolar crest, and because there is no clinical radiographic equivalent which allows examination of the full depth of the mandible in the midline.

Antero-posterior projections

The slices were laid flat on the film and were thus viewed in an antero-posterior direction. Lines were drawn across from the following landmarks:

- alveolar crest - ac,
- midpoint of the mental foramen - mf,
- lower border of the mandible - lb, and the internal surface lower border cortex to a line along which they were all measured (figure 3.1). Care was taken that the midpoint of the mental foramen was the same on the two projections, especially where it occupied a position on the crest of the ridge where it was important to distinguish it from
the inferior dental canal. Thus measures of mandibular height were obtained from the lower border to the mental foramen (which has been taken to represent basal bone height in some studies), from the lower border to the alveolar crest (total mandibular height), and from the mental foramen to the alveolar crest (in some studies taken as alveolar bone height). The cortical thickness of each slice was also measured.

![Figure 3.1 Radiographic measurements](image)

**Photography**

The specimens were photographed on a tilting table using a 35 mm camera with an 80 mm macro lens. Two photographs were taken on both sides of every specimen at a tilt of 5° each side of the normal, with all other settings remaining the same. The stereophotographs produced were viewed on a Stereosketch (Hilger and Watts, England) where outlines of each cut surface of the slices were traced onto paper and cut out. This procedure allowed the true two dimensional area of each cut surface to be determined by active reference to the three dimensional image (Howell et al 1986). The mean cross sectional areas of the slices were then calculated from the mean weight of the cut-outs of the two sides. All the photographs for each group of specimens were taken under the same conditions, ie all mental foramen, all midline mandibular and all the post-cranial bone slices. Because each specimen was photographed with a millimetre scale it was possible to work out for each group the weight of paper that corresponded to an area of 10*10 mm. Thus each group could be calibrated and once corrected for differences in magnification, accurate comparisons between the groups could be made.

The bone slices were weighed on a pan balance (Sartorius, Göttingen, Germany) accurate to 0.01 g, and were reweighed over a period of days until a constant reading for the bone was obtained. Teeth, where present, were removed prior to weighing the slices.

The volume for each slice was then calculated by multiplying the calculated area by the mean thickness of the slice as measured at three sites using digital callipers accurate to 0.01 mm (Mitutoyo, Japan). It was then possible to establish the true 'apparent' density of the specimens in g/cm³.
Analysis

The mandibular material was analysed for sex, age, dental and skeletal status using Minitab™ (Minitab, Inc) statistical software.

For dental status, the mental foramen specimens were separated into completely dentate ('d' in figures), partially dentate ('p' - having no more than one molar tooth) and edentate ('e'). Since the partially dentate group all had anterior teeth, the midline specimens were only separated into dentate and edentate, with no separate group for the partially dentate. On a few occasions too little of a specimen was available to determine the number of teeth.

For determining a relationship with skeletal status, only the mandibular sections from individuals with more than one skeletal site represented were used. The two sample t-test was used to assess the differences between whole groups of data; Student's paired t-test was used to reveal trends between different sites within an individual; and Pearson's linear correlation coefficient was used in assessing the relationship of one variable to another, as well as for correlating between the measurements from the lateral and the antero-posterior radiographic projections. In the graphs, the regression line is only shown where a significant correlation exists. Unless otherwise stated, standard error of the mean (sem) is presented.

Results

Apparent density measurements

Mental foramen (MF) site

A total of 44 individuals was analysed. The details of the sample are as in table 3.1. In addition, there were two neonates of unknown sex, which were excluded from the analysis of the adult material.

Table 3.1 Number, age (in years) and sex distribution of MF specimens

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>mean</th>
<th>median</th>
<th>sd</th>
<th>sem</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>42</td>
<td>74.9</td>
<td>78.0</td>
<td>13.1</td>
<td>2.0</td>
<td>35 - 96</td>
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<tr>
<td>male</td>
<td>25</td>
<td>70.2</td>
<td>72.0</td>
<td>14.1</td>
<td>2.8</td>
<td>35 - 91</td>
</tr>
<tr>
<td>female</td>
<td>17</td>
<td>80.7</td>
<td>82.0</td>
<td>9.2</td>
<td>2.2</td>
<td>56 - 96</td>
</tr>
</tbody>
</table>

The males were significantly younger than the females (p<0.006), but there was no difference in apparent density between the groups (table 3.2, overleaf), nor was there if the edentulous, partially dentate and dentate individuals were compared (means (sem): 1.28 (0.05) g/cm³, n=21; 1.17 (0.08) g/cm³ n=7; 1.16 (0.57) g/cm³, n=7).
Figure 3.2 Bone apparent density against age - mental foramen site
Figure 3.2 shows the plots of apparent density against age for the mental foramen site. There is no significant correlation between apparent density and age at this site (all: \( r=0.13, p=0.31 \); male: \( r=0.32, p=0.11 \); female \( r=-0.06, p=0.81 \)), even when the sample is divided into dentate, edentate or partially dentate groups (dentate: \( r=-0.48, p=0.28, n=9 \); edentate: \( r=0.26, p=0.29, n=19 \); partially dentate: \( r=0.38, p=0.28, n=10 \)). However, if the youngest male is removed from the analysis, the males do show an increase in density with age (\( r=0.51, p<0.02 \), regression equation: \( y=0.01x+0.56 \)); the section for this male came from a site slightly posterior to that used for the rest of the sample and included the socket for the mesial root of the lower first molar.

The mean apparent density of the dentate group \((1.16 (0.57) \text{ g/cm}^2, n=7)\) was no different to that of the edentulous \((1.28 (0.05) \text{ g/cm}^2, n=21)\) or that for the partially dentate \((1.17 (0.08) \text{ g/cm}^2, n=7)\) individuals. The mean age for the dentate individuals (69 years) was significantly lower than for the edentulous (81 years) but not for the partially dentate (73 years).

The cross sectional area of the mandible reduced with age (\( r=-0.50, p<0.001 \)) and with loss of the teeth. Edentulous and partially dentate mandibles were significantly smaller (\( p<0.001 \) and \( 0.005 \)) than dentate mandibles (mean areas (sem): 1.42 (0.16), 1.77 (0.10) and 2.58 (0.19) cm\(^2\) respectively).

Not all the mandibles had been radiographed from the lateral aspect before sectioning, therefore the radiographic analysis was performed using the measurements obtained from the slices postsectioning. However, for those that had measurements from both, the correlation between the measurements from the two projections was tested and found to be satisfactory (cortical thickness \( r=0.79, p<0.001, n=17 \); basal bone height \( r=0.79, p<0.001, n=15 \); total bone height \( r=0.99, p<0.0001, n=17 \)) (table 3.3).

### Table 3.2: Apparent densities of adult MF specimens (g/cm\(^2\))

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>mean</th>
<th>median</th>
<th>sd</th>
<th>sem</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>42</td>
<td>1.23</td>
<td>1.17</td>
<td>0.23</td>
<td>0.04</td>
<td>0.81 - 1.77</td>
</tr>
<tr>
<td>male</td>
<td>25</td>
<td>1.25</td>
<td>1.17</td>
<td>0.23</td>
<td>0.05</td>
<td>0.90 - 1.63</td>
</tr>
<tr>
<td>female</td>
<td>17</td>
<td>1.22</td>
<td>1.17</td>
<td>0.24</td>
<td>0.06</td>
<td>0.81 - 1.77</td>
</tr>
</tbody>
</table>

### Table 3.3: Mean results from antero-posterior radiographic measurements (mm)

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>mean</th>
<th>sd</th>
<th>sem</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>cortical thickness</td>
<td>37</td>
<td>3.46</td>
<td>0.95</td>
<td>0.16</td>
<td>1.50 - 5.00</td>
</tr>
<tr>
<td>basal bone height</td>
<td>35</td>
<td>13.30</td>
<td>2.53</td>
<td>0.43</td>
<td>6.00 - 17.50</td>
</tr>
<tr>
<td>whole bone height</td>
<td>37</td>
<td>23.47</td>
<td>7.82</td>
<td>1.29</td>
<td>6.50 - 34.50</td>
</tr>
<tr>
<td>alveolar bone height</td>
<td>35</td>
<td>10.11</td>
<td>6.15</td>
<td>1.04</td>
<td>0.50 - 19.50</td>
</tr>
</tbody>
</table>
Figure 3.3A Bone apparent density against cross-sectional area
- mental foramen site

Figure 3.3B Bone apparent density against cortical thickness
- mental foramen site
The radiographic measurements of bone height were all highly correlated (p<0.0001): basal bone height (lb to mf) with total bone height (lb to ac) r=0.81; basal bone height with alveolar bone height (mf to ac) r=0.64; and total bone height with alveolar bone height r=0.97.

The graph for apparent density against area at the mental foramen site is shown in figure 3.3a. The significant negative correlation (r=-0.45, p<0.003) could arise as the shrinking mandible is increasingly occupied by a relatively greater cortical bone fraction. The cortical thickness measurements are positively correlated with apparent density (r=0.43, p<0.02) (figure 3.3b), but have no correlation with the cross sectional area (r=0.17, p=0.34, n=32) or the measures of mandibular bone height. However, there is a good correlation between cross sectional area and the measurement from the inferior border of the mandible to the middle of the mental foramen (r=0.74, p<0.0001) and to the alveolar crest (r=0.88, p<0.0001), as well as from the mental foramen to the alveolar crest (r=0.83, p<0.0001).

As would be expected from the above, there is a negative correlation between the apparent density and the measurement from a) the inferior border of the mandible to the middle of the mental foramen (r=-0.62, p<0.002), b) the inferior border of the mandible to the alveolar crest (r=-0.64, p<0.001), and c) the mental foramen to the alveolar crest (r=-0.59, p<0.001), as there is between apparent density and area as a whole.

**Midline (MD) site**
Twenty eight individuals were examined. The details of the age and apparent density values are shown in tables 3.4 and 3.5. The ages of the males and females differ significantly (p<0.05), but there was no difference in the apparent densities.

**Table 3.4 Number, age & sex distribution of MD mandibular specimens**

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>mean</th>
<th>median</th>
<th>sd</th>
<th>sem</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>28</td>
<td>72.4</td>
<td>76.5</td>
<td>16.8</td>
<td>3.2</td>
<td>19 - 92</td>
</tr>
<tr>
<td>male</td>
<td>18</td>
<td>67.7</td>
<td>73.0</td>
<td>18.9</td>
<td>4.5</td>
<td>19 - 86</td>
</tr>
<tr>
<td>female</td>
<td>10</td>
<td>80.8</td>
<td>82.5</td>
<td>6.9</td>
<td>2.2</td>
<td>70 - 92</td>
</tr>
</tbody>
</table>

**Table 3.5 Apparent densities of MD specimens (g/cm²)**

<table>
<thead>
<tr>
<th></th>
<th>n=</th>
<th>mean</th>
<th>median</th>
<th>sd</th>
<th>sem</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>all</td>
<td>28</td>
<td>1.34</td>
<td>1.33</td>
<td>0.22</td>
<td>0.04</td>
<td>0.97 - 1.59</td>
</tr>
<tr>
<td>male</td>
<td>18</td>
<td>1.39</td>
<td>1.35</td>
<td>0.24</td>
<td>0.06</td>
<td>1.03 - 1.91</td>
</tr>
<tr>
<td>female</td>
<td>10</td>
<td>1.26</td>
<td>1.28</td>
<td>0.18</td>
<td>0.06</td>
<td>0.97 - 1.59</td>
</tr>
</tbody>
</table>
Figure 3.4 Apparent density against age - mandibular midline

\[ r = 0.53 \]

Figure 3.5 Bone apparent density - mandibular midline against mental foramen site

\[ r = 0.64 \]
The plots of apparent density against age for the midline can be seen in figure 3.4. In the male there is a positive correlation between apparent density and age ($r=0.53$, $p<0.03$, $y=0.01x + 0.94$) in the female there is a much narrower age range and no significant correlation is seen ($r=-0.44$, $p=0.21$). The correlation between apparent density and age for males is even greater if only dentate individuals are analysed ($r=0.91$, $p<0.002$, $y=0.01x + 0.98$). The positive correlations between density and age at the midline and at the mental foramen both have a gradient of 0.01.

As with the mental foramen site, midline cross sectional area decreases with increasing age ($r=-0.46$, $p<0.02$). Likewise, as it decreases, there is an increase in the apparent density ($r=-0.48$, $p<0.02$). However, there is no significant relationship between area and age when the sample is analysed for dentate or edentate status. The mean area for the dentate individuals was significantly greater ($p<0.05$) than the edentulous (means (sem): 2.68 (0.16), n=14; and 2.08 (0.19) cm$^2$, n=14 respectively). There was no significant difference in mean age (dentate 67.60 (5.38) and edentulous 77.93 (2.13) years).

The mean apparent density of the edentulous individuals was greater than that for dentate individuals (mean (sem): 1.43 (0.07); 1.26 (0.04) g/cm$^3$).

Comparing the sites within one mandible, there is a significant correlation between the apparent density at the mental foramen and the midline ($r=0.64$, $p<0.001$, $y=0.63x+0.59$) (figure 3.5) being greater at the midline (means (sem) MF 1.19 (0.04); MD 1.34 (0.04) g/cm$^3$, $p<0.001$ paired t test). The same is also true of area ($r=0.87$, $p<0.0001$; MF 1.72 cm$^2$; MD 2.30 cm$^2$, $p<0.0001$).

**Postcranial bones**

Fourteen individuals with a mean age of 80.36 years were analysed, 9 female and 5 male. The causes of death and ages are presented in table A.1 in the appendix. The mean areas and apparent densities for the postcranial bones are shown in table 3.6. The fourth lumbar vertebra has a significantly lower apparent density ($p<0.0001$) than both the iliac crest and the femoral neck. Iliac crest areas are not given, as the depth to which the sections were taken was not standardised.

<table>
<thead>
<tr>
<th></th>
<th>L4</th>
<th>FN</th>
<th>L4</th>
<th>IC</th>
<th>FN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mean</strong></td>
<td>8.66</td>
<td>8.25</td>
<td>0.24</td>
<td>0.54</td>
<td>0.48</td>
</tr>
<tr>
<td><strong>sd</strong></td>
<td>1.15</td>
<td>1.92</td>
<td>0.07</td>
<td>0.16</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Figure 3.6 Area against apparent density for mandibular and postcranial sites

- **L4**
  - Slice area (cm²)
  - Apparent density (g/cm³)

- **IC**
  - Slice area (cm²)
  - Apparent density (g/cm³)

- **FN**
  - Slice area (cm²)
  - Apparent density (g/cm³)

- **MF**
  - Slice area (cm²)
  - Apparent density (g/cm³)
  - Correlation: r = 0.60

- **MD**
  - Slice area (cm²)
  - Apparent density (g/cm³)
Correlation between variables for cranial and postcranial bones

The correlations of the apparent density plots of the different bones against each other are shown in Table 3.7. The correlations between the post-cranial bones are high, but they relate poorly to the bone apparent density values obtained for the mandible.

<table>
<thead>
<tr>
<th></th>
<th>MF</th>
<th>MD</th>
<th>L4</th>
<th>IC</th>
<th>FN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF with</td>
<td>-</td>
<td>0.56</td>
<td>0.16</td>
<td>0.02</td>
<td>0.17</td>
</tr>
<tr>
<td>MD with</td>
<td>-</td>
<td>-0.05</td>
<td>-0.24</td>
<td>-0.04</td>
<td></td>
</tr>
<tr>
<td>L4 with</td>
<td>-</td>
<td>-</td>
<td>0.87</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>IC with</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.80</td>
<td></td>
</tr>
</tbody>
</table>

Only the post-cranial bones were significantly correlated with each other. Lumbar vertebra with iliac crest p<0.0001, lumbar vertebra with femoral neck p<0.002 and iliac crest with femoral neck p<0.001.

Graphs of area against density for each site are shown in Figure 3.6. Table 3.8 shows the correlations; the mental foramen site was significant (p<0.05, y=-2.05x+5.14).

<table>
<thead>
<tr>
<th></th>
<th>MF</th>
<th>MD</th>
<th>L4</th>
<th>IC</th>
<th>FN</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF with</td>
<td>-0.60</td>
<td>-0.58</td>
<td>-0.23</td>
<td>-0.26</td>
<td>-0.52</td>
</tr>
</tbody>
</table>

Morphological observations

Mental foramen site

The variation in size, shape and structure for the 14 individuals with bone samples from multiple skeletal sites can be seen in Figure 3.7 (overleaf). Even from this small sample, it can be seen how the apparent density of the bone can be independent of area, height or size.

The location and condition of the inferior dental canal is variable. The trabecular thinning may be most noticeable either below or above the canal (ie in the alveolar or in the basal bone). However, the superior portion of the medulla more frequently has smaller marrow spaces than inferior to the inferior dental canal with the occurrence of trabeculae apparently ending in free space being more common in the lower compartment.
Figure 3.7

Photographs of 2 mm thick bone sections from mental foramen region of mandible

Dental status (e - edentate, p - partially dentate, or d - dentate), age (in years), sex, and net apparent density (g/cm$^3$) from left:

top row p86F 1.43 e85F 1.20 e74M 1.63 e79F 1.42 e83F 1.59
middle row e82F 0.98 e86F 0.99 e79M 0.98 p79M 1.09 e96F 1.17
bottom row scale (cm) e69M 1.11 e77F 1.13 d72M 1.17 p78F 1.28

These specimen numbers for these mandibles (as they appear in table A.1 in appendix 1) are as follows:

1230 1206 1197 1216 1217
1200 1188 1199 1219 1183
1218 1229 1221 1214

Note the range in sizes of the mandible from top right to bottom left and that trabecular quality seems to behave independently of the amount of cortical bone. The sections second from the left in the bottom and top rows have a large cross sectional area with a robust looking cortex but with sparse trabeculae. The section at the left of the middle row has a thinnish cortex but thick trabeculae. The section top right consists entirely of cortical bone.

Note also the variable location and condition of the inferior dental canal, and how the trabecular thinning may be more noticeable either below or above the canal. However, the superior portion of the medulla more frequently has smaller marrow spaces than inferior to the inferior dental canal with trabeculae apparently ending in free space being more common in the lower compartment. Scale in cm.
**Midline site**

At the midline there is generally a much higher proportion of cortical to trabecular bone as is reflected in the apparent density measurements, but here the main bulk lies lingually, rather than towards the lower border as occurs at the previous site (figure 3.*, over page). The labial cortex is often considerably more porous than the lingual (as is seen to a lesser degree at the mental foramen: Atkinson & Woodhead 1968, von Wowern & Stoltze 1980, Jager et al 1990).

The trabeculae sometimes have a strong horizontal component attaching slightly inferiorly on the labial side, in some cases having a very ladder-like arrangement (figure 3.*). In contrast to the mental foramen region, the marrow spaces are usually larger nearer to the alveolus than to the lower border.

At the level of the genial tubercles there is frequently an extra thick strut of bone that may span the full thickness of the medulla. It can be seen from figure 3.* that this probably houses a neurovascular bundle, since it is hollow and opens as a foramen on the lingual surface (figure 3.*, section 1221 on bottom right). Other foramina are frequently present, usually on the lingual, but occasionally on the buccal side.

In younger mandibles, the trabeculae exist as plates (figures 3.10 & 3.11), but with increasing age the marrow spaces may be enlarged and finer, often disordered, trabeculae are seen. However, these do not always appear to have arisen through the remodelling of the pre-existing trabeculae, but instead may represent sites of woven bone formation. Note the particularly exuberant example in the 70 year old female (figure 3.12).

**Postcranial bones**

The postcranial bones were of a range of sizes and a range of trabecular morphologies. In several of the lumbar vertebrae and femoral necks it was possible to see microfracture microcalluses in the trabecular regions. The quality of the postcranial bones does not seem to have a direct bearing on the amount of reduction that has occurred in the mandible. For example, the smallest mandible comes from an individual with relatively good trabecular bone in the three postcranial sites. On the other hand, the individuals who had mandibles which are of a reasonable size could show evidence of osteoporosis in the other bones. Figures 3.13 and 3.14 show the mental foramen slices of the mandible with the corresponding postcranial bone sections. The bone area is generally larger than for the females.
Figure 3. Midline mandibular slice from 72 year old male (specimen 27, table A.2 in appendix 1), showing:
- the strong directional component to the trabeculae which attach lower on the labial than on the lingual cortex;
- the large hollow strut (actually a mass of compact bone with Haversian canals) of bone opposite the genial tubercle;
- that the lingual alveolar bone may consist entirely of compact bone;
- and areas with extremely fine trabeculae
Figure 3.
Photographs of 2 mm thick sections from midline region of the mandible.

Age, sex and apparent density (g/cm$^2$) for each individual from left to right is:

<table>
<thead>
<tr>
<th>Top row</th>
<th>Middle row</th>
<th>Bottom row</th>
</tr>
</thead>
<tbody>
<tr>
<td>82F 1.34</td>
<td>74M 1.91</td>
<td>83F 1.40</td>
</tr>
<tr>
<td>86F 1.20</td>
<td>79M 1.32</td>
<td>85F 1.34</td>
</tr>
<tr>
<td>78F 1.27</td>
<td>69M 1.22</td>
<td>72M 1.29</td>
</tr>
</tbody>
</table>

These specimen numbers for these mandibles are as follows:

<table>
<thead>
<tr>
<th>Top row</th>
<th>Middle row</th>
<th>Bottom row</th>
</tr>
</thead>
<tbody>
<tr>
<td>1200</td>
<td>1197</td>
<td>1217</td>
</tr>
<tr>
<td>1230</td>
<td>1199</td>
<td>1219</td>
</tr>
<tr>
<td>1218</td>
<td>1229</td>
<td>1221</td>
</tr>
</tbody>
</table>

Note the variations in morphology from consisting almost entirely of compact bone to those having a larger trabecular component. In the midline of many mandibles there is a large strut of bone running from the lingual downwards towards the labial cortex (see central mandible), this usually was seen to contain a soft tissue bundle (as in the mandible at the right of the bottom row).

In contrast to the mental foramen region, the marrow spaces are usually larger nearer to the alveolus than to the lower border.
Figure 3.10 Near midline slice from 35 year old male (A40.2i in table A.2) showing the comparatively small marrow spaces and the socket surrounded by compact bone (cf figure 3.11).
Figure 3.11 Midline slice from 48 year old male. Note the indistinct boundary between cortical and trabecular bone, and the perforated socket wall.
Figure 3.12 Near midline slices from 70 year old female mandible (specimen 305), showing:
- the horizontal and downward component to the broad trabeculae in the superior part of the medullary cavity;
- the very many irregular small trabeculae (as described by Parfitt 1962);
- the labial cortex is thinner than the lingual; the 'cribriform' plate of the tooth socket seems to consist almost entirely of compact bone.
Figure 3.13

Bone slices from mandible, lumbar vertebra, iliac crest and femoral neck of three females.

Mental foramen mandibular (MF), fourth lumbar vertebral (L4), iliac crest (IC) and femoral neck (FN) slices from three females aged 83, 78 and 77 years (specimen numbers 1217, 1214 and 1229).

The top row shows the L4s, the second row the ICs, the third row the FNs and the bottom row the MF slices.

In the left hand column (83 year old, 1217), note how the postcranial bones show fairly good trabecular bone quantity and yet the individual has a very small mandible.

The central column (78 year old, 1214) shows finer trabeculae in a partially dentate female who has a mandible of medium size. This individual had the femoral neck slice with the lowest apparent density for the whole sample examined.

The right column (77 year old, 1229) shows severe trabecular disturbances (note especially the implant holes in the FN slice) in an individual with a mandible of large cross section.

The areas and apparent densities for the slices are as below:

<table>
<thead>
<tr>
<th></th>
<th>1217 (left)</th>
<th>1214 (mid)</th>
<th>1229 (right)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4 cm²</td>
<td>8.78</td>
<td>7.00</td>
<td>8.22</td>
</tr>
<tr>
<td>IC cm²</td>
<td>3.06</td>
<td>3.44</td>
<td>4.11</td>
</tr>
<tr>
<td>FN cm²</td>
<td>6.69</td>
<td>6.31</td>
<td>8.61</td>
</tr>
<tr>
<td>MF cm²</td>
<td>0.40</td>
<td>1.67</td>
<td>3.07</td>
</tr>
<tr>
<td>L4 g/cm³</td>
<td>0.31</td>
<td>0.16</td>
<td>0.21</td>
</tr>
<tr>
<td>IC g/cm³</td>
<td>0.78</td>
<td>0.41</td>
<td>0.46</td>
</tr>
<tr>
<td>FN g/cm³</td>
<td>0.64</td>
<td>0.44</td>
<td>0.38</td>
</tr>
<tr>
<td>MF g/cm³</td>
<td>1.59</td>
<td>1.42</td>
<td>1.13</td>
</tr>
</tbody>
</table>
Figure 3.14

Bone slices from mandible, lumbar vertebra, iliac crest and femoral neck of three males.

Lumbar vertebra, iliac crest, femoral neck and mental foramen slices from three males aged 74, 79 and 69 years respectively.

The individual on the left (74 years, 1197) had the largest lumbar vertebra and femoral neck of the whole group (see table below), with good quality trabecular bone, but note the size of the mandible.

The middle row shows a more osteoporotic postcranial bone appearance, with a mandible of medium size and quality (79 years, 1219).

On the right are the bones from the individual with the largest mandible (69 years, 1218), these are of good quality.

Note particularly the thickness of the iliac crest and compare it with the female examples shown in the previous figure.

The areas and apparent densities for the slices are as below:

<table>
<thead>
<tr>
<th></th>
<th>1197 (left)</th>
<th>1219 (mid)</th>
<th>1218 (right)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4 cm²</td>
<td>10.56</td>
<td>10.17</td>
<td>10.31</td>
</tr>
<tr>
<td>FN cm²</td>
<td>12.59</td>
<td>10.69</td>
<td>10.47</td>
</tr>
<tr>
<td>MF cm²</td>
<td>0.87</td>
<td>1.70</td>
<td>3.33</td>
</tr>
<tr>
<td>L4 g/cm³</td>
<td>0.25</td>
<td>0.14</td>
<td>0.26</td>
</tr>
<tr>
<td>IC g/cm³</td>
<td>0.43</td>
<td>0.32</td>
<td>0.42</td>
</tr>
<tr>
<td>FN g/cm³</td>
<td>0.38</td>
<td>0.36</td>
<td>0.48</td>
</tr>
<tr>
<td>MF g/cm³</td>
<td>1.63</td>
<td>1.09</td>
<td>1.11</td>
</tr>
</tbody>
</table>
Discussion

This chapter looked at the bone structure of two regions of the adult human mandible and how its density (weight per unit volume) relates to a variety of factors. These include: the site and size of the bone; the age, gender and skeletal status of the individual; and some simple radiographic measures.

Bone slices were taken from the midline and mental foramen regions of the mandible, the fourth lumbar vertebra, the femoral neck and the iliac crest. These skeletal sites were selected because they have been much investigated in the study of osteoporosis: the first two because they are a common site of fracture, and the iliac crest because it is used as a biopsy site in the diagnosis and study of metabolic bone diseases.

The dissecting room material presented a particular problem in cleaning because it had been in fixative (made up of 12.5 litres (l) industrial methylated spirit, 2.5 l 80% phenol solution, 1.5 l formalin and 3.0-3.5 l glycerine), for between one and two years, which seemed to render the soft tissues much more resistant to the usual digestion methods. Many strategies for cleaning the soft tissue off bone have been described (Hall & Russell 1933, Mahoney 1966, Grayson 1967, Boyde & Jones 1974, Snyder et al 1975, Boyde 1984, Coy 1987, Krüger 1988, Wiltshire 1989), but are either ineffective upon phenol-formaldehyde fixed tissue or may damage the bone. Johan (1924) described a special 'antiformin' method utilising a hot solution of sodium carbonate and calcium hypochlorite with aqueous sodium or potassium hydroxide. This technique, however, can have a detrimental effect upon the quality of the bone surface (Ivings 1989), especially since some advocate that its use is followed by brushing with a medium wire brush (Chauhan 1989). Thus the Johan method may provide adequate results for the preparation of whole skeletons or bones for teaching purposes, but would not be suitable for electron microscopic study. Furthermore, the strongly alkaline and heat conditions would probably render the bone partially anorganic.

A variety of alternative cleaning techniques was therefore tried including: aqueous sodium hydroxide, hydrogen peroxide, and sodium hypochlorite (bleach). The most consistently good results for dissecting room material were obtained using bleach (5% available chlorine) diluted to 1:3 to 1:10 parts water on sections of mandible (with or without sonication) for 2-30 hours, which removed sufficiently little of the organic matrix within the bone to prevent its becoming brittle. However, this technique was not adopted since making the specimen partially anorganic may affect the apparent mineralization density of the specimen as detected by quantitative backscattered electron analysis (see next chapter), insofar as there is a difference between the mean atomic composition of PMMA and the organic matrix of bone. This study therefore selected the technique of prolonged washing followed by the use of an enzyme detergent solution.
Relatively thick sections of bone were used to give some indication of the internal bony architecture and to minimise the loss of trabeculae. This then necessitated a stereo method for the area assessment so that the true surface of the bone could be observed.

*Mandibular findings*

The mandibular midline and body differ very much in structure, and also in the way they change with age. At both sites, a decrease in the cross sectional area occurs with age which cannot be attributed to anything other than the presence or absence of the teeth. As the bone decreases in size it becomes increasingly dense, however at the midline, the apparent density increases even in dentate individuals. This therefore does not merely reflect an increasing proportion of cortex as the bone gets smaller.

It is possible that there is a significant difference in the strains experienced at the midline between dentate and partially dentate individuals. Daegling et al (1992) found the torsional rigidity of the mandible to change upon the loss of the teeth which might have a secondary effect upon what happens at the midline, which is one of the areas to experience the highest strains during function (Hylander 1979, Karioth et al 1992). So it is possible that the increase seen in the dentate males is, in fact, influenced by the decreasing number of posterior teeth with age.

The densities and areas of the midline slices correlated well with those from the mental foramen region of the same mandible, but differed significantly, indicating that the structure of the bone changes markedly from one site to another. This will be further addressed in chapter 4.

The radiographic measures seem fairly reliable indicators of cross sectional area. This implies that the mandible changes more in height than it does in width on becoming edentulous. The whole mandibular height and the distance from the inferior border to the mental foramen and from the mental foramen to the alveolar crest and from the inferior border to the alveolar crest was correlated, and it therefore seems unlikely that the 'basal' bone height can be a measure of previous mandibular height (as used by Wical & Swoope 1974a), as it too decreased as the whole mandible decreased in height.

Cortical thickness correlates with apparent density (and has been used as a measure of this in previous studies). Cortical thickness was not correlated with height or area in this study, although density was correlated with area, and both area and density were correlated with bone height. (It may be that measuring to the nearest 0.5 mm was not adequate, but it was felt that this was the limit of accuracy that could be obtained.)
The differences in the width of the mandibular slices show how this may be an essential variable to take into account when assessing information derived from lateral projections of the mandible (see especially the middle two mandibles in the bottom row of figure 3.7 which otherwise have similar heights). This would not have been allowed for in studies such as that performed by Ulm et al (1994) which used dual photon absorptiometry in the assessment of the bone mineral content in the region below the inferior dental canal.

Since this is a cross sectional study with relatively few individuals, one has to be careful with drawing conclusions, particularly as there are no younger females. It is unknown when these individuals lost their teeth or what additional local factors were present before death, yet the postcranial bones did yield some useful and interesting information.

**Postcranial correlation**

Of the four bones studied, the fourth lumbar vertebra had the lowest density, reflecting the very small amount of cortex that this bone has. Conversely, the mandible consists mainly of cortical bone and is considerably denser than the others, as reported by Tammisalo & Kiminki (1969).

The most interesting finding in this study is the apparent lack of correlation between mandibular cross sectional size and density with density at other skeletal sites. This might be partly due to the different proportions of cortical and trabecular bone at these sites, and it may be that a correlation would become evident if the trabecular bone was considered independently. However, this would be better done on a sample of mandibles that did not show such a vast range in morphologies.

**Conclusions**

This chapter has shown how the use of a simple photographic technique can yield useful information on mandibular trabecular architecture and how it relates to the rest of the skeleton. The use of smoothly cut specimens has given much clearer pictures than those obtained by previous workers (see, for example, Lautenbach (1972) who broke, rather than sectioned, the specimens). This technique also enables a larger field of view to be examined than is possible by light microscopy, but raises many further questions on the mechanism of the changes that occur in the mandible. It can be concluded that, if the systemic environment has an influence on mandibular cross sectional size and structure, then it is unlikely that it is the major factor (Dyer & Ball 1980), or that systemic factors do not have the same effect on mandibular as on postcranial bones.

The next chapter uses a more sophisticated mode of analysis to study the mineralization density of the bone tissue that makes up these different skeletal regions.
CHAPTER 4

MINERALIZATION DENSITY OF THE MANDIBLE AND ITS VARIATION WITH SITE, SEX, AGE, DENTAL AND SKELETAL STATUS

The main constituents of bone tissue are: protein (mainly type I collagen), mineral and water. The degree to which the mineral may be substituted for water is variable (Richelle 1964) and tends to increase with the increasing age of any particular packet of bone. Since, even in the adult skeleton, bone is constantly being turned over, the tissue consists of regions with different levels of mineralization. Thus a measure of the mineralization density can give an indication of the rate of bone turnover, or the efficiency of the mineralization process. This aspect of density measurement can therefore provide information about events occurring at the tissue level.

It is possible to study the relative quantities of particular levels of mineralization density using x-ray microanalysis on fractionated bone samples (Grynpas et al 1986). However, the fact that the bone is fractionated removes the opportunity for detailed anatomical investigation. Microradiography, in which radiographs of thin bone slices (around 100 µm thick) are examined microscopically, does provide a more detailed structural analysis of the distribution. However, slices of constant thickness (which are required for reliable quantitative information) are difficult to prepare and, even at 80-100 µm, contain too great a volume of tissue to provide images of high resolution. For these reasons it is better to use a technique that can be used on thick sections of bone, but that only samples a thin surface layer (Boyde et al 1993). As mentioned in chapter 2, quantitative backscattered electron analysis in a scanning electron microscope (BSE-SEM) can be readily applied to bulk specimens. Under normal operating conditions only a small volume of the tissue surrounding each point of electron beam impact will be sampled - for an accelerating voltage of 20 kV on 40% mineralized bone, for example, it is estimated that the majority of the electrons will be returned from a volume of less than 1 µm deep and 2 µm in radius (for details see Howell & Boyde 1994). This obviously allows far superior tissue sampling to microradiography. However, care has to be exercised wherever the bone has an interface with the embedding medium, since there will be a partial volume effect due to averaging the signal apparently from the bone with that from the neighbouring medium.

Many factors other than the mineral density of the tissue may affect the backscattered electron signal and have to be controlled for. Microscope factors include the working distance, accelerating voltage, spot size, filament current, emission current, the detectors used, the focus and the brightness and contrast settings. For the specimen itself, the
most important factors are the surface topography, the embedding medium, the quality of the embedding, and the avoidance of charging/discharging. However, if all of these parameters are carefully controlled, then the signal intensity relates exactly with the mineralization density. Combined with a suitable standard for calibration purposes, the corrected values become truly quantitative and comparable between batches (Boyde et al 1995a).

Unlike the reduction in structural density which has long been recognised to occur with aging, the bone tissue may become more highly mineralized with age (Reid & Boyde 1987, Boyde et al 1995b). Using clinical imaging techniques it is possible that the increasing mineralization density may mask to a degree the falling bone density and the thinning of the cortices.

This chapter looks at changes in mineralization density with sex, age and site. Since it is not always clear what is being tested when using the huge variety of techniques that apparently detect bone mineral density, this chapter also looks at the relationship between the apparent density and the mineralization density.

**Materials and Methods**

**Cranial bone**

In this thesis the broader definition of cranial bone, as used in Gray's Anatomy (Soames 1994), as including all bones of the head and facial skeleton, is used. Mandibular slices adjacent to those used for the apparent density analysis in chapter 3 were selected. In addition pieces of fresh mandibular bone were obtained from operations for the removal of unerupted or partially erupted third molar teeth. These fragments were produced by the 'lingual split' technique in which a chisel, rather than a bur, is used to cleave off a portion of lingual cortical plate (the lingual tuberosity, Edwards 1954) to provide a path of removal for the molar. For comparison with these fragments, one slice from the posterior molar region of each dissecting room mandible was also selected. Therefore the whole mandibular cross sections represented were in the region of: i) the midline (MD specimens); ii) the mental foramen (MF specimens); and iii) the lingual split (LS specimens). The smaller surgical specimens are indicated as 'Is' specimens.

Small pieces of parietal and a few pieces of occipital bone were also collected. These came from the margins of post mortem calvarial incisions and from dissecting room cadavers.

**Postcranial bone**

One slice was selected from each of the postcranial sites as detailed in chapter 3; that is, from the fourth lumbar vertebra, from the iliac crest and from the femoral neck.
Apart from the fresh bone fragments which were directly fixed in 70% ethanol prior to dehydration in 100% ethanol, all of the bone slices were cleaned as detailed in chapter 3, washed in distilled water, and dehydrated in ethanol prior to being embedded for mineral density determination.

**Embedding**
The slices were transferred from 100% ethanol to a minimum of two changes of freshly distilled methylmethacrylate (MMA) and embedded by placing into MMA which had been activated by the addition of 1 g per litre azo-iso-butyronitrile (AIBN), polymerised at 40°C.

**Final preparation**
The poly(methylmethacrylate) (PMMA) embedded specimens were sectioned on a band saw and ground on wet carborundum paper to expose the specimen at the surface of the block. For the previously unsectioned lingual split specimens, the face of the bone which corresponded with the whole sections of mandible was exposed. For the calvarial material (CA), the cut face exposed for examination lay in a supero-inferior direction where orientation was possible. The block face was then micromilled on a Polycut E (Reichert-Jüng, Germany) micromilling machine to produce a flat surface (Boyde 1984). The base of the block was made parallel to the block face both to reduce the need for refocussing required at different regions within the same specimen during microscopy, and to eliminate tilt of the surface within each field.

**Mounting the specimens**
The specimens were mounted onto a square raft of an aluminium alloy 80 mm*80 mm using small pieces of double sided adhesive carbon tape. To minimise the risk of charging, a continuous ring of conducting carbon paint was first traced around the sides of each block immediately adjacent to the face of interest. This track was continued down the sides of the block to make contact with the carbon tape attachments. Once the raft was full of specimens, a layer of evaporated carbon was applied (figure 4.1, overleaf).

The distribution of mineralization densities within the mineralized tissues was determined by BSE-SEM and digital image analysis (Boyde et al 1995a,b, Boyde & Jones 1996).

**Microscopy**
The rafts of specimens were viewed on an automated stage in a Zeiss digital scanning microscope (DSM 962) controlled by a Kontron IBAS computer (Kontron, Elektronik, Munich, Germany). The microscope was equipped with an annular solid state BSE detector (KE Electronics, Toft, Cambridge, UK: Boyde et al 1995b). Fields of view
Figure 4.1 Raft of carbon coated, micromilled blocks of PMMA-embedded cranial specimens ready for analysis by BSE-SEM. The aluminium raft is 8 cm square. Note the space at the bottom for the standard. The map of the raft was necessary to aid location in the microscope. It was possible to examine more than 40 specimens in one run using this technique.
were chosen on the specimens, with the microscope operating in secondary electron mode. With whole bone cross sections, fields from all aspects of the bone were selected, taking care not to allow adjacent fields to overlap. For each mandibular slice, between 5 and 10 fields of view were chosen depending upon size. For each of the postcranial bones, fields from both cortical and trabecular zones were recorded.

The working distance was kept constant at all times (17 mm) with focus being achieved by moving the specimen stage in the Z axis, avoiding altogether any alteration of the final lens current which would have affected the specimen-detector geometry. Focus was checked at 2000x original magnification in secondary electron mode and 512*512 pixel fields were recorded at a nominal 33x (giving field dimensions of 2.70 mm) and at 44x for the calvarial samples (field dimensions 2.025 mm). The time taken for selection of all the fields allowed the filament and electronics to warm up. Saturation, being the point at which any increase in the filament current will cause no increase in the number of electrons emitted by the filament, was ensured and each field was automatically captured by the automated system using a slow scan in BSE mode at an accelerating voltage of 20 kV, emission current 70 μA and a filament current between 3.40 and 2.79 A depending on the filament age. Each image took approximately 30 seconds to capture. For each run a 'standard' was included which allowed the system to be calibrated thus enabling comparisons to be made between different runs in the microscope. The standard consisted of two monosubstituted halogenated dimethacrylates (Davy 1994, \(C_{22}H_{23}O_2Br\), mean BSE coefficient according to the procedure given by Lloyd (1987) = 0.1159 to \(C_{22}H_{23}O_2I\), mean BSE coefficient 0.1519) with different apparent densities which just span the normal densities for bone (Boyde et al 1995a). The standard was imaged at the beginning of each run, after every twenty images, and again after the last field had been recorded.

Data analysis
The images were edited to eliminate any artefactual features, debris, or overlap with the other fields. The histograms of the edited images were stretched to 256 grey levels covering the range (Br-I) between the values for the low and high density standards. To correct for any instrumental drift during the run, each image was stretched by linear interpolation between the values from the two consecutive standard fields between which it was taken.

The mean, standard deviation and median of each stretched image histogram were recorded, the values given lying on a scale where 0 represents black and 255 represents peak white (highest possible mineralization density considered). The standard deviations were checked for similarity and the means per individual were calculated.
To facilitate further analysis, the histogram bins were pooled (Boyde et al. 1995b). Three different steps of dividing the images were tried. The data was first separated into 16, and then 8, equally sized bins to analyse the different histogram shapes and distributions. This was found to be unnecessarily complex, and it was decided to use 4, unequally sized, bins that correspond to bone tissue with low (grey levels 0-149), medium (150-174), high (175-199) and very high (200-255) mineralization level following the scheme used by Boyde et al. (1995b). This last method was felt to reflect the biological situation better. Since more than one image was taken for most of the specimens, the mean value in each bin (given as a percentage) for each individual was calculated.

Analysis was performed using Minitab™ (Minitab, Inc) and FigP (FigP Software Corporation, Durham, NC, USA) statistical software. The two sample t-test was used to compare columns of data; Student's paired t-test was used to reveal trends between different sites within an individual; and Pearson's linear correlation coefficient was used in assessing the relationship of one variable to another (the correlation coefficient, r, is given with a p value relating to the correlation, therefore two p values are given, one for the t-test and one for the r value). Unless otherwise stated, the mean data and the standard error of the mean (sem) are presented in the graphs and tables.

The data was analysed, as in the previous chapter, for age, sex and for dental status.

**Analysis with sex and age**

Only the cranial material was analysed for age. Since all the mandibular bone representing the younger age groups was from the lingual split surgical procedure, only the fields most closely corresponding to this region were selected from the whole bone cross sections (in edentulous mandibles this was taken as the region of the mylohyoid ridge). This was necessary because the mineralization density measurements between this and other sites around the cortex differed.

**Analysis with site**

**Mandible**

The data was compared between different points on the slices and between different locations within the mandible. Thus the inferior, buccal, alveolar and lingual cortices and the trabecular zones of the slices were compared, as were the lingual split, the mental foramen and the midline regions of each mandible. (The term 'alveolar' is used to denote the most superior aspect of the mandibular body, in edentulous regions being represented by the crest of the ridge.) The data was also analysed for dental status as in the previous chapter.
Postcranial bones
The mineralization density values were compared for cortical and trabecular regions. For this purpose, all of the measurements from the cortical and trabecular zones were summed for each slice, which meant that the cortical bone also included areas of calcified fibrocartilage and Sharpey fibre bone.

Comparison between cranial and postcranial bone
Means for the whole bone cross sections of the individuals with more than one skeletal site represented were compared.

Morphological imaging
The same specimens were studied using BSE-SEM to study and record relevant morphological detail over a range of magnifications. Here, the more important criterion was to produce high quality images, rather than high quality measurement data, and a 1024*1024 pixel matrix was used, recording the images directly on the Zeiss DSM 962 system without the external IBAS computer control. These are the images used to illustrate this chapter (figures 4.7-4.19); these were archived as photographic negatives.

Results
Mandible
A total of 893 mandibular analytical images was recorded (and archived on optical disks): 317 from the lingual split, 328 from the mental foramen and 248 from the midline regions. Of the 76 specimens representing the Is site of the mandible, one was severely cracked as a result of the surgery and was eliminated, leaving 75 individuals ranging in age from 16 to 92 years. The 38 specimens from individuals under 40 years of age were all by-products of minor oral surgical procedures. Two specimens (48 and 49 years) were collected post mortem over 20 years ago, and ten specimens from individuals between 54 and 75 years were from major surgical resections. The remainder were from dissecting rooms. Further details of the sample are shown in table 4.1 and in appendix 1.

<table>
<thead>
<tr>
<th>Table 4.1 Number, age and sex distribution of 'Is' mandibular specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>n=</td>
</tr>
<tr>
<td>mean age (yrs)</td>
</tr>
<tr>
<td>median age</td>
</tr>
<tr>
<td>age range</td>
</tr>
</tbody>
</table>

The standard deviations (sd) in the histograms of the images fell in a narrow range (24.2 to 35.3). This meant that the images showed a similar distribution in grey levels, thus justifying grouping of the data for further analysis.
Variation with sex

There were no significant differences in the mean mineralization density measurements between the sexes (table 4.2), even if only those individuals over the age of 50 were compared.

Table 4.2. Means and sem of the mean mineralization densities of the Is groups

<table>
<thead>
<tr>
<th></th>
<th>male</th>
<th>female</th>
<th>all</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean grey level in Br-I range</td>
<td>167.51</td>
<td>164.04</td>
<td>166.00</td>
</tr>
<tr>
<td>sem</td>
<td>1.85</td>
<td>2.70</td>
<td>1.42</td>
</tr>
</tbody>
</table>

Variation with age

The mean mineralization densities of the lingual split region of the mandible showed interindividual spread. However, there is a clear increase in the mean mineralization density with age (with a regression equation of the form \( y=0.33x + 149.92 \), where \( y=\)density and \( x=\)age), the correlation \((r=0.70)\) being highly significant \((p<0.0001)\). The mean mineralization density for the 39 individuals 16-50 years of age was significantly lower \((p<0.0001)\) than for the 35 individuals of 51-92 years, means \((\text{sem})\) 158.20 (1.63) and 174.71 (1.27) respectively. If the younger subgroup is further divided to examine the differences in the lower age brackets, a significant difference \((p<0.05)\) was seen between the age ranges of 15-24 \((\text{mean (sem)}\) 153.92 (2.31) \(n=7)\) and 25-34 \((\text{mean (sem)}\) 160.73 (2.03) \(n=17)\), but no significant change was seen over 40 years of age.

The four bin data (figure 4.2) shows how the rising mean is accounted for by a smaller percentage of bone falling into the lower two bins with increasing age. Correlations for the whole sample are:

<table>
<thead>
<tr>
<th>Bin</th>
<th>Regression Equation</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (low)</td>
<td>( y=-0.34x + 36.19 )</td>
<td>( r=-0.65 )</td>
</tr>
<tr>
<td>2 (med)</td>
<td>( y=-0.22x + 46.97 )</td>
<td>( r=-0.60 )</td>
</tr>
<tr>
<td>3 (high)</td>
<td>( y=0.33x + 18.06 )</td>
<td>( r=0.71 )</td>
</tr>
<tr>
<td>4 (vhigh)</td>
<td>( y=0.22x + -1.19 )</td>
<td>( r=0.65 )</td>
</tr>
</tbody>
</table>

All being highly significant \((p<0.0001)\).

Analysis by site

Lingual split equivalent (LS) slices

The mean mineralization densities of the following sites were calculated from 32 whole cross sections from the posterior body of the mandible. The mean age was 74.88 ranging from 48-92 years (table 4.3, overleaf).
Figure 4.2 Variation in percentage occupation of four unequal mineralization density fractions with age - lingual split site only
Table 4.3 Mean and sem of the mean mineralization densities of the LS groups

<table>
<thead>
<tr>
<th></th>
<th>inferior</th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>31</td>
<td>32</td>
<td>25</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>mean grey level in Br-I</td>
<td>177.75</td>
<td>179.93</td>
<td>177.55</td>
<td>175.82</td>
<td>173.01</td>
</tr>
<tr>
<td>range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sem</td>
<td>0.90</td>
<td>0.95</td>
<td>1.43</td>
<td>1.40</td>
<td>2.31</td>
</tr>
</tbody>
</table>

Student's paired t-tests reveal that, for each individual, the buccal bone is significantly more highly mineralized than all the other sites. More details are shown in tables 4.4 and 4.5.

Table 4.4 Student's paired t-tests of LS slices

<table>
<thead>
<tr>
<th></th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>inferior against</td>
<td>0.01</td>
<td>-ns-</td>
<td>-ns-</td>
<td>0.02</td>
</tr>
<tr>
<td>buccal against</td>
<td>0.05</td>
<td>0.001</td>
<td>-ns-</td>
<td>0.001</td>
</tr>
<tr>
<td>alveolar against</td>
<td>0.02</td>
<td>-ns-</td>
<td>0.0001</td>
<td>-ns-</td>
</tr>
<tr>
<td>lingual against</td>
<td>-ns-</td>
<td>0.0001</td>
<td>0.0001</td>
<td>-ns-</td>
</tr>
<tr>
<td>-ns- = not significant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5 Pearson's linear correlation coefficient of LS slices

<table>
<thead>
<tr>
<th></th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>inferior with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td>0.66</td>
<td>0.38</td>
<td>0.67</td>
<td>0.48</td>
</tr>
<tr>
<td>p&lt;</td>
<td>0.0001</td>
<td>-ns-</td>
<td>0.0001</td>
<td>-ns-</td>
</tr>
<tr>
<td>buccal with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td>0.70</td>
<td>0.70</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>p&lt;</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>alveolar with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td>0.64</td>
<td>-ns-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p&lt;</td>
<td>0.01</td>
<td>-ns</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lingual with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td></td>
<td></td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>p&lt;</td>
<td></td>
<td></td>
<td>0.005</td>
<td></td>
</tr>
</tbody>
</table>

No site showed a significant correlation with age or sex.

Mental foramen (MF) slices

A total of 43 mandibles was analysed in the region of the mental foramen: two were omitted because the data were insufficient. The ages ranged from 48-96 years with a mean of 73.79 years, there were 24 males (mean age 70.75, range 48-88) and 15 females (mean age 77.31, range 50-96). There were no significant differences in age or mean mineralization densities between the sexes. The mean and sems are shown in table 4.6.
Table 4.6 Mean and sem of the mean mineralization densities of the MF groups

<table>
<thead>
<tr>
<th></th>
<th>inferior</th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>40</td>
<td>41</td>
<td>41</td>
<td>41</td>
<td>23</td>
</tr>
<tr>
<td>mean</td>
<td>180.06</td>
<td>177.11</td>
<td>174.30</td>
<td>178.08</td>
<td>173.82</td>
</tr>
<tr>
<td>sem</td>
<td>0.62</td>
<td>0.71</td>
<td>0.77</td>
<td>0.69</td>
<td>1.22</td>
</tr>
</tbody>
</table>

Paired t-tests showed the mean mineralization density of the inferior cortex to be significantly greater than that of all the other sites (p<0.0001, except the lingual where p<0.001). Pearson's linear correlation coefficient showed the inferior cortex to be correlated with the buccal (r=0.66, p<0.0001), alveolar (r=0.64, p<0.0001) and lingual (r=0.66, p<0.0001) but not the trabecular sites. The mandibular trabeculae usually make up so tiny a proportion of the slice in an effectively infinitely thin section from this region that often no trabecular field was recorded (hence n=23). Furthermore, the proportion of edge pixels (voxels) will be significantly higher. A volume which includes partially bone and partially osteoid or PMMA will obviously return a lower value than a voxel which hits square on and in well mineralized bone.

The buccal region, in turn, had a higher mean mineralization density than the alveolar and trabecular regions (p<0.001 and p<0.005) and these were positively correlated (r=0.61, p<0.0001, and r=0.46 p<0.05). The mean mineralization density of the buccal region did not differ significantly from that of the lingual site but was positively correlated (r=0.61, p<0.0001). The lingual was greater than the alveolar region (p<0.0001, r=0.59 and p<0.0001) and the trabecular regions (p<0.01) but not correlated. The alveolar and trabecular regions were weakly correlated (r=0.57, p<0.01) but were not significantly different.

At the mental foramen site, the bone from the inferior cortex therefore consists of more highly mineralized bone than the lingual and buccal, which in turn are more highly mineralized than the alveolar and trabecular regions, and all, except the lingual with the trabecular region, are correlated.

Apart from a weak negative correlation for the trabecular site (r=-0.49, p<0.05; y=-0.22x + 190.49), none of the sites showed a relationship between the mineralization density and age of the individual.

Midline (MD) slices
A total of 28 mandibular midlines were analysed (mean age 73.79, range 19-92). The 10 females were significantly older (p<0.05) than the males (mean age 80.60, range 70-92: mean age 70.00, range 19-86) (see tables 4.7-9, over page).
Table 4.7 Mean and sem of the mean mineralization densities of the MD groups

<table>
<thead>
<tr>
<th></th>
<th>inferior</th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>27</td>
<td>28</td>
<td>28</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>mean</td>
<td>174.46</td>
<td>173.05</td>
<td>171.13</td>
<td>174.58</td>
<td>173.78</td>
</tr>
<tr>
<td>sem</td>
<td>1.14</td>
<td>1.42</td>
<td>1.53</td>
<td>1.23</td>
<td>1.28</td>
</tr>
</tbody>
</table>

Table 4.8 Student's paired t-tests of MD slices

<table>
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<th>lingual</th>
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</tr>
</thead>
<tbody>
<tr>
<td>inferior against</td>
<td>0.05</td>
<td>0.0001</td>
<td>-ns-</td>
<td>-ns-</td>
</tr>
<tr>
<td>buccal against</td>
<td>0.01</td>
<td>0.05</td>
<td>-ns-</td>
<td>-ns-</td>
</tr>
<tr>
<td>alveolar against</td>
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<td>0.01</td>
<td></td>
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<tr>
<td>lingual against</td>
<td></td>
<td></td>
<td>-ns-</td>
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</table>

Table 4.9 Pearson's linear correlation coefficient of MD slices

<table>
<thead>
<tr>
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<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>inferior with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td>0.87</td>
<td>0.91</td>
<td>0.87</td>
<td>0.77</td>
</tr>
<tr>
<td>p&lt;</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>buccal with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td></td>
<td>0.90</td>
<td>0.87</td>
<td>0.83</td>
</tr>
<tr>
<td>p&lt;</td>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>alveolar with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td></td>
<td></td>
<td>0.85</td>
<td>0.82</td>
</tr>
<tr>
<td>p&lt;</td>
<td></td>
<td></td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>lingual with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>r=</td>
<td></td>
<td></td>
<td></td>
<td>-ns-</td>
</tr>
<tr>
<td>p&lt;</td>
<td></td>
<td></td>
<td></td>
<td>0.73</td>
</tr>
</tbody>
</table>

Unlike the previous two mandibular regions tested, the mean mineralization densities of each of the sites was significantly positively correlated with age: inferior cortex r=0.49, p<0.01 (y=0.19x + 160.63); buccal r=0.57, p<0.002 (y=0.29x + 152.01); alveolar r=0.49, p<0.01 (y=0.26x + 151.81); lingual r=0.62, p<0.001 (y=0.26x + 155.01) and trabecular r=0.43, p<0.05 (y=0.19x + 159.82). This group of midline slices did, however, show a better age range for the individuals.
Relationship between sites of the same mandible

Mandibles with more than one site represented (i.e., LS, MF, MD) were cross-correlated (table 4.10 overleaf). The mean mineralization density of the inferior and buccal cortices was significantly greater at the lingual split and the mental foramen sites than at the midline. The density of the alveolar bone was greatest at the lingual split site, and that for the lingual cortex was greatest at the mental foramen site. There was no significant difference in the mineralization density of the trabecular region between sites.

See figures 4.3 a & b (page 94) for summary diagrams showing the pattern of the mineralization density variation around the mandible.

Relationship with dental status

The results are shown in table 4.11 (overleaf). In the LS region, the lingual cortex of partially dentate mandibles was significantly more highly mineralized than in either edentulous (p < 0.05) or dentate mandibles (p < 0.02). This was also true of the inferior cortex of partially dentate compared with dentate mandibles (p < 0.05). Dentate individuals were significantly younger than the partially dentate in this sample (mean ages of 63.80 and 78.57 respectively, p < 0.05).

In the mental foramen region, the partially dentate values were significantly greater than the edentulous at both the alveolar and the lingual regions (p < 0.05 and p < 0.005). Dentate mandibles showed no significant differences from either the edentulous or the partially dentate.

For the midline, the partially dentate group was included with the dentate group. There was a difference in the alveolar value between these two groups which just reached significance (p < 0.05), the partially dentate value being the higher. However, the numbers in the groups were very small (n=7 and 6 respectively). The same level of significance (p < 0.05) was found between the buccal and the alveolar sites when comparing all mandibles with teeth with those without.
Table 4.10: Student's paired t-tests and Pearson's linear correlation coefficients between MD, MF and LS sites of the same mandible

<table>
<thead>
<tr>
<th>Site</th>
<th>n=</th>
<th>Paired t-test</th>
<th>r=</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inferior Cortex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD with MF</td>
<td>22</td>
<td>0.0001</td>
<td>0.49</td>
<td>0.05</td>
</tr>
<tr>
<td>MD with LS</td>
<td>19</td>
<td>0.02</td>
<td>0.36</td>
<td>-ns-</td>
</tr>
<tr>
<td>MF with LS</td>
<td>25</td>
<td>-ns-</td>
<td>0.31</td>
<td>-ns-</td>
</tr>
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</table>

**LS and MF greater than MD**

<table>
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<th>Site</th>
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<th>Paired t-test</th>
<th>r=</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD with MF</td>
<td>23</td>
<td>0.001</td>
<td>0.49</td>
<td>0.02</td>
</tr>
<tr>
<td>MD with LS</td>
<td>21</td>
<td>0.0001</td>
<td>0.46</td>
<td>0.05</td>
</tr>
<tr>
<td>MF with LS</td>
<td>26</td>
<td>-ns-</td>
<td>0.37</td>
<td>-ns-</td>
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**LS and MF greater than MD**

<table>
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<th>r=</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
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<td>Alveolar Cortex</td>
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<td></td>
<td></td>
</tr>
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<td>MD with MF</td>
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<td>-ns-</td>
<td>0.17</td>
<td>-ns-</td>
</tr>
<tr>
<td>MD with LS</td>
<td>17</td>
<td>0.001</td>
<td>0.16</td>
<td>-ns-</td>
</tr>
<tr>
<td>MF with LS</td>
<td>20</td>
<td>0.05</td>
<td>0.28</td>
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</table>

**LS greater than MF and MD**

<table>
<thead>
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<th>r=</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lingual Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MD with MF</td>
<td>23</td>
<td>0.05</td>
<td>0.29</td>
<td>-ns-</td>
</tr>
<tr>
<td>MD with LS</td>
<td>20</td>
<td>-ns-</td>
<td>-0.08</td>
<td>-ns-</td>
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<tr>
<td>MF with LS</td>
<td>25</td>
<td>0.02</td>
<td>0.55</td>
<td>0.005</td>
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**MF greater than MD and LS**

<table>
<thead>
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<th>Paired t-test</th>
<th>r=</th>
<th>p&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medulla</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD with MF</td>
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<td>-ns-</td>
<td>0.74</td>
<td>0.01</td>
</tr>
<tr>
<td>MD with LS</td>
<td>9</td>
<td>-ns-</td>
<td>0.20</td>
<td>-ns-</td>
</tr>
<tr>
<td>MF with LS</td>
<td>9</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
</tr>
</tbody>
</table>
Table 4.11 Mean and sem BSE signal intensity (in Br-I range) values for dentate, partially dentate and edentate mandibles

<table>
<thead>
<tr>
<th>LS REGION</th>
<th>inferior</th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>edentate n=18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>176.65</td>
<td>178.26</td>
<td>176.84</td>
<td>173.91</td>
<td>168.18</td>
</tr>
<tr>
<td>sem</td>
<td>1.01</td>
<td>1.24</td>
<td>2.62</td>
<td>1.77</td>
<td>1.87</td>
</tr>
<tr>
<td>part dentate n=7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>180.52</td>
<td>183.43</td>
<td>179.08</td>
<td>181.59</td>
<td>177.64</td>
</tr>
<tr>
<td>sem</td>
<td>2.06</td>
<td>1.62</td>
<td>1.65</td>
<td>2.66</td>
<td>4.05</td>
</tr>
<tr>
<td>dentate n=6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>174.09</td>
<td>178.20</td>
<td>177.99</td>
<td>172.10</td>
<td>182.00</td>
</tr>
<tr>
<td>sem</td>
<td>1.18</td>
<td>1.52</td>
<td>0.79</td>
<td>0.66</td>
<td>0.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MF REGION</th>
<th>inferior</th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>edentate n=19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>179.47</td>
<td>176.51</td>
<td>173.14</td>
<td>176.92</td>
<td>172.17</td>
</tr>
<tr>
<td>sem</td>
<td>1.04</td>
<td>1.17</td>
<td>1.04</td>
<td>0.84</td>
<td>1.49</td>
</tr>
<tr>
<td>part dentate n=6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>181.71</td>
<td>179.29</td>
<td>178.48</td>
<td>182.70</td>
<td>177.64</td>
</tr>
<tr>
<td>sem</td>
<td>0.94</td>
<td>0.95</td>
<td>2.18</td>
<td>1.32</td>
<td>0.00</td>
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<tr>
<td>dentate n=9</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>mean</td>
<td>179.46</td>
<td>176.11</td>
<td>172.90</td>
<td>177.64</td>
<td>175.47</td>
</tr>
<tr>
<td>sem</td>
<td>1.38</td>
<td>1.71</td>
<td>1.88</td>
<td>1.81</td>
<td>2.57</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>MD REGION</th>
<th>inferior</th>
<th>buccal</th>
<th>alveolar</th>
<th>lingual</th>
<th>trabecular</th>
</tr>
</thead>
<tbody>
<tr>
<td>edentate n=15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>172.40</td>
<td>169.59</td>
<td>167.37</td>
<td>171.94</td>
<td>171.04</td>
</tr>
<tr>
<td>sem</td>
<td>2.39</td>
<td>2.58</td>
<td>2.81</td>
<td>2.34</td>
<td>2.32</td>
</tr>
<tr>
<td>dentate n=13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>176.10</td>
<td>176.05</td>
<td>174.38</td>
<td>176.87</td>
<td>175.94</td>
</tr>
<tr>
<td>sem</td>
<td>0.56</td>
<td>0.97</td>
<td>0.97</td>
<td>0.76</td>
<td>1.17</td>
</tr>
</tbody>
</table>
Figure 4.3 Summary diagrams showing sites of highest and lowest mineralization densities in the mandibular regions tested. The light shading in A represents the sites of highest mineralization density: inferirolingually at the midline, inferiorly at the mental foramen region and buccally at the posterior site. The heavy shading in B shows that the site with the lowest mineralization density corresponds with the alveolar crest in the anterior mandible, but shifts towards the lingual tuberosity posteriorly. The dotted lines indicate sites not tested, but define an area which may be anticipated to show high and low mineralization levels respectively.
Calvarial bone
A total of 133 fields from 49 individuals was examined. The mean age of the sample was 69, the ages ranging from 0-93 years (table 4.12). In addition there were two neonates of unknown sex.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
<td>19</td>
<td>17</td>
<td>11</td>
</tr>
<tr>
<td>mean age (yrs)</td>
<td>69</td>
<td>78</td>
<td>67</td>
</tr>
<tr>
<td>range</td>
<td>46-89</td>
<td>33-93</td>
<td>37-88</td>
</tr>
</tbody>
</table>

The standard deviations for the images ranged between 19 and 28 greyscale units, except for the two neonates whose images had standard deviations of 31-33 greyscale units. The mean for the whole population was 167.86, sem 1.06, range 136.93 to 179.47. There was only a significant correlation in the mean data with age (r=0.41, p<0.005) if the two neonates were included in the analysis. The four-bin histograms, however, do show a shift (upwards then downwards again) in the bin in which the greatest percentage of bone falls (figure 4.4).

Postcranial bone
A total of 260, 240 and 262 images were recorded from the fourth lumbar vertebra, the iliac crest and the femoral neck respectively. The mean age of the 14 individuals was 80.07 yrs, range 69-92.

There was a good correlation between the mean mineralization densities of the summed cortical and trabecular zones of the slices (L4 r=0.71, p<0.01; IC r=0.84, p<0.001; FN r=0.84, p<0.001). Only in the lumbar vertebra was there a significant difference between the two zones (p<0.001 paired t-test) (table 4.13).

<table>
<thead>
<tr>
<th></th>
<th>L4 cort</th>
<th>L4 trab</th>
<th>IC cort</th>
<th>IC trab</th>
<th>FN cort</th>
<th>FN trab</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean</td>
<td>153.93</td>
<td>158.85</td>
<td>155.63</td>
<td>154.82</td>
<td>160.37</td>
<td>161.37</td>
</tr>
<tr>
<td>sem</td>
<td>1.20</td>
<td>1.40</td>
<td>1.86</td>
<td>1.22</td>
<td>1.30</td>
<td>1.37</td>
</tr>
</tbody>
</table>
Figure 4.4 Four mineralization density fraction histograms for different age groups - parietal bone

- 0 yrs n=2
- 60s n=4
- 30s n=2
- 70s n=20
- 40s n=3
- 80s n=11
- 50s n=4
- 90s n=2

Percentage falling into each bin: low, med, high, vhigh
The four bin data in Table 4.14 show where the differences in the mean densities for the cortical and trabecular zones of the lumbar vertebra arises. Although there is a significantly higher percentage of bone falling into the highest density bin for cortical bone, this is more than outweighed by the higher percentages falling into bins 2 and 3 for the trabecular region, which gives the trabecular region a higher mean mineralization density overall (as shown in Table 4.13).

Table 4.14. Distribution of mineralization densities for cortical and trabecular zones in L4 four bin data as a percentage of the total

<table>
<thead>
<tr>
<th></th>
<th>Bin 1</th>
<th>Bin 2</th>
<th>Bin 3</th>
<th>Bin 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cort %</td>
<td>37.88</td>
<td>39.14</td>
<td>17.30</td>
<td>5.91</td>
</tr>
<tr>
<td>Trab %</td>
<td>25.91</td>
<td>46.05</td>
<td>24.15</td>
<td>3.80</td>
</tr>
<tr>
<td>SEM</td>
<td>2.30</td>
<td>1.02</td>
<td>1.53</td>
<td>0.37</td>
</tr>
<tr>
<td>Paired T-Test</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.002</td>
</tr>
<tr>
<td>R</td>
<td>0.70</td>
<td>0.58</td>
<td>0.71</td>
<td>0.29</td>
</tr>
<tr>
<td>P</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01</td>
<td>-ns-</td>
</tr>
</tbody>
</table>

Comparison between cranial and postcranial bones

The means and standard errors of the different sites for each individual are shown in figures 4.5 a&b overleaf, the shaded bars represent the three mandibular sites.

The mean values for the whole group for each bone are given in Table 4.15 (page 100), with the paired t-tests and correlation results in Tables 4.16 and 4.17 respectively (on page 100).

The calvarial mean mineralization density was significantly different from all the other body sites, being of a lower value than the mandible, but a higher level than the postcranial bones. The mandibular sites showed no significant differences from each other, but were significantly greater than all other sites. The lumbar vertebra and iliac crest do not differ significantly, but both have a significantly lower mineralization density than the femoral neck site (Table 4.16, page 100).

Calvarial mean mineralization density was found to be weakly correlated with two mandibular sites, and with the femoral neck value. The mandibular sites were not correlated with any of the postcranial sites which, amongst themselves, were correlated. The midline mean mineralization density was not correlated with that of any other skeletal site examined.
Figure 4.5A Mean and sem greyscale values for different skeletal sites of each individual studied (whole bone cross sections)
Figure 4.5B  Mean and sem greyscale values for different skeletal sites of each individual (continued)
Table 4.15 Mean mineralization densities of whole bone cross sections

<table>
<thead>
<tr>
<th></th>
<th>CA</th>
<th>LS</th>
<th>MF</th>
<th>MD</th>
<th>L4</th>
<th>IC</th>
<th>FN</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=</td>
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<td>11</td>
<td>14</td>
<td>9</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>mean</td>
<td>170.13</td>
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<td>178.00</td>
<td>174.87</td>
<td>155.41</td>
<td>155.18</td>
<td>160.67</td>
</tr>
<tr>
<td>sem</td>
<td>1.46</td>
<td>1.63</td>
<td>1.29</td>
<td>1.02</td>
<td>1.18</td>
<td>1.48</td>
<td>1.28</td>
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Table 4.16 Significance of differences between sites - paired t-tests

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<th>MD</th>
<th>L4</th>
<th>IC</th>
<th>FN</th>
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</thead>
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<td>CA</td>
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<td>0.0001</td>
<td>0.05</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>LS</td>
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<td>-ns-</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>MF</td>
<td>-ns-</td>
<td>0.0001</td>
<td>0.0001</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MD</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4</td>
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<td>-ns-</td>
<td>-ns-</td>
<td>0.001</td>
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<td></td>
</tr>
<tr>
<td>IC</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
<td>0.001</td>
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</tr>
<tr>
<td>FN</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
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</tbody>
</table>

Table 4.17 Pearson's linear correlation coefficient between sites

<table>
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<th>MD</th>
<th>L4</th>
<th>IC</th>
<th>FN</th>
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</thead>
<tbody>
<tr>
<td>CA</td>
<td>r= 0.68</td>
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<td>0.56</td>
<td>0.16</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>p&lt; 0.05</td>
<td>0.02</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
<td>-ns-</td>
</tr>
<tr>
<td>LS</td>
<td>r= 0.64</td>
<td>0.47</td>
<td>0.05</td>
<td>-0.04</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>p&lt; 0.05</td>
<td>-ns-</td>
<td>-ns-</td>
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Graphs of mean four bin data for the sites are shown in figure 4.6 (over page). Note that the peaks occur in the third bin ('high', after Boyde et al 1995b) for the mandibular, but not the other skeletal sites.

**Bone apparent density against mineralization density**

There is no significant correlation between the bone apparent density as measured in g/cm$^3$ and the level of mineralization as determined from the backscattered electron analysis for the mandibular (MF and MD regions $r=0.30$, $n=38$ and 27 respectively), or for the postcranial bones ($r=0.30$, 0.15 and 0.43 for the L4, IC and FN respectively).

As found with the mandibular material, there is no relationship between the mean backscattering coefficient and apparent density or cross sectional area in the fourth lumbar vertebra, the iliac crest or the femoral neck.

**Morphological observations**

**Mandible**

BSE-SEM imaging demonstrates well the variation in mineralization densities that occur in bone as shown in figures 4.7 to 4.19 (pages 104-129). At all sites examined, the mandible was seen to consist largely of secondary osteonal bone (figure 4.7). The diameters of the Haversian canals in the buccal cortex tended to be greater than in the lingual cortex as reported by Atkinson & Woodhead (1968), von Wowern & Stoltze (1980) and Jager et al (1990), and as observed in the last chapter.

Examination of the mandibular images showed that the higher mean mineralization densities measured in this bone were attributable to an overall increase. However, more specifically, high degrees of mineralization was seen in the cement lines (figure 4.8), the appositional lamellar bone (figure 4.7a), interstitial lamellar bone, regions with mineralized osteocyte lacunae (figure 4.9) or mineralized Haversian canals (figure 4.10) and at the sites of woven bone formation. Extensive areas of Sharpey fibre bone were seen particularly in the fundus area beneath the tooth apices (figure 4.12); it was not seen to the same extent in edentulous mandibles.

Regions with less dense bone were also noted at some sites within the mandible. This occurred particularly at some bony prominences (figure 4.11) which may correspond to sites of muscle attachment. The reason that the bone is less dense here may be because: a) this site is having to remodel more rapidly than the rest as the muscle attachment moves inferiorly as the ridge recedes; b) turnover is at a more normal level here due to the functional stimulus of the attachment, with the other regions becoming increasingly hypermineralized; or c) remodelling here has occurred at a time when full mineralization.
Figure 4.6 Four mineralization density fraction histograms for different skeletal sites - all individuals pooled.

CA - parietal bone
LS - posterior mandible
MF - mental foramen site
MD - mandibular midline
L4 - fourth lumbar vertebra
IC - iliac crest
FN - femoral neck
could not be realised for some reason. In the particular example shown in figure 4.11b 'a)' seems to be the most likely explanation, taking into account the signs of continued apposition on the inferior aspect of this process, and the fact that the central portions of the osteons have mineralized to a level similar to that of the adjacent bone.

Mineralized osteocyte lacunae are a frequent finding in the aging mandible (Pudwill & Wentz 1975). Figure 4.9a shows how extensive the regions are that may be affected. In addition, the canaliculi were also completely mineralized (Atkinson & Hallsworth 1983), even when it was not so evident in the lacunae themselves (figure 4.14a). The mineralization of the blood vessel canals was also a fairly frequent finding (figure 4.12), but the surrounding osteocyte lacunae did not always appear to be mineralized. Mineralization of Haversian canals was seen in both the alveolar and the basal bone.

Sometimes a line of demarcation was seen in the region of the crest of an edentulous ridge. Figures 4.11a show this at low and higher power. The osteocyte lacunae adjacent to the region of more highly mineralized bone have an unusual appearance.

Postcranial bones

All of the postcranial specimens showed regions of extra dense tissue in their cortices (Boyde et al 1995b). This occurred mostly at the end plates of the lumbar vertebrae, at the crest of the iliac crest samples and in the cortex of the femoral neck slices. It is recognised because it is more highly mineralized than the surrounding bone (figure 4.16), although overall the mean mineralization density is similar to the trabecular bone, due to the bone surrounding the extra dense tissue being less highly mineralized than other bone. The extra dense tissue may be: a) Sharpey fibre bone, which is likely to be the case in the cortices of the lumbar vertebrae and femoral neck; b) calcified fibrocartilage as occurs at the rim of the lumbar vertebrae and the femoral neck; c) calcified articular (hyaline) cartilage, as at the endplates of the lumbar vertebrae; or d) calcified growth plate cartilage remnants.

Regions of less well mineralized bone were occasionally seen on the trabecular surfaces (figure 4.17a), but more frequently an appearance of defective mineralization was seen in the cortical regions (figure 4.17b).

Another observation in the postcranial trabecular bone, was the occasional occurrence of very highly mineralized cement lines (figures 4.18 and 4.19b). These probably represent regions that have undergone a prolonged resting period.
Normal appearances

20 kV, BSE-SEM micrographs of micromilled, PMMA-embedded blocks of bone from the cortex of the mandibular midline. These images resemble those obtained by microradiography. The brightness of the image is dependent upon the density and the composition (mean atomic number) of the material. Gain and contrast levels have been adjusted such that PMMA in the background is black and the most highly mineralized bone is white.

These two images typical of mandibular bone show the large proportion of the cortex that may be taken up by secondary osteonal bone; this contrasts with the conclusions of Lautenbach (1972) who concluded that the mandible contained very few Haversian systems: figure 4.7B shows that the interstitial lamellar bone fraction is lower than the osteonal bone fraction.

Figure 4.7A shows the inferior cortex from a 67 year male (specimen 308 in table A.2, appendix 1). Highly mineralized circumferential lamellae which have not been replaced by secondary osteons can be seen at the top of the field.

The mineralization density fraction histogram for the analytical image corresponding to this particular field had:
- 5.75% falling in the low (0-149),
- 39.51% in the medium (150-174),
- 46.35% in the high (175-199), and
- 8.38% falling in the very high (200-255) range.

The mean mineralization density for the whole field is 174.53, on a scale of 0-255.

Figure 4.7B illustrates the different levels of mineralization seen in the osteons. Note also the presence of some highly mineralized cement lines, particularly in the bottom left corner of the image (78 year male, specimen 47, basolinguval cortex).

The analytical image area corresponding to this field was studied further to give the following analyses:
- number of hits on interstitial bone = 48236
- number of hits on whole bone, excluding cracks and Haversian canals = 106873
giving an interstitial bone fraction of 45.13%.
Highly mineralized cement lines

Highly mineralized cement lines were seen in all body sites examined, yet no site matched the extent to which these were seen in some individuals in the mandibular midline. Here they occurred particularly in the more trabecular regions and contributed to the high mean mineralization density values obtained for this site, although occasionally the mineralization levels in the interstitial bone reached a higher value.

**Figure 4.8A** 20kV BSE-SEM from the midline of an 82 year old female (specimen 1200). Note that the highly mineralized cement lines are arranged around the marrow spaces.

**Figure 4.8B** 20kV BSE-SEM from the mid-labial aspect of the midline of a 78 year female (specimen 1214), showing that the continued apposition, as evidenced by these cement lines, must be having a consolidating effect upon the bone. This would account for the increasing apparent density that was seen to occur in the mandible with increasing age.

The four bin histogram for the analytical image from this particular site had:

- 7.74% falling in the low (0-149),
- 29.08% in the medium (150-174),
- 48.89% in the high (175-199), and
- 14.16% falling in the very high (200-255) range.

The mean grey level (on the scale of monobrominated standard = 0, monoiiodinated standard = 255) for the whole field is fairly high at 176.87.
The presence of extensive areas of bone in which the osteocytes were mineralized also contributed to the high mean values for the mineralization densities recorded in the mandible.

**Figure 4.9A** 20kV BSE-SEM taken towards the crest in the buccal cortex in the mental foramen region of a 79 year male (specimen 1219). This image shows well the extensive areas of bone that may be occupied by mineralized osteocyte lacunae (white spots). There are several infilled and mineralized Haversian canals. The bone surrounding most of the mineralized lacunae is fairly old, as indicated by its whiter appearance than the adjacent vital osteons.

In the corresponding analytical field, the division into four bins gave:
- 6.90% low,
- 34.09% medium,
- 46.67% high, and
- 12.19% very high.

The mean greylevel value was 176.55.

**Figure 4.9B** Shows the same site as above at a higher magnification showing that each white spot in the above micrograph represents a mineralized osteocyte. Parallel scratches are due to imperfect micromilling.
The mineralization of Haversian canals was also a fairly frequent finding.

**Figure 4.10A** 20kV BSE-SEM of basolinguval cortex of 78 year male mandibular midline block (specimen 47). The lacunae surrounding the mineralized central canal are not mineralized. This, however, does not imply that the osteocytes were vital, since the occlusion of the vessel must have blocked access to nutritional exchange. There was a fairly high level of turnover in the bone surrounding the field shown.

**Figure 4.10B** 20kV BSE-SEM of buccal cortex near ridge crest of mental foramen region of the mandible of a 79 year male (specimen 1219). The canaliculi of the osteocytes in the interstitial bone are mineralized: it would be difficult to envisage how the osteocytes concerned could survive.
Figure 4.11

Sites with lower levels of mineralization

Regions of less well mineralized bone were found to occur more frequently at some sites in the mandible. These occurred especially at the alveolar crest, but also occasionally at the site of a bony prominence.

Figure 4.11A,B Low (left) and higher power (right) BSE-SEMs showing a line of demarcation in the region of the crest of an edentulous ridge of an 82 year female (specimen 1200). Note the unusual appearance of the osteocyte lacunae, which are more numerous, larger and have wider canaliculi, than in the adjacent region of more highly mineralized bone.

Figure 4.11C 20kV BSE-SEM at 33x original magnification, of 78 year old male (specimen 47) showing a bony prominence on the labial aspect of the mandibular midline with a lower mineralization density than the surrounding bone. This may be the site of attachment for the mentalis muscle. Bone may be less highly mineralized here may be because the bone is having to remodel more rapidly than the adjacent bone whilst the muscle attachment moves inferiorly with the recession of the ridge, or because the turnover is higher here as a result of the functional stimulus of the attachment (either on the bone itself or on the blood supply).

The mineralization levels determined from the corresponding analytical image in this image were much lower than at other sites of the mandible:

- 39.90% low,
- 40.43% medium,
- 17.76% high, and
- 1.90% very high.

The mean greylevel value was 153.26.
20kV BSE-SEM micrographs of mental foramen region of two dentate mandibles showing the large areas of bone near the tooth sockets which contain large numbers of Sharpey's fibres

**Figure 4.12A** 86 year male (specimen 23). Here the fibres are seen in longitudinal section in the socket side of the alveolar bone proper on the buccal side of the tooth. Note the reversal line halfway down the field which shows the shape of the once-resorbing front. There is a thin surface layer on the bone that seems to lack any structural features, but elsewhere intrinsic fibres can be seen.

**Figure 4.12B** 57 year old male (specimen 158 ). Fibres in transverse or oblique section at the fundus of the tooth socket. The fibres were much more numerous here than at any other site around the tooth. Note the nonmineralized fibre cores shown by the dark stellate features.

Extrinsic fibre bone was not seen to the same extent in edentulous mandibles.
Figure 4.13

Fine bone structure I

Higher magnification images showing osteocytes, their canaliculi and collagen orientation patterns.

Figure 4.13A 20kV BSE-SEM of the bone beneath the apex of an incisor of a 92 year female (specimen 29). Note the porous appearance is caused by the osteocyte canaliculi being cut in cross section.

Figure 4.13B 20kV BSE-SEM, 500x of trabecular bone near the base of the mandibular midline of an 82 year female (specimen 1200). See again the large number of nonmineralized osteocyte lacunae and canaliculi (compare with next figure), and the pattern of the fibres.
These images show at high magnification the reduction in porosity of the bone that may occur with mineralization of the osteocytic lacunae and canaliculi.

**Figure 4.14A** 20kV BSE-SEM of bone in the midline of the mandible of a 69 year male (specimen 1218) (labial cortex near to ridge crest) with osteocytic lacunae which have undergone differing stages of mineralization. The triplet of mineralized lacunae towards the right of the field is obvious but at the top of the field (above the leftmost of the three) is a lacuna where any mineralized infill has been plucked out by the milling process to expose the subjacent bone matrix which has the same level of mineralization as the surrounding tissue. A mineralized 'pearl' can be seen in the lacuna at the very top of the image. By contrast, the lacuna to the bottom left of the image shows no signs of mineralization, although the canaliculi surrounding it may be mineralized. Note, in the lower compartment of bone in the image, that some mineralized canaliculi have been cut obliquely. (Nonmineralized lacunae in regions where all or most of the surrounding lacunae and canaliculi are infilled may never be properly embedded by PMMA - this is probably the case for the lacuna at the top left hand side of the field.)

**Figure 4.14B** 20kV BSE-SEM inferior cortex of midline mandibular slice from 85 year old male (specimen 35). A reversal line can be seen running down the right hand side of the field where partial resorption and replacement of the nonvital bone with live bone has occurred. The canaliculi and the visible osteocytic lacuna in the new bone are unmineralized.
Figure 4.15

Fine bone structure III

**Figure 4.15A** 20kV BSE-SEM, inferior cortex of mandibular midline of an 82 year female (1200) showing a central region of bone containing mineralized osteocytic canaliculi which has been partially resorbed and replaced by new live bone. However, it is unknown whether it was dead at the time it was resorbed, or whether the remodelling so compromised the blood supply to the central section that it subsequently died.

**Figure 4.15B** 20kV BSE-SEM 1000x of interstitial lamellar bone near the alveolar crest of the mental foramen region of the mandible in a 78 year female (specimen 1214). The collagen fibre arrangement within the lamellae is particularly well illustrated.
Postcranial bones

Figure 4.16

Highly mineralized tissue in postcranial bones

All of the postcranial bones examined showed regions of densely calcified tissue in their cortices. This occurs mostly at the end plates of the vertebrae, at the crest of the iliac crest slices and around most of the cortex of the femoral necks.

Figure 4.16A BSE-SEM 74x of the rim of lumbar vertebral slice of a 77 year female (specimen 1229) showing the thinness of the 'cortex' of this bone and the fairly narrow range of mineralization densities in the bone underlying the calcified fibrocartilage at the site of the insertion of the annulus fibrosis.

Figure 4.16B 20kV BSE-SEM 50x of calcified fibrocartilage in the cortex of the femoral neck from a 69 year male (specimen 1218) which has been incompletely replaced by bone of lower mineralization density in the centre of the field. Note how the bone shows a limited range of mineralization densities, as in the micrograph above. The mineralization density histogram for the image analysed in this field was:

18.79% low,
48.16% medium,
24.08% high, and
8.62% very high.

The mean greylevel value was 165.14.
Figure 4.17

Regions of low mineralization

Regions of less well mineralized bone were occasionally seen on the trabecular surfaces of the postcranial bones, but more frequently an appearance of defective mineralization is seen in the cortical regions.

Figure 4.17A 20kV BSE-SEM micrographs, of trabecular bone in the femoral neck of a 79 year male (specimen 1199). Note the less well mineralized region on the trabecular surface facing the right hand side of the image. The distribution of the mineralization density fractions in the analytical image corresponding to this field differs significantly to those for the mandible and are as follows:

- 22.02% low,
- 50.42% medium,
- 24.69% high, and
- 2.84% very high.

The mean value was 161.14

Figure 4.17B 20kV BSE-SEM, of the anterior cortex of the fourth lumbar vertebra of an 85 year old woman (specimen 1206). This site was quite often seen to have osteons with the appearance shown here. There is a region of defective mineralization within the osteon, but the central portion seems to be fairly well mineralized. Examination of the secondary electron image confirmed that this area was not a region damaged by the grinding and milling processes.
Highly mineralized cement lines

Highly mineralized cement lines were occasionally seen in the trabecular regions of postcranial bones. These differ from those seen in the mandible (figure 4.8) in their relative brightness with respect to the surrounding bone and in their location. In the postcranial bones they tended to be singular, and much rarer than in the mandible.

Figure 4.18A 20kV BSE-SEM of a trabecula in the femoral neck of a 69 year male (specimen 1218). Note the well mineralized cement line in the centre of the trabecula. This obviously represents an extremely long-period resting line. Whether it is a resting-resorbed or a resting-whilst-otherwise-forming line is difficult to determine because resorbed trabeculae frequently have a rather smooth profile. In either event, such lines only prove an extreme temporal uncoupling of new formation to any prior resorption and cast doubt on the wisdom of a close adherence to the resorption-formation story. The new bone which has been deposited at this line appears to be well attached to the older part of the trabecula.

Figure 4.18B BSE-SEM 200x of the femoral neck of an 83 year old female (specimen 1217). Here, it appears that the right hand extent of the line has been partially resorbed and replaced with newer bone.
**Figure 4.19**

**Highly mineralized cement lines II**

Higher magnification BSE-SEM micrographs of highly mineralized cementing lines in the midline of the mandible and the trabecular portion of the femoral neck.

**Figure 4.19A** BSE-SEM 120x of cement lines in the mandibular midline of a 92 year female (specimen 29).

Here they were seen frequently, and seemed to occur during the sequential apposition of bone, and not at the site of prior resorption (ie they are resting and not reversal lines).

**Figure 4.19B** BSE-SEM 1000x of the femoral neck of a 69 year male (1218). Here it can be seen that the line has a more diffuse side (towards the top of the image) and a better demarcated side.
Discussion

Since the widespread introduction of clinical bone densitometry using absorptiometry, many investigations have been carried out on the bones most obviously of interest in the study of osteoporosis, but fewer studies have been performed on bones of the head (von Wawern 1988, Klemetti et al 1993, Ulm et al 1994). Karlsson et al (1995), in a study of over 300 subjects, found there to be a significant loss of 'bone mineral' over the age of 20 years in the upper part of the skulls of males, and in the femoral necks of both sexes. Klemetti et al (1993) found the 'bone mineral density' of the buccal cortex of the mandible to correlate with that of the femoral neck as well as that of the lumbar spine. These results differ from the findings presented here. However, both of these studies used dual energy x-ray absorptiometry which may be influenced both by the mineralization density and by the thickness and porosity of the tissue: and a correlation between the cortical porosity of the mandible and the femur have previously been noted (Dyer & Ball 1980).

Since it is unknown which parameter of bone density is being measured by these clinical imaging techniques, the present study was undertaken to determine whether there is any relationship between the mineralization density of the bone of the mandible with that of other skeletal sites, and to determine the changes with aging.

Mandible

Because the largest age series was available for the 'lingual split' sample region, the best demonstration of an increase in the mineralization density with age was found in the bone lingual to the site of the mandibular third molar. This may be a real finding common to all bone, and was substantiated by the results found for the midline, but it has to be borne in mind that it is possible that in young individuals, particularly those who require removal of their third molars, other factors are affecting the bone in this region (including the continuing lingual cortical drift). Third molars are usually removed whilst in the process of eruption so that the bone turnover may be expected to be increased at this site. However, the findings would tend to reflect the clinical perception that tooth extraction may be more difficult in the elderly and the fact that greenstick fractures occur in the young. The region tested was very small and may sometimes have included the site of attachment of the mylohyoid muscle which may further complicate the picture with regard to turnover, both because the sites of muscle attachment often seemed to have a different mineralization density, and also because the activity of the muscles of the floor of mouth and tongue may change upon the individual becoming edentulous (Klemetti 1994c).

The whole bone cross sections showed that different aspects of the mandible have different mean mineralization densities. This agrees with the findings of previous studies (Manson & Lucas 1962, Atkinson & Woodhead 1968, von Wawern & Stoltze 1977,
Jager et al 1990) who found larger Haversian canal sizes, indicative of at least the resorption phase of remodelling, in the alveolar and buccal cortices, especially in the elderly. Yet, using computed quantitative microradiography, Hobson and Beynon (1988) found no difference in the mineral density between buccal and lingual sites in the mandible of six specimens, but their technique of measurement cannot have been as sensitive as the one described here because the whole section thickness of 80-100 µm was sampled.

In general, the present study found that the mineralization density tended to reduce the more superior the site sampled on the mandible, with the alveolar portion having the lowest apparent density at the mental foramen and midline regions. In the posterior mandible (LS region), it was the supero-lingual aspect of the slice (ls site) that had the lowest density with the alveolar portion not differing significantly from the inferior cortex. This may be because the site taken as alveolar in the posterior mandible was, in fact, nontooth-bearing and was therefore unaffected by the factors affecting turnover and producing lower mean mineralization densities at tooth bearing sites. Correlation between the sites was good, except for the alveolar and trabecular regions. As mentioned, there may be some additional factors to consider for the alveolar portion compared with the other aspects of the mandible (and may be influenced by the presence of the attached gingiva even in the edentulous), and the trabecular regions were often represented by a very small amount of bone, showing a large standard deviation in the mineralization density measurements in the posterior and mental foramen regions where there was less tissue available to sample. Sometimes the trabeculae, together with other regions of the mandible, had clearly noticeable cement lines: it was the presence of these that tended to have the largest effect upon the percentage of bone recorded in the highest bin of data.

The regions of highest density seemed to shift from the lingual/inferior region at the midline to the inferior cortex at the mental foramen and to the buccal cortex at the most posterior site sampled (ie the LS slices). This is very interesting because the sites of low density bear some relationship to where bone is lost postextraction, and the sites of high density to where the highest principal strains have been predicted to occur during biting from finite element analysis models (Korioth et al 1992). Obviously the most bone is lost from the alveolar region (lowest density): anteriorly, bone is additionally lost from the buccal aspect, and posteriorly from the lingual aspect. Whether the higher mineralization density retards the resorption of that region (Jones et al 1995), or whether the effect is secondary, in that less resorption is occurring at these sites due to the original tooth position, cannot be certain. On balance, however, the latter may be the more readily accepted view, since the pattern of resorption following tooth loss has traditionally been attributed to the tooth position, with the thinner cortical plate having the preference for
removal. Yet the reason that one cortical plate is thinner than the other at different sites of the mandible could be a result of the functional differences, and hence have a prior effect upon the requirements of the bone. This then raises the issue of the effect of dental status, which is the only way a study like this can in any way extrapolate to what had been happening in life.

Effect of dental status

In the posterior mandible, the mineralization density of the lingual cortex of partially dentate individuals was significantly greater than in the dentate or edentulous. The inferior cortex portion of the partially dentate was also greater than in the dentate, but the latter were younger.

Partially dentate individuals can still generate high strains with their remaining teeth (Helkimo et al 1976) and the highest compressional forces are experienced on the lower border during biting (van Buskirk et al 1988). However, tooth loss reduces the torsional rigidity of the mandible (Daegling et al 1992), which must be compensated for in another way. The mechanical properties do not improve by increasing the diameter of the bone, since edentulous or partially dentate mandibles reduce in size: therefore, to withstand the same forces, any improvement must either be achieved through an increase in the proportion of cortical bone or via a change in its material properties (as was seen here).

The reduction in size that occurs in the partially dentate and the edentulous means that the sites taken as representing the alveolar crest and the mean values measured from the buccal and lingual cortices might be expected to be higher than in the dentate, merely because they will be sampling a more inferior, and hence more well mineralized, area of the mandible. Yet at the mental foramen region, the partially dentate had higher mean mineralization densities than the edentulous in the alveolar and lingual regions, with the dentate showing no differences to either the edentate or the edentulous. It therefore seems that the differences in the levels of mineralization between the groups of individuals with differing dental status could truly represent an adaptive change to the altered functional requirements.

At the midline, the labial and alveolar sites (where the net resorption occurs after the loss of the teeth) of those individuals with teeth were more highly mineralized than those without. Postextraction resorption is akin to disuse atrophy in that the resorption does not just take place from the periosteal or endosteal surfaces, but from within the Haversian systems, which is probably the mechanism which results in the change of mineralization density with tooth loss. However, caution must be exercised in drawing too many conclusions from the small numbers of individuals left after they had been separated into the different groups for dental status.
With the exception of the alveolar region, all of the sites showed some positive correlation between the different regions, with adjacent regions (i.e., MD with MF, and MF with LS) having closer associations. Thus the inferior, buccal and trabecular sites of the midline were correlated with the respective sites at the mental foramen region; and the lingual split and mental foramen regions were correlated over the buccal and lingual cortices. Only in the buccal site were all regions correlated; it was the buccal region of the mandible that Klemetti et al. (1993) found to correlate best with postcranial bone density.

The factors studied in this and the previous chapter suggest that the mandible not only adapts to its changing functional demands by altering its internal architecture and external form, but that it may enhance its mechanical properties by varying its level and pattern of mineralization. The determination of this by such an accurate method has not been tested previously and it would be very interesting and exciting to test this hypothesis further.

It may transpire with subsequent studies that it is possible to use the pattern of the mineralization density variations around bones to give information on the functional strains perceived in the different sites. This would be particularly useful for a complex bone such as the mandible which may experience a vast range of strains, complicated by the presence of the teeth, and for which the creation of models for reliable analysis of strains and stress is difficult (Ralph & Caputo 1975, Korioth et al. 1992).

*Calvarial bone*

Excluding neonates from the sample, there was no correlation between mineralization density and age. However, neonates had significantly lower mean mineralization densities than the rest of the population, which implies that there is a change in the mineralization density with age, but that it must occur sometime in the first two decades of life (for which no samples were available in this study) in agreement with Jones & Boyde (1995). The reason that the variation in mineralization density with age differs from the mandible, which continued to show changes in the third decade and possibly beyond, may be partly due to the fact that the cranial vault reaches its adult size at a much earlier stage than the mandible; there are differences in strains experienced by the two bones (further discussed in chapter 5), and; the calvaria is not influenced to such an extent by dental changes.
**Correlations between cranial and postcranial bones**

The postcranial bones all came from elderly individuals. This helped to reduce interindividual differences attributable to aging, but may not be very representative of the healthy skeleton.

The calvarial bone had a lower mean mineralization density than the mandibular sections, but higher than the rest of the skeletal sites tested. The mandibular slices were significantly more highly mineralized than all the other sites. This is interesting because the three postcranial sites had regions of very highly mineralized tissue in their cortices, yet the immediately adjacent bone is significantly less highly mineralized than the bone in the trabecular regions (table 4.14). This helps to emphasise the importance of using standards in quantitative microscopy, and also indicates that the mean mineralization density can give a misleading picture of the mineralization density distributions unless account is taken of the local anatomy. Therefore the four-bin analysis becomes increasingly interesting when comparing between bones from different sites. The four bin analysis (figure 4.6) showed how the mandible had a larger proportion of bone falling into the two bins with the highest mineralization levels.

Why there was correlation between the mean mineralization density of the calvarial bone and that of the posterior mandible and mental foramen sites, but not for the midline, is uncertain, but it may relate to the different development of the mandibular midline to the other mandibular regions, the fact that it is subject to a large amount of flexing (Korioth et al 1992) during the 'wishboning' of the mandible described by Hylander (1979), or because there were too few specimens available from the midline to show a trend. The calvarial mineralization density did not compare well with that for the lumbar vertebra and iliac crest, but the skull fields were almost entirely cortical, whereas the lumbar vertebra and iliac crest regions have a substantial trabecular component which would create a greater partial volume effect.

There were no significant differences between the overall mineralization densities of the three mandibular sites, which were all significantly more highly mineralized than the calvaria. The calvaria, in turn, was significantly more highly mineralized than the postcranial sites. The femoral neck had a higher mean mineralization density than the iliac crest and lumbar vertebra.

The differences between cranial and postcranial bone are very interesting, and raise the question as to what accounts for this dissimilarity, as well as to what purpose they may serve. The levels of stored growth factors are known to vary throughout the skeleton and could have an influence in the turnover of the bone at these sites (Baylink et al 1993). It may be that maintaining the material properties of postcranial bone by
preventing it from reaching its highest mineralization density is more important for its locomotory role than it is for the mandible. Therapies for osteoporosis, such as fluoride, which might be expected to affect the mineralization density of bone, have not always produce the desired fall in fracture incidence, despite their success at increasing the 'bone density'. Yet, in osteoporotic states, fractures occur in the postcranial but not in the cranial skeleton. This may be due to the different proportions of trabecular and cortical bone that these regions have. The collagen fibre arrangement in bone cortices, including the mandible (Bromage and Boyde, in preparation) is well ordered and arranged primarily to resist tensional forces (Riggs et al 1993). This same network of continuous reinforcing fibres cannot operate to the same extent in bones which consist mainly of trabecular bone, which may therefore have to guard against becoming too well mineralized.

Traditionally osteoporosis has been described as a disease affecting bone quantity and not quality. This is largely to distinguish it from osteomalacia in which there is an increase in the quantity of unmineralized osteoid. Yet aging and osteoporosis are be associated with higher mineralization densities. In addition, osteoporotic bone may contain an increased number of osteocytes per unit volume, which has been attributed to each osteoblast producing a smaller amount of bone matrix (Mullender et al 1996), which may favour the attainment of higher level of mineralization.

Comparison of apparent density and mineralization density
There is no significant correlation between the apparent density as measured in g/cm³ and the level of mineralization as determined from the backscattered electron analysis. This provides evidence that bone quantity acts independently of bone quality.

Morphological observations
In all body sites, highly mineralized resting lines are seen. No site, however, matches the scale to which these were seen in some individuals in the mandibular midline. Here they occurred particularly in the trabecular regions and contributed to the high mean mineralization density values obtained here, although occasionally the mineralization levels in the interstitial bone reached a higher value. The nature and cause of the highly mineralized lines in the postcranial bones is unknown, but they most likely represent a prolonged resting period in bone turnover in which the resorption and formation is poorly coupled.
Summary of findings

1. The alveolar crest has a lower mineralization density than other regions of the mandible (Landini 1991).

2. The pattern of the regions with lower mineralization density in the mandible seems to match the pattern of bone loss that follows becoming edentulous.

3. The regions of highest mineralization density in the mandible mirror the sites thought to experience the highest stresses.

4. The mandible is more highly mineralized than, and its mineralization level is not correlated with that in, the bone from the postcranial skeleton.

5. Bone apparent density behaves independently of mineralization density.

It is interesting that the regions of lower mineralization density are most affected by resorption. This may be cause or effect, since the lower density may be the first signs of the increased turnover which ultimately leads to loss. Yet it is known that substrates with lower mineralization densities may be more rapidly resorbed by osteoclasts (Jones et al 1995).

The following chapters investigate what effects the aging and anatomical features seen in this chapter may have upon osteoclastic resorption. These are: the effect of the bone origin, cranial or postcranial (chapter 5); the effect of extrinsic fibre bone, which can be found in relatively large amount in the dentate individuals, but must disappear at some point following tooth loss (chapter 6); and lastly, the vitality of the osteocytes, which may be substantially changed (chapter 7).
CHAPTER 5

THE EFFECT OF BONE ORIGIN UPON OSTEOCLASTIC RESORPTION

The previous chapters have highlighted some of the anatomical differences between bones of cranial and postcranial origin. There is an increasing number of papers in the literature concerning other interesting features of cranial as opposed to postcranial bone, covering such areas as its properties as a grafting material (Smith & Abramson 1974, Zins & Whitaker 1983, Moskalewski et al 1988, 1991, Scott & Hightower 1991, Scott et al 1994), its rate of healing after grafting (Kusiak et al 1985, Sasano et al 1995), and its response to mechanical stress (Mackie et al 1994). These differences have been attributed both to cranial bone's origin as a 'membrane bone' (Zins & Whitaker 1983), and the different functional demands upon it. (Chapter 7 discusses the difficulties that may be encountered in the interpretation of grafting experiments.)

The evolutionary origin of bones that are often referred to as membrane bones is distinct from that of the other bones of the skeleton. Like teeth, they evolved from the tissues that originally formed the scales or other skin appendages of primitive fishes, instead of from the cartilaginous backbone and associated structures (Smith et al 1994). Since some appendicular bones may embryologically develop in membrane, albeit a condensation of perichondrium, and since some cranial bones may be associated with cartilage, it is more accurate to refer to membrane bones and cartilage bones as being of 'dermal' and 'endoskeletal' origins. The bones of modern vertebrates that are of dermal origin include: the bones of the cranial vault (Couly et al 1993), the bones of the face (including mandible and maxilla) and the clavicle; the remainder are endoskeletal.

If one considers only the bones of dermal origin, there is still a large variation in functional requirement; the calvaria may be argued to have a mainly protective role, whereas the mandible and maxilla have to withstand the often substantial forces generated during mastication. Although the calvariae of some animals are subject to high strains (Jaslow & Biewener 1995 propose that peak strains in excess of 1000 με may occur at the base of goats' hornscores during fighting), Hylander et al (1991) found that primate crania experience strains at a level lower than that which is considered necessary to maintain bone in the appendicular skeleton. They recorded in vivo strains of the order of 100-500 με in the frontal bones of monkeys during incision and mastication: in the human, however, mastication has been found to cause smaller strains in the cranial vault than grimacing (Hillam et al 1995).

Few in vivo studies have been done on strains generated in the mandible. Dechow et al (1995), in a study of the stress shielding properties of bone plates on the body of the
monkey mandible, reported strains in excess of 2000 με during maximal electrical stimulation of the masseter and temporalis muscles. In the ex vivo passive loading of human mandibles Daegling et al (1992) recorded peak principal strains between -900 με (negative values refer to compression) and 500 με. This is also within the range reported for the human condylar neck (Throckmorton et al 1992). In postcranial bones from a variety of animals, normal loading results in strains between 150 and 1000 με with peak levels in long bones reaching in excess of 3000 με (Rubin & Lanyon 1984, Skerry & Lanyon 1993).

The reason that dermal bone may be maintained when subjected only to low strains is unknown. This chapter therefore addresses whether there is a difference in the sizes of resorption pits made when equivalent osteoclasts are cultured upon bone of dermal and endoskeletal origins in an in vitro system.

Methods
Slices measuring 6 x 7 x 0.7 mm were cut parallel to the external surfaces of the frontal bone and the first phalanx (cranial surface) of an adult horse of unknown sex in its second decade, and the smoother surface of each slice was marked. The bone had been refrigerated for approximately one day prior to fixation in 70% ethanol and sectioning. The slices were defatted by refluxing for three days in 50:50 chloroform:methanol in a Soxhlet apparatus, and were dried from ethanol after sonication in distilled water.

Culture
Long bones dissected from two 15-day chick embryos were cleaned of adherent soft tissues and cartilages and washed with phosphate-buffered saline. The shafts of the bones were transferred to Eagle's minimum essential medium containing glutamax-1 (GIBCO BRL, Life Technologies), 10% foetal calf serum, L-Glutamine and Gentamycin (50 μg/ml) and were cut into small fragments and triturated to dislodge osteoclasts from the bone. The large bone fragments were allowed to settle. The overlying cell suspension was transferred to four slices of each bone origin. The cells were allowed to settle for one hour in an incubator in a 5% CO₂ atmosphere at 37°C, after which non-adherent cells were rinsed off with fresh medium and 2 mls additional medium was added. The four cranial bone slices were cultured in one dish, and the four postcranial slices in another. After 24 h, the slices were cleaned of cells, air-dried from absolute ethanol, and glued to glass slides.

Measurements
The osteoclastic resorption lacunae were mapped and measured using a video-rate reflection confocal laser scanning microscope system (Lasertec Corporation, Japan 1LM2W), using a 40x/0.95NA objective with a coverslip attached to it. In this system a
He-Ne laser beam ($\lambda=633$ nm) is broadened to a line using a cylindrical lens, which is scanned in the field direction by an acousto-optical device. The signal corresponding to each point in the line derives from a single detection element in a linear diode array. Thus images are acquired at full standard TV-rate. For each pit, or collection of pits, the microscope is set to move between two focus levels in the z-axis, between which it grabs 256 plane height images. At each level the data is compared with the data stored at the same pixel in the previous frame. The image produced may be viewed as a map, which retains the value of the focus at which the maximum signal intensity was found for each pixel, or a max image which retains the maximum intensity found during the z scan (Boyde & Jones 1992, 1995, Jones et al 1992, Jones & Boyde 1993).

The software automatically calculates the volume and area, taking the surface reference level to be the average height that falls under a trace binary 5-6 pixels wide and 5-10 pixels from the edge of the pit (figure 5.1a, overleaf) (Boyde et al 1990; Boyde & Jones 1995). In instances where it is difficult to estimate the true extent of the pit from the map image (for example if they had a shelving profile) the max image can be examined (figure 5.1b, overleaf).

Where possible the volumes and areas of over 320 pits per slice were measured. All pits with volumes greater than 10,000 $\mu$m$^3$ were excluded from the analysis, as these were likely to be the work of more than one osteoclast.

Roughness measurements of the slabs were made at random sites over the surface of the tissue using the same lens as for the volume and area measurements. For this, the software takes the area between a smoothed and an unsmoothed line following the surface contour of the tissue, and divides it by the maximum height that the two deviate in each segment; smooth surfaces have a number that approximates to zero. For each field examined the roughness is automatically calculated along 30 lines within the image, hence the tables show the number of sites measured as $n=x*30$.

After the measurements, one slice of each origin was embedded in PMMA, micromilled, and carbon coated for determination of the distribution of mineral densities by electron backscattering (Boyde et al 1995b) as in the method described in chapter 4. Four fields calibrated as an area of 2802$\mu$m*2658 $\mu$m were analysed on each slice at a nominal instrumental magnification of 33x, and using 512*512 pixels.

Statistical analysis
Analysis of all data was performed using Minitab statistical software.
Figure 5.1a Confocal micrograph of osteoclastic resorption pits in sperm whale cementum showing the 'map' image where the greatest depth is represented by black and the highest points as white. A trace binary, representing the slice surface from which the measurements are calculated surrounds each pit at a distance of 6 pixels.

Figure 5.1b 'Max' image of the same field. The max image can be examined to help determine the extent of a pit at the drawing round stage. Note that the pits appear to be inverted when examined in this mode.
Results
Numerous pits were present upon the bone slices (figures 5.2 a&b, overleaf). However, curling of the frontal bone slices upon drying made measurement impossible in some regions.

The results obtained from the analysis of the four slices from each group are shown in table 5.1. Since the slices were not seeded in pairs, the statistical analysis is only shown in the graph of pooled data (figure 5.3, overleaf). The median volumes and the volume:area ratios of the 860 pits in cranial bone were significantly smaller than the 1284 pits in leg bone (p<0.05 and 0.0001 respectively), but the areas were significantly greater (p=0.0001). The Mann-Whitney test was used since the data showed a skewed distribution. The exclusion of pits over 10 000 μm³ removed 28 and 38 pits from the analysis of the cranial and postcranial bone respectively.

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Figure 5.2 BSE-SEM micrographs of osteoclastic resorption pits in frontal (top) and phalangeal (bottom) equine bone slices.
Figure 5.3 Histogram showing sizes of resorption pits in cranial and postcranial bone mean values with standard errors for pits <10,000μm$^3$

- **p<0.0001
- *p<0.05
Figure 5.4 Pseudo-coloured quantitative BSE-SEM micrograph of equine frontal an phalangeal bone slices. The white and pink ends of the scale represent the most highly mineralized bone as is seen in the phalangeal bone - the yellows and browns are less highly mineralized.
The pseudocoloured images obtained using BSE-SEM show distinct differences between the nature of the substrates presented to the osteoclasts (figures 5.4 a&b). The frontal bone has a less homogeneous appearance, with many bone packets punctuated by bright resting and reversal lines indicative of a considerable amount of previous turnover (figures 5.5 a&b overleaf). In contrast, the phalangeal slices show a more even appearance in the mineralization density distribution, with fewer new bone packets. The areas of new bone, however, appear to be poorly mineralized relative to the highly mineralized background in which it is still possible to see areas with large osteocyte lacunae. Both sets of images show a mixture of transversely, obliquely and more tangentially cut Haversian systems. On average, the cranial slices were rougher than the leg slices (table 5.2), but fewer measurements could be made on the former due to their curvature.

<table>
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<tr>
<td>median</td>
<td>0.31**</td>
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</table>

**p<0.0001 Mann-Whitney

The mineralization density was greater in the postcranial bone (mean and median (standard deviation) BSE coefficients, cranial 0.1348, 0.1360 (0.0032); phalanx 0.1392, 0.1410 (0.0036)).

Discussion

This study addressed possible differences in the resorbability of cranial and postcranial bone in vitro where mechanical effects due to different functional requirements, which may account for some of the previously reported differences in the behaviour of the bones from the two origins in vivo, have been removed.

The mean depths, volumes and the areas of the resorption pits made in the bones of the two origins were significantly different, with the cranial bone having pits of a smaller mean volume. This agrees with those in vivo studies which suggest that cranial bone grafts remain for longer periods than postcranial grafts (Smith & Abramson 1974), despite an initial faster revascularisation (Kusiak et al 1985) and slower osteogenetic response (Sasano et al 1995). There may be a number of reasons for this difference.
Figure 5.5a  BSE-SEM image of equine frontal bone showing many bright reversal lines indicative of previous turnover.

Figure 5.5b shows the appearance of the phalanx, with the new bone showing up very prominently against the more homogeneous background. Note the numerous large osteocyte lacunae in the primary bone. Original magnification 33x.
The bone slices were obtained from the frontal bone and the cranial (anterior) surface of first phalanx (or pastern) of the same adult horse. They had to be taken parallel to the external surface of the bone as the frontal bone is thin (in fact, probably far thinner than bone that would be selected as a grafting material) and would not provide a large enough area upon which to culture osteoclasts if sectioned in a perpendicular direction. The horse was killed by a captive bolt, making a hole one inch above the intersect of a line running from the medial canthus of each eye to the contralateral ear. The frontal bone fragment came from a region infero-anterior to this hole and showed no macroscopic or microscopic signs of fracture. In the adult the pastern is a straight bone that is approximately 100 mm in length and is composed of a thick cortex and a very densely trabecularised medulla. The two bones are therefore very different, as suits their distinct functions. The excessive curving upon dehydration after culture of the frontal slices may reflect a different collagen fibre orientation to the other slices. The arrangement of the collagen fibres might have an effect upon resorption as will be seen in the next chapter. This curving occurred despite the use of a greater thickness of slice than is normally used for resorption assays in this laboratory (600-700 μm compared with 200-300 μm) let alone other laboratories (100 μm - Collins & Chambers 1991). The effects of contained collagen fibres and slice roughness is discussed further in the next chapter.

The implications of the mean mineralization density value of bone has been discussed in chapter 4. The reason that the frontal bone has a lower mean mineralization density than the phalangeal bone may result from a higher rate of turnover in the part studied or to a slower rate or lower endpoint for mineralization. The BSE images show the cranial bone site to have more evidence of turnover than at the phalangeal site in this experiment. Where new bone is seen in the phalanx, it appears to be less highly mineralized than the new bone packets in the cranium because it contrasts markedly with the surrounding bone. The quantitative technique used shows that the new bone in the phalanx is, in fact, mineralized to a similar degree as the cranial bone, but that the mature bone is more highly mineralized. This, therefore, shows a different pattern in the mineralization density differences between cranial and postcranial bone than was seen for humans in the last chapter. This may result from the use of the cranial rather than the caudal surface of the phalanx, which may differ greatly in their structure (as was shown for the equine radius by Riggs et al 1993), or it could represent the difference between being in secondary osteonal bone and in circumferential lamellar bone, and may not be the case if the whole bone cross sections were considered. If this experiment was to be repeated using the same bones, it would be better to have three sets of slices, with the additional one coming from the caudal surface of the phalanx.
It is known that the volume resorbed by osteoclasts is inversely proportional to the degree of mineralization of the substrate (Jones et al 1995). In this case, it would be expected that the less highly mineralized cranial bone would have had larger resorption lacunae than the phalanx, which was not the case. Thus, clearly it is not a difference in the mean mineralization density that accounts for difference in the resorption of the two substrates.

In addition, it is clear from the amount of previous turnover as seen in the BSE images (figure 5.5a) that resorption does occur readily within this part of the frontal bone in vivo. In contrast, the phalanx showed less evidence of prior turnover (figure 5.5b) yet had larger resorption lacunae in vitro.

One factor that may account for the shallower, wider shape of the pits in cranial bone may be related to the frequency of the cement lines. The abrupt change in mineral density that occurs at these lines may act as a hindrance to resorption, even in a bone which overall is less highly mineralized. Alternatively it may be due to different levels of contained growth factors or proteinases (Délaissé et al 1987).

Even if there was a difference in the physical characteristics of cranial bone, it would not necessarily explain why the bone can be maintained in a lower stress environment, since in vivo resorption occurs readily in the normal turnover of the bone. The fact that low peak strains have been recorded in the cranial vault does not necessarily imply that higher strains do not occur, although Mackie et al (1994) have reported that calvarial osteocytes respond to stress in a different manner from osteocytes from other regions.

This study emphasises that a measure of pit area alone will not be a reliable index of the amount of tissue destroyed. The significance of the difference between the areas resorbed for each type of bone in the present study is greater than those which have been taken to indicate a difference between experimental and control groups in earlier studies (Alam et al 1992), yet there was only just a significant difference in the mean volume. This further emphasises the conclusions regarding pitfalls in pit measurement considered by Boyde & Jones (1991).

**Conclusion**

This chapter has shown that there is a difference between the in vitro resorption of equine cranial and leg bone. The former exhibited a relative resistance to resorption even though it was less highly mineralized, which should have favoured its removal.
CHAPTER 6

THE EFFECT OF SUBSTRATE COLLAGEN FIBRE ORIENTATION UPON THE SHAPE OF OSTEOCLASTIC RESORPTION PITS MADE BY CHICK OSTEOCLASTS IN VITRO

As discussed in chapter 4, regions of bundle bone are more abundant in the alveolar bone of dentate individuals than they are in edentulous mandibles. Sharpey's fibres do also occur at some locations in basal bone at the sites of muscular or ligamentous attachments. In contrast to alveolar bone, these areas (most notably the mylohyoid, and geniohyoid attachment sites) often become prominent in the edentulous mandible as it remodels, and may require surgical reduction. This disproportion in the amount of remodelling after loss of the teeth reflects the differing functional requirements of the bone at these sites, since even these 'resistant' muscle attachment sites will exhibit changes in pathological states affecting muscular function (Mouly 1959, White et al 1977, Pogrel 1988, Cawson et al 1996), in addition the activity of the tongue may increase in the edentulous state (Klemetti et al 1994c). It is unknown, however, whether the presence of extrinsic fibres may have any effect upon the ease of resorption of the tissue; for this reason the resorption of a substrate containing extrinsic collagen fibres was studied.

Background

In vitro assays of osteoclastic resorption by cells isolated from bone use slices of mineralized tissues upon which the cells are seeded. The substrate is then examined to assess the resorptive activity of the cells under the varying experimental conditions. Dentine is usually used as a substrate because it is relatively uniform, which facilitates pit identification and measurement (Boyde et al 1984). It is known that the volume resorbed by osteoclasts is inversely proportional to the degree of mineralization of the substrate (Jones et al 1995), but the question as to whether the orientation of collagen (and mineral) may affect the rate of resorption of bone-like tissues has been largely ignored.

The predominant collagen fibre orientation in the mid-shaft of human long bones is statistically more nearly parallel with the long axis (Portigliatti-Barbos et al 1987, Carando et al 1991). A comparison of the depths, volumes and areas of resorption pits in longitudinal and transverse sections of mid-shaft human femur, however, failed to reveal any influence of slice orientation on the shape or size of resorption pits (Boyde & Jones 1992). This may have been due to a relative lack of preferred orientation in this tissue or an inadequacy in the number of pits measured. A better experimental model might be derived from other choices within bone or some other mammalian mineralized
tissue. For example, the anisotropy of collagen fibre orientation may be such that the fibres are predominantly parallel, at least for the extrinsic fibre component, in Sharpey fibre bone at the insertions of tendons and ligaments. Physiological resorption occurs in the restructuring of such sites. Exaggerated resorption may occur after reduction in the functional loading. Notable examples of this include the remodelling that is seen after a muscle has been denervated (Carter & Harkness 1995), or the resorption of alveolar bone that occurs after tooth loss. In this study, designed to determine whether the shape of resorption pits produced by osteoclasts in culture is influenced by the organisation of collagen fibre bundles, dental cementum was chosen as a substrate. Cementum was favoured for two reasons: firstly, the fractional content of extrinsic (Sharpey's) fibres in cementum can be higher than in other bundle bone, so that any effect the fibres may have upon resorption should be pronounced; and, secondly, it is highly anisotropic. This means that if cut in two, carefully selected, mutually perpendicular planes it should be possible to obtain a substrate that presents two very different orientations of fibres at the surface.

Method
The experimental method was similar to that detailed in the previous chapter. In this instance, slices of sperm whale (Physeter catodon) cementum were used. Male sperm whale mandibular teeth are large and have a thick layer of cementum. Although the extrinsic fibre bundles may show areas of decussation (Boyde 1971) it is still possible to prepare sections in which the predominant extrinsic fibre orientation is more perpendicular or parallel to the surface (figure 6.1). Flat slices 600-800μm thick were cut in each orientation (being called Group I and Group II respectively) using a water-cooled diamond saw (Isomet Low Speed Saw, Buehler Ltd, USA 60204). All slices were obtained from the same sperm whale tooth and at the same horizon.

Osteoclasts were obtained from pre-hatch chick embryos as in the previous chapter, except that no antibiotic was added to the medium. The cell suspension was transferred to the slabs of sperm whale cementum (SWC), alternate aliquots of approximately 150 μls being applied to Groups I and II which were kept under identical conditions. After 24 hours of culture the slices were immersed in water to ensure osmotic disruption of cells, and further cleaned by treatment with an alkaline protease solution (Terg-A-Zyme, Alconox Inc. New York) for 1-4 hours to remove the residual demineralized collagen fringe within the resorption pits. They were then sonicated in distilled water, transferred to ethanol, air dried and then attached to slides with double-sided adhesive tape. A perspex slide was then placed over the slabs and a weight of 1.5 kg was applied to keep them flat until measurement. The volumes and areas of all pits on the slices were measured using the Lasertec 1LM2W at a magnification of 40x. The mean depth (volume:area ratio) was calculated for each pit from the volume and area data, as was a
Figure 6.1 BSE-SEM of block of sperm whale cementum cut and polished to produce two facets at 90° which imitate the Group I (bottom left) and Group II (right) substrates used in this chapter. The nonmineralized cores of the extrinsic fibres appear dark. Fieldwidth 500 microns (fold page along the white line in the image to reconstruct in three dimensions).

Figure 6.2 Processed BSE-SEM image of milled, embedded sperm whale cementum slice. Note how the fibre boundaries have been enhanced with an edge-finding filter to aid the calculation of the percentage occupation of the tissue with extrinsic and intrinsic fibres. See also the pits in profile at the surface of the slice.
three dimensional form factor (3DFF). This compares the pit shape to that of a hemisphere of the same projected area was also calculated using the formula $3DFF = \frac{V_p}{V_h}$; where $V_p$ is the pit volume and $V_h = \frac{2}{3}\text{area} \times (\text{area}/\Pi)$. This numerical index of shape is not influenced by the overall pit size and returns a value of 1 for pits with a hemispherical shape; those less than 1 will have a more shallow profile. The surface roughness of each slice was also measured. After measurement the slices were embedded and viewed, as described below, for verification of the fibre orientation.

Reproducibility was tested by the 'blind' remeasuring of 148 pits. In addition, for the first experiment an elongation ratio was calculated for a random selection of over 65 pits from each group to determine whether there was any increased tendency for osteoclast translocation along the extrinsic fibre axis in Group II. For this purpose, confocal images were acquired using the >515 nm autofluorescence excited by 488 nm radiation of an Odyssey video rate laser scanning confocal microscope (Noran Inc. Middleton WI 53562 USA). These images were captured using a Noran TN8502 image analysing computer. The elongation ratio was taken as the ratio of the length to the width of each pit as measured perpendicular to each other in the surface plane.

To see if the results obtained could be attributed to a physico-chemical effect caused by the orientation of the mineral within the tissue, one slice from each orientation was placed in 0.1N HCl for thirty minutes, prior to being dehydrated and embedded. Care was taken never to let the slices dry in air to avoid shrinkage of the demineralized collagen fringe. The blocks were polished to expose the profile of the slices as before and measurements were made of the thickness of the demineralized zone at multiple points.

**Embedding**

An additional pair of slabs was cultured as above but the added cells were preserved in situ. They were washed in warm (37°C) phosphate-buffered saline and fixed in 2.5% glutaraldehyde in a 0.15 M sodium cacodylate buffer initially at 37°C, for two hours. The slabs were rewashed and post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 1½ hours to stain (ie increase the mean atomic number and density of) the surface cell layer, dehydrated with alcohol, and embedded in PMMA containing 5% styrene (Boyde 1984). The embedded samples were then polished perpendicular to the slab surface and coated with carbon by evaporation. These samples allowed observations to be made of the pit profiles, the depth of the demineralized collagen fringe and the surface topography, using BSE-SEM. Each sample was repolished and recoated several times to section more pits. By stretching the image and by filtering with an 'edge-finding' filter (Canny) with the Kontron image analysis equipment it was possible to improve the definition of the extrinsic fibre boundary (figure 6.2, previous page).
enabled a calculation of the proportion of the cement occupied by intrinsic and extrinsic fibres using a 100 point intersection stereological grid.

Experiment 1
One slab from each orientation, measuring approximately 20 mm by 15 mm, was used. After measurement the slabs were embedded and viewed as described above for verification of the fibre orientation, and for quantification of the proportion of extrinsic to intrinsic fibre cement.

Experiments 2-4
Four slabs (approximately 5-10 mm by 5 mm) from each orientation were prepared and seeded in pairs, alternating the groups to receive the first aliquot. The paired slabs were randomised before examination, but it was not possible to perform the measurements completely blind since the orientation of the fibres upon the slice surface was usually obvious.

Results
The areas, volumes and mean depths for experiments 1-4 and for the pooled data from all four experiments are shown in table 6.1 (overleaf). Table 6.2 shows the 3DFF results. The marked difference between the means and medians for area and volume in all the tables is an indication of the skewed distribution of the pit sizes. For this reason, the Mann-Whitney was the test selected to assess significance.

It is necessary to measure between 300-500 pits from each slice before a steady reading of cumulative mean data for areas, volumes and mean depths (figures 6.3-5, pages 160-162) is obtained. It can be seen that the shapes of the cumulative mean volume and area graphs are similar. This fairly constant ratio (distinct for each group) is reflected in the cumulative mean depth graphs (figure 6.6, page 163), which plateaus earlier at around 150-200 pits. (This data is presented in the order of acquisition of the pits, where a constantly changing mean may partly reflect a difference from one region of the slice to another, either in terms of surface topography, or unevenness of cell seeding.) Randomisation of the order after pooling the results from all four experiments (figure 6.7, page 164), again reveals a similar trend.

In all cases where it was measured, the surface roughness, although variable across a slice, was significantly greater in Group I than in Group II (mean range Group I=0.33-1.02; Group II=0.24-1.98) (table 6.3).
### Table 6.1 Area (A), volume (V) and mean depth (V/A) data - all pits

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### Table 6.2 - Three dimensional form factor (3DFF)

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### Table 6.3 Roughness

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*p<0.05, **p<0.01, ***p<0.0001. In this table n=number of sites measured.
Experiment 1

282 resorption lacunae were measured in Group I, and 290 in Group II. The pits in Group I were significantly smaller (both in area and volume) yet deeper than those in Group II, having a more hemispherical shape. This did not appear to reflect a greater tendency for the osteoclasts to spread along the direction of the extrinsic collagen fibres in Group II, since the elongation ratios showed no significant differences between the groups (means of 1.39 and 1.31, n=68 & 69).

Experiment 2 (slices A & B)
Insufficient numbers of pits were obtained to yield consistent findings between the individual seeded pairs. Only when all groups were summed (n=232 and 326 for Groups I and II respectively) was a larger mean depth found in Group I, in agreement with the first experiment. (These differences are, however, accounted for by the third row of data alone, if the data is summed without this group of results - no significant difference is seen.) No significant differences were found in area and volume (means for volume appear much larger in Group I, but the medians are virtually identical). There was no significant difference in form factor, but it was greater in Group I than in Group II.

Experiment 3 (slices C & D)
No significant differences in area, volume or their ratio were found in the summed data. However, very few pits were obtained (n=180 and 126).

Experiment 4 (slices E & F)
In contrast to the first experiment the volume as well as the mean depth was greater in the first group, and no difference was found in the areas (n=491 and 217). The 3DFF was again significantly greater for Group I than Group II.

Pooled Results
There was a large range in the sizes of pits (areas: I=27-21658 μm², II=34-18328 μm²; volumes: I=63-286919 μm³, II=44-114526 μm³; mean depths: I=0.54-22.26 μm, II=0.71-12.15 μm). The scatter plots of volume against area (figure 6.7, page 164) show the wide overlap of the two sample groups. (However, the statistically significant intergroup differences in the mean depth holds at all sizes of pit.) For both groups I and II, over 85% of the pits were less than 2500 μm² or 30000 μm³, reducing to 37.5% and 25.9% respectively for pits less than 250 μm² or 3000 μm³. At all levels the difference in mean depth is significant with group I being greater than group II. The situation with volume and area is more complex. The pooled results show the area of the pits from Group II to be significantly larger than Group I; yet this difference only showed up in experiment 1 and three pairs of slices from experiments 3 and 4 (although it did not show up in the summed results of experiments 3 and 4). There are also two pairs of
Figure 6.3 Cumulative mean pit areas for each experiment
**Figure 6.4** Cumulative mean pit volumes for each experiment

**Expt 1**
- Group I
- Group II

**Expt 2**
- Group I
- Group II

**Expt 3**
- Group I
- Group II

**Expt 4**
- Group I
- Group II
Figure 6.5 Cumulative mean pit depths for each experiment
Figure 6.6 Cumulative mean values for pooled data randomised order

- Mean area (\(\mu m^2\))
  - Group I
  - Group II

- Mean volume (\(\mu m^3\))
  - Group I
  - Group II

- Mean depth (\(\mu m\))
  - Group I
  - Group II

Number of pits
Figure 6.7 Volume against area plots for all experiments
Pooled data

Inset of above

Inset of above

Inset of above

area (\(\mu m^2\))
slices (experiments 2 and 4) that show larger areas to occur in Group I. The volumes of the pits was significantly larger in either groups on several occasions; overall, the summed results show no difference in mean values, but the median value was greater in Group II even though the range was narrower. The form factor was considerably greater in Group I.

Reproducibility: there was no significant difference between the areas, volumes or mean depth measurements between the original and remeasured group of pits (p=0.92, 0.95 and 0.69 respectively - one way analysis of variance).

**Scanning electron microscopy**

The embedded slices with cells preserved provided information on the depth of the demineralized collagen fringe in the slices, and illustrated the considerable amount of decussation of the extrinsic fibres. The orientation of the intrinsic fibres also became apparent, tending to lie perpendicular to the predominant extrinsic fibre orientation (figure 6.8, overleaf).

Examination of the embedded slices confirmed that the predominant collagen fibre orientation is very different between the groups, and that in Group I, where the extrinsic fibres meet the surface, they may be associated with a mound of tissue (figure 6.9, overleaf). This accounts for the increased roughness of this group of slices.

Direct SEM and confocal microscopy of the surface showed that sometimes a Sharpey's fibre was left proud in the bottom of the pit (figures 6.10 & 6.11). However, apart from some rounding off at the corners, the depth of demineralisation of the slice surface by 30 minutes of treatment with dilute hydrochloric acid seemed to be fairly even and largely unaffected by the individual structural features of the substrate, with no significant difference between the thickness of the demineralized layer of each orientation (means of 35 μm and 36 μm for Groups I and II, n=23 and 36 (figure 12). However, a zone of partially demineralized matrix underlying the demineralized layer was more noticeable in the Group I slices.

The proportion of the slices made up of Sharpey's fibres was calculated as 62%, 19% of which was the volume occupied by the unmineralized cores. Cementocyte lacunae accounted approximately 1%, and the intrinsic fibres 37% (figure 6.2).
Figure 6.8  BSE-SEM of embedded sperm whale cementum slice viewed sideways on, showing the profile of a resorption pit. The outline of the cell surface layer, which has cracked away from the slice surface during embedding, was made visible by osmication. Note the great depth of the demineralized collagen fringe in the pit.

Figure 6.9  BSE-SEM at the same scale as above, showing profile of sperm whale cementum surface. Note the unevenness of the surface where the fibres reach the cut edge of the slice.
Figure 6.10 Confocal micrographs (Lasertec) showing exposed fibres in the base of the pit on a Group II slice: a is the map image, b is the max image.
Figure 6.11  Confocal micrograph (Lasertec) showing that the unmineralized extrinsic fibre core often remained proud relative to the mineralized fibre periphery in the base of pits on the Group I substrate.
Figure 6.12 BSE-SEM of micromilled, embedded, partially demineralized sperm whale cementum slice showing the demineralized surface layer towards the top of the picture on a Group I surface. The thickness of the demineralized zone was measured at multiple points on the substrates of both orientations - no intergroup differences were seen in thickness of the totally demineralized layer.
Discussion
This study was carried out to determine whether the extrinsic collagen fibre arrangement of a substrate may affect the size or shape of resorption lacunae in vitro; and hence whether it might affect the pattern of resorption in vivo or be of significance to those who use anisotropic substrates such as bone for resorption assays.

As mentioned in the previous chapter, it has been shown that the volume resorbed by osteoclasts is controlled by the degree of mineralization of the substrate (Jones et al 1995). This raises difficulties in designing a study to compare the resorption of substrates containing, and not containing, extrinsic fibres since any variation in the overall mineralization density of the tissue may mask or enhance other differences. For this reason it was thought easier to investigate whether the orientation of extrinsic fibres within a single substrate could affect the shape and size of resorption pits. Sperm whale cementum was chosen as a test substrate because of its high proportion of included principal periodontal ligament fibres. The perpendicularly oriented slices (Group I) were taken near to the external surface of cementum where the fibres are more ordered. The other slices spanned the full thickness of the cementum (from periodontal ligament to dentine) and consisted of a more variable content of fibres from one end of the slice to the other, although all were more or less parallel to the slice surface. This was as close as could be obtained to eliminating any effect of the mineral component of the substrate.

The use of a protease (Terg-A-Zyme®) to clean the slices prior to measurement is useful because it removes the collagen fringe, allowing an assessment of the amount of work carried out by the acid, reducing the sensitivity to different modes of drying, and probably giving a clearer reflected image which is required in the measurement stages. It has the advantage over hydrogen peroxide, which may itself be acidic, and hypochlorite which may weaken the slice. It may, however, affect the centres of some of the incompletely mineralized fibres, so its use was limited to a few hours.

The results indicated that resorption lacunae tended to be deeper when the predominant extrinsic fibre orientation was perpendicular to the slice surface (Group I). However, the differences between volumes and areas were less consistent and may be more dependent upon cellular, rather than substrate factors, and purely represent 'normal variation'. The differences observed in mean depth may be for several reasons.

Surface roughness
Cutting or polishing of an anisotropic material usually results in features lying parallel being preferentially removed to those that lie perpendicular to the surface. Since the intrinsic fibres generally lie at around 90° to the extrinsic fibres, this phenomenon would have occurred to some extent in both of the orientations of cementum investigated.
Overall, however, Group I slices had a rougher surface than Group II. This may have affected the measured depth of pits in two main ways:

a) by restricting the spreading of the osteoclasts either directly, or as a secondary effect by favouring the retention of osteoblasts on the surface. This may result in a compensatory increase in the mean depth of pit, with the resorptive capacity of the osteoclasts remaining unaltered. However, a simple analysis of surface pit shape showed no apparent difference between the groups.

b) because of its influence upon measurement. If the osteoclasts in Group I did tend to settle in the valleys of the slice surface, depth measurements may have been overestimated since they were calculated from the average height of the slice surface, the majority of which consists of extrinsic fibres which in some areas may protrude with the same order of magnitude as the pit depth. In addition, the cells will be resorbing the intrinsic fibre cement that may be less highly mineralized. Both of these factors would tend to increase the volume of pits in Group I, which was not the ultimate trend seen.

**Orientational effect of the tissue**

Since the extrinsic fibres make up the majority of the volume of the tissue, it follows that there will be more features lying perpendicular to the surface in Group I than in Group II. The structure of a substrate may affect resorption *in vitro*; often the shape of resorption pits in bone appears to be affected by the presence of lamellar boundaries which may represent the transition between collagen-rich and collagen-poor regions (Marotti 1993) or at least the transition between areas with different orientations of the intrinsic collagen fibres. In Group II, not only will the cells be presented with bands of highly mineralized tissue, with smaller bands of intrinsic fibre cement and nonmineralized cores, but the osteoclasts will be dissolving across the width of the fibre, starting at any point on the rota of: intrinsic fibre cement, highly mineralized extrinsic fibre periphery, poorly or nonmineralized extrinsic fibre core, and back to periphery etc. In Group I, the osteoclast will be able to follow along one or more fibres for the full depth of the pit. The collagen orientation will have some bearing upon the mineral orientation, and arrangements of crystals may show a preferred direction of dissolution upon exposure to acids. In addition, crevice corrosion may add to the effect; acids released by the osteoclasts may track along the edge of the nonmineralized core of the Sharpey's fibres and account for the appearance seen in figure 6.11b, which shows the greatest depth of the pit to be immediately adjacent to the nonmineralized fibre core.

In addition, higher levels of osteopontin may be associated with extrinsic collagen fibre bundles where it may play a role in the functional attachment of the fibres. Due to its association with high degrees of mineralization it may have an effect upon resorption (McKee & Nanci 1995).
Conclusions
This study suggests that the orientation of extrinsic fibres within a substrate can alter the
shape of osteoclastic resorption pits, and that the choice of substrate when undertaking
an osteoclastic resorption assay is very important. Although no report to date has used
cementum as a substrate, the shafts of long bones may contain extrinsic fibres. This is
particularly true when using, for example, bovine cortical bone slices in which extrinsic
fibres may be found surprisingly deep within the cortex, having escaped turnover due to
cortical drift during growth. Using sequential and adjacent slices for experimental and
control substrates may be acceptable, but it is important to avoid the temptation to use
slices of unknown origin. This finding is probably worth bearing in mind for people
doing assays looking for small effects, using slices of anisotropic material such as bone,
especially if they have been prepared by other people and are selected at random. In
addition, this study also emphasises the importance of measuring many pits, and of
checking whether a constant value has been reached.
CHAPTER 7

THE RESORPTION OF VITAL AND DEVITALIZED BONE IN VITRO:
SIGNIFICANCE FOR BONE GRAFTS

In normal circumstances the mandible experiences tension along its superior border
during biting (van Buskirk et al 1988), but in denture wearers there is likely to be an
element of compression during mastication. Rubin and Lanyon (1984) have suggested
that the osteocytes at particular sites around a bone may differ in their response to stress.
This might mean that an area, such as the lower border of the mandible that habitually
experiences compression would respond differently to the alveolar crest in this situation.
Yet, in chapter 4 it was seen that whole areas of mandibular bone may contain nonviable
osteocytes, which further complicates the picture. The way these areas of nonviable
osteocytes may affect the turnover of the tissue is uncertain, but an immense amount of
work has been done on the healing of nonvital bone in the form of bone grafts.

Although the literature concerning the fate of bone following grafting is vast (for reviews
see Urist 1953, Peer 1955, Chase & Herndon 1955, Burchardt 1983, Schweiberer et al
1989), its interpretation is complicated by the number of variables in the grafting
techniques used (table 7.1), and little is known about the importance of the cellular
events that follow implantation. The survival of the cells within the graft does not seem
to be one of the essential factors for bringing about clinical evidence of union (Chase &
Herndon 1955; Chalmers 1967; Frame et al 1982). The majority of cells are not thought
to survive very long even in fresh autogenous bone grafts, which, after microvascularly
reconstructed grafts, may be considered to provide the best chance for cellular survival
(Chase & Herndon 1955, Jonck 1981). Some studies which report survival of the graft
bone cell population make no allowance for the fact that new cells may be arriving via
the bloodstream (Puranen 1966, Berggren et al 1982). To overcome this problem,
research on bone graft vitality has concentrated upon the osteocyte as the ultimate
indicator of whether bone may be considered to be alive. Unfortunately, the duration for
which osteocytes are reported to survive once separated from their blood supply is as
varied as the methods by which their survival has been determined, from as little as four
hours to in excess of two weeks having been reported (Peer 1955, Gray & Elves 1981,
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</table>

**Table 7.1 Factors that may influence bone graft healing**

- Bassett 1972; Burchardt 1983
- Haggerty 1977; Gray & Elves 1981
- Albee 1944
- Albee 1944; Stevenson 1973
- Killey et al 1966; Habal 1994
- Chalmers 1967; Gray & Elves 1979
- Horwitz 1949
- Bassett 1972
- Gray & Elves 1979
- Peer 1955
- Smith & Abramson 1974;
- Kusiak et al 1985
- Gray & Elves 1979
- Pavlova & Vyalcev 1969;
- Plezia et al 1983; Kamijou et al 1994
- Narang et al 1982; Burchardt 1983
- Ely 1924
- Wangerin et al 1986
- Gonçalves & Merzel 1976
- Narang et al 1982
- Burchardt 1983
- Albee 1944; Eriksson et al 1984
- Berggren et al 1981; Beppu 1989;
- Treble et al 1990
- Berggren et al 1981
- Orell 1937
- Knize 1974; Gray & Elves 1979
- Evans et al 1991
- Bell et al 1985
- Berggren et al 1982;
- Moran & Wood 1993
- Deleu & Trueta 1965
- Peer 1955
- Habal 1994
- Evans et al 1991
- Smith & Abramson 1974
- Smith & Abramson 1974;
- Habal 1984; Kusiak et al 1985
- Wangerin et al 1986
- Smith & Abramson 1974
- Gray & Elves 1981
- Gray & Elves 1979
- Plezia et al 1983
- Tonna 1973;
- Thorogood & Craig Gray 1975
- Peer 1955
- Hunter 1750s; Habal 1994
- Burchardt 1983
Although fresh bone is favoured for grafting procedures (Urist 1953, Gray & Elves 1979, Hockley et al 1990), it is limited in availability and its harvest requires a prolonged operation with the risk of increased blood loss (Montgomery et al 1990). The alternative of using one of the various forms of dead bone (particularly frozen or freeze-dried) may result in slower healing (Urist 1953, Peer 1955), which some have attributed to a resistance of dead bone to the resorption which may precede new bone formation (Plezia et al 1983, Kamijou et al 1994).

Since it is known from in vitro resorption assays that osteoclasts are capable of resorbing a wide variety of mineralized tissues (Jones et al 1984, Jones et al 1995), and that the presence of live osteocytes within bone may have an inhibitory effect upon osteoclastic activity (Maejima et al 1995), this explanation seems improbable. Instead, this 'resistance' may reflect the slower rate of revascularization which is known to occur in dead bone (Ray 1972, Burchardt 1983).

This chapter addresses whether any difference exists between the resorption of bone containing live osteocytes and those damaged by heating or freezing in an in vitro situation where immunological and vascular effects can be ignored.

Materials and Methods
Slices of live bone 600-800 μm thick were cut from freshly removed adult rabbit femora using a low speed diamond impregnated circular saw (Isomet, Buehler Ltd, Illinois, USA 60204). An extremely light load was used, and the specimens were kept moistened with phosphate buffered saline (PBS) at all times to minimise cellular damage. As the slices were collected, they were assigned to alternate sets and the marrow was removed with tweezers. The total time between death of the animal and the slices being seeded with cells was less than one hour (hr). Three experiments were undertaken.

Experiment 1
Eight sections were taken, alternate slices being placed into either sterile PBS (Set 1) or distilled water (Set 2) at 20°C. After 20 minutes (min) the slices from the latter group were subjected to three freeze-thaw cycles of immersion in liquid nitrogen for 2 min, followed by thawing at room temperature in distilled water (Ray 1972, Plezia et al 1983).

Control and treated slices were paired and soaked for 45 min in medium containing 40 ml Eagle's minimum essential medium (EMEM), 0.5 ml L-glutamine, 1.0 ml ABAM (penicillin (100 iu/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml)), and 600 μl gentamycin (150 μg/ml), at 37°C and in an atmosphere of 5% CO₂ in air. This initial soaking stage was omitted for the subsequent two experiments as infection was not found to be a problem.
The paired slices were transferred to dry, sterile dishes and seeded with chick bone cells including osteoclasts (after Jones et al 1984). These were obtained by chopping the shafts of long bones, cleaned of overlying periosteum, in EMEM, L-glutamine and gentamycin (50 µg/ml), each pair being kept in the same dish to ensure identical culture conditions (1a with 2a, 1b with 2b etc).

The cells were allowed to settle for one hour in an incubator. Loose cells were then washed off with medium and 2 mls of fresh medium were added. At the end of the culture period, the cells were removed by washing in distilled water and sonicating, and the bone slices were soaked for 1 hr in a detergent protease solution (Terg-A-Zyme®, Alconox Inc.) in order to remove any demineralized collagen fringe and facilitate measurement. The slices were washed, dehydrated in 100% ethanol, air dried, and mounted on glass slides for pit measurements.

**Experiment 2**
This was conducted as Experiment 1, but instead of the prior soaking stage in antibiotic containing medium, Sets 1 and 2 were washed in warm (37°C) sterile PBS before seeding with bone cells.

**Experiment 3**
This experiment used nine bone slices, three sets of three that were designated 1 and 2 as in the previous two experiments, but with an additional Set 3. Set 3 was left in distilled water at 20°C for at least 10 min prior to being transferred to, and maintained in, distilled water for 10 min at 53-58°C in a water bath. For Set 2, each freezing period was increased to 10 min.

To increase the number of pits found, the slices were cultured separately in 16 mm wells and completely overlaid with medium containing bone cells. After the one hour settling period, the culture was continued for a further 24 hr without changing the medium. One drop of extra FCS was added to two groups of slices to increase the 'stickiness' of the cells. At the end of culture, prior to randomisation and coding of the slices, 3 groups of slices were examined in order to determine the cell status in the sets, as detailed below.

**Measurements**
The volumes and areas of all the osteoclastic resorption lacunae were measured 'blind' on coded slices using a reflection confocal scanning laser microscope system (1LM2W Lasertec Corporation, Japan; software by SIS Münster Germany) with a 40x/0.95 NA objective with a coverslip attached to it (Jones et al 1992). The mean depth per pit was calculated as the volume:area ratio. The mean surface roughness of each slice in the first experiment was measured in order to check whether it varied between the groups.
Cell status determination
Two fluorescent stains, ethidium bromide (5\mu g/ml) and dimethylamino-styryl-n-methylpyridinium iodide (DASPMI 100\mu g/ml) (Haughland 1992) were selected for use. Ethidium bromide stains the nuclei of dead cells and DASPMI stains mitochondria in living cells. The stains were first tested upon cultured cells and waste human bone fragments (from mandibular third molar removal and from neurosurgical operations) which had been collected and stored in EMEM or PBS. They were applied for 20-30 min at varying time intervals postoperatively, either to whole fragments or after sectioning the bone into 600-800 \mu m thick slices.

The effects on cellular staining of the different treatment regimes used in this experiment were also assessed using living osteoblasts and equivalent cells, cultured as monolayers on plastic. These were subjected to the same heating or freezing processes and stained as above. Since positive staining is more difficult to identify in osteocytes retained \textit{in situ} than in a monolayer of cells cultured in a dish, the appearance of DASPMI staining was tested further upon fresh, heated and frozen rabbit bone slices, as well as chick bones that had been chilled for 1 year.

After the 24 hr culture period, surface cells were swept off and the slices were transferred to PBS. Two groups, each containing one slice from sets 1, 2 and 3, were selected and either stained by the addition of a few drops of ethidium bromide or DASPMI for 15-20 min; a third group of three slices was left unstained as a measure of autofluorescence. Each of the slices was then stabilised with plasticine and viewed whilst still submersed in PBS using an Odyssey video-rate laser scanning confocal microscope (Noran Inc., Middleton, WI, 53562, USA) with a LOMO 40/0.75 water immersion objective. Images were recorded in both reflection and fluorescence (>515 nm fluorescence excited by 488 nm radiation) channels and captured using a Noran TN8502 image analysing computer. This procedure only took a few minutes and helped to distinguish osteocytes from any remaining surface cells since it allowed visualisation of their lacunae.

Results
Measurements
The median values for the areas, volumes, and mean depths (volume/area) of the resorption pits are shown in table 7.2 (overleaf), presented for individual experiments, and with the results for all experiments pooled. In experiments 1 and 2, too few pits were obtained to make reliable individual statistical evaluations. In all, over 2300 pits were measured (n=833, 872 and 668 for Sets 1, 2 and 3 respectively). The differences between the means and medians for area and volume indicated a skewed distribution of the pit sizes, so the Mann-Whitney test was used to assess significance. In the controls
(Set 1) the areas and volumes were significantly smaller (p<0.0001) than those of the other sets. The volumes and mean depths of the pits in Sets 2 and 3 did not differ significantly from each other (volume p=0.0598; mean depth p=0.192; area p=0.0023). The volume:area ratio (mean depth) was also smaller in the pits of Set 1 than in the other two groups (p<0.03) as demonstrated in the graphs of the mean data (figure 7.1).

| Table 7.2 Effect of bone cell vitality upon resorption: median values for pit sizes |
|-----------------------------------------------|--------|--------|--------|
| Expt 1                                       | n=     | area μm² | volume μm³ | mean depth μm |
| Set 1                                        | 227    | 214     | 1217      | 5.88          |
| Set 2                                        | 146    | 343     | 2044      | 5.38          |
| Expt 2                                       |        |         |           |                |
| Set 1                                        | 109    | 260     | 1217      | 6.10          |
| Set 2                                        | 38     | 292     | 1541      | 5.30          |
| Expt 3                                       |        |         |           |                |
| Set 1                                        | 497    | 206     | 1449      | 6.95          |
| Set 2                                        | 688    | 309     | 2343      | 7.37          |
| Set 3                                        | 668    | 259     | 1823      | 7.17          |
| Pooled                                       |        |         |           |                |
| Set 1                                        | 833    | 215     | 1402      | 6.44          |
| Set 2                                        | 872    | 311     | 2301      | 6.83          |
| Set 3                                        | 668    | 259     | 1823      | 7.17          |

There was no significant difference between the sets in the estimated total volume of tissue resorbed per slice.

**Cell Status**

The characteristic speckled appearance of DASPMI staining is more reliably shown in the cultured cells than in the bone pieces (figures 7.2&3, over page). It was clear that they were destroyed by both the heating (figure 7.2c) and the freezing processes: in the latter group no intact cells remained at all. The range of time periods over which live cells could be demonstrated within the human mandibular and cranial bone was large. For chilled specimens stored in PBS, some live cells could be demonstrated after as much as 5 days; for EMEM stored material it was up to ten days postremoval. For specimens stored at room temperature no staining was seen after 48 hours. It was noticeable that those specimens which demonstrated the longer survival times, kept either at room temperature or refrigerated, were thin pieces of bone (<500 μm).

The status of the osteocytes within the treated rabbit bone was more difficult to determine than that of the cultured bone cells, because the lacunae present a narrow
Figure 7.1 Histograms showing mean data and sem for resorption pits in live, frozen and heated bone

- **Mean area (µm²)**
  - Set 1: 300
  - Set 2: 600
  - Set 3: 900
  - * p<0.0001
  - * p<0.05

- **Mean volume (µm³)**
  - Set 1: 3000
  - Set 2: 6000
  - Set 3: 9000
  - * p<0.0001
  - * p<0.05

- **Mean depth (µm)**
  - Set 1: 6
  - Set 2: 6
  - Set 3: 9
  - * p<0.05
  - * p<0.001

* = significantly greater than Set 1 (Mann-Whitney)
Figure 7.2

**Appearances of stains for indicating cell vitality**

These confocal micrographs, taken using the fluorescence channel of an Odyssey videorate laser scanning confocal microscope with a LOMO 40/0.75 water immersion objective, show the distinct appearances of DASPMI, ethidium bromide and autofluorescence in culture cells.

**Figure 7.2A** Live cultured osteoblasts stained with DASPMI. Note the nonstaining nucleus and the more particulate appearance of the stain in the spread cells.

**Figure 7.2B** Cultured cells stained with ethidium bromide. Ethidium bromide stains the nuclei of dead cells.

**Figure 7.2C** Autofluorescence of cultured osteoblasts subjected to heating. These cells had been stained with DASPMI but since they are dead the DASPMI staining is negative. For the detection of autofluorescence, the brightness settings of the microscope must be increased to at least 1.5x that required for visualisation of either fluorescent dye.
Figure 7.3  Confocal fluorescence image of DASPMI-stained lingual split bone fragment 45 minutes after removal from the body. Note the nonstaining area within the central fluorescent cell which represents the nucleus. See that the stain of the other two brightly labelled cells does not appear to be particulate - this is because the cells are slightly out of focus because they lie outside the optical section imaged here.
profile in the orientation examined. Some fluorescent staining could be seen within the osteocyte lacunae in all experimental groups, but in most cases it was nonspecific with the 'cell' appearing shrunken and having no clearly distinguishable nucleus. In the live bone group (Set 1) it was occasionally possible to recognise a nonstaining area within a stained lacuna (figure 7.3) that may have represented the nucleus of a live cell. Autofluorescence produced a similar appearance to the DASPMI staining but required a much higher gain (~1800 rather than 1200).

Discussion
This study was undertaken to determine whether there is a difference between the resorption of 'live' and 'dead' bone in vitro. This is of possible relevance to in vivo grafting procedures, and the physiological turnover of aging bone tissue.

It was found that resorption pits were larger on bone that had been treated previously by heating or freezing than on bone kept at room temperature prior to culture. These results are in contrast to in vivo reports which suggest that dead bone shows a resistance to resorption (Plezia et al 1983, Kamijou et al 1994) despite the presence of osteoclasts, but agree with the few reports that frozen or boiled bone shows superior healing to fresh bone (Ely 1924, Pavlova & Vyalcev 1969).

It is generally considered that the more readily a graft is resorbed and replaced with new bone the better it is (Chalmers 1967), since dead bone may be prone to fracture (Burchardt 1983, Goldberg et al 1987). Provided there is no ensuing infection or immunological rejection (Treble et al 1990), rapid resorption will be coupled with the apposition of new bone. This facilitates rapid stabilisation at the graft site, and eventual remodelling of the graft and new bone means that the functional requirements at the graft site are met.

In contrast to the use of ethidium bromide, the DASPMI staining technique proved successful on human bone, even though mitochondrial numbers reduce as osteocytes age (Tonna 1973, Kukletová et al 1982). The technique requires few processing steps, is simple and allows the rapid sampling of a large amount of tissue. It therefore has advantages over transmission electron microscopy, and could add qualitative information to biochemical measures of cellular activity. However, the presence of functioning mitochondria may not give a direct indication of the nuclear status of the cells. One factor that may complicate this technique is the change in permeability that bone undergoes when it dies (Ramp et al 1994), which would have to be taken into account when staining the cells.
The duration of osteocyte survival as reported here is much longer than that reported by James & Steijn-Myagkaya (1986) who found electron microscopical evidence of irreversible damage in osteocytes following four hours of ischaemia. In their study, the bone pieces were subjected to ischaemia at body temperature, whereas here the cells were encouraged to stay alive by placement in EMEM and by chilling; it is known that other cells may remain vital for very long periods in culture. However, determining exactly when a cell may be considered to be dead is an area causing problems in current research into apoptosis (the initial stages of which are reversible).

The heating and freezing procedures used in this experiment were sufficient to kill cells cultured in a dish. However, although it was difficult to be certain of the status of the osteocytes in the rabbit bone fragments after heating and freezing, temperatures in excess of 47°C for one minute have been found to kill bone in vivo (Eriksson & Albrektsson 1984). Such temperatures are readily exceeded in many bone surgical procedures (Eriksson et al 1984). Jonck (1981) has suggested that bone cells die by the increasing hypertonicity of the tissue electrolytes during the slow freezing that occurs in bone. The use of distilled water may have counteracted this to some extent, but was intended to hasten cell death in the small experimental specimens. However, the fragments used were much smaller than bone fragments that may be frozen and used clinically.

The apparent resistance to resorption that has been found with frozen bone in vivo may be attributable to an impaired vascularization that occurs after bone has been frozen (Ray 1972, Burchardt 1983), rather than an inherent difference in the 'resorbability' of the tissue. This explanation also harmonizes with the 'latent period' which Urist (1953) described prior to the incorporation of frozen bone. Bone grafts tend to revascularise along existing vascular channels starting at the graft-host junction, and the accuracy of fit and the lining up of the Haversian systems of the graft with the surrounding host bone have been found to be important factors in healing (Albee 1944, Peer 1955, Burchardt 1983). The vasculature is considered to be of such importance in bone graft healing, that it has been suggested that the original functioning blood vessel density needs to be re-established before resorption will occur (Eriksson & Albrektsson 1980). Evans et al (1991) demonstrated that even irradiated grafts, typically associated with poor healing, would unite provided the vasculature was intact. The boiling of bone is known to coagulate the contents of the Haversian canals (Burchardt 1983) and obstruction of the vascular channels by necrotic tissue might also occur in frozen bone, either of which would hinder the ingrowth of new vessels.

What factors might account for an increased amount of resorption of dead bone compared with live? Several variations in the handling of the experimental sets may have altered their susceptibility to osteoclasia. During the freezing and heating processes, a
smeared layer of blood and cutting debris created during sectioning would be removed, so presenting a different surface to the seeded osteoclasts, which might allow them to adhere and start resorbing more quickly. This potential difference was countered as much as possible by washing the untreated slices vigorously in phosphate buffered saline. If the procedures followed with the frozen and heated sets altered the bone surface so that cellular adherence to it and/or spreading upon it was enhanced, this may have allowed a longer resorbing time for the cells, and hence resulted in larger pits. Nevertheless, similar differences would be produced in the preparation of graft bone for clinical use. Eriksson and Albrektsson (1984) concluded from their *in vivo* study using a bone growth chamber that 'a temperature of more than 47°C was shown to consistently give rise to bone resorption and fat-cell degeneration.'

Recent work that might furnish an explanation of increased resorption on dead bone is that performed by Maejima et al (1995). They suggest that a factor released by bone containing live osteocytes has an inhibitory effect upon osteoclasts. Such a factor has not yet been identified.

**Conclusions**

Thus, rather than failing to resorb, dead bone may show an enhanced resorption *in vitro*. In this respect, its use in bone grafting is not contraindicated. This is probably a desirable feature in the normal turnover of bone as it would counter the accumulation of dead bone in the skeleton. However, the presence of dead osteocytes may accentuate the bone loss in the edentulous mandible if the resorption is not coupled to formation.
CHAPTER 8

Discussion

This thesis has addressed two main areas of interest with respect to mandibular bone structure and turnover: firstly, how mandibular density is affected by aging and how it compares with the general skeletal status; and secondly, how certain anatomical features of the mandible may affect its subsequent resorption.

The main findings are:

1. The mineralization density (as determined by quantitative electron backscattering) and the apparent density (dry weight per unit volume) of bone tissue vary independently of one another.

   This would imply that clinical imaging techniques that use the radiation-stopping ability as a measure of bone density (and which cannot distinguish between changes in bone quantity and levels of mineralization) may not be entirely reliable. This is of particular importance in the assessment of fracture risk, where it is already known that therapies (such as fluoride) that increase the radiographic bone density do not necessarily reduce fracture incidence.

2. The mineralization and apparent densities of the mandible increase with age.

   The increase in both of these aspects of density may explain why bone appears to become less pliable in the elderly. This probably increases the likelihood of fracture of the alveolar bone during tooth extraction, which may prolong healing and lead to larger amounts of bone dying than in the young. This will also be contributed to by any decrease in the vascularity of the tissue with age, which will be the subject of further study.

For osseointegrated dental implants, mandibles with a high apparent density are favoured over those with a low apparent density yet, since an increasing mineralization density is thought to be indicative of a reduction in bone turnover, it may be preferable if the mineralization density is on the lower side. Thus one might expect that there would be a optimum period of implant placement when the apparent density is increasing, but before the peak mineralization density is reached. However, the importance of one aspect may far outweigh the other, for example the mechanical stability provided by a mandible with thick cortices may be more important for the initial stages of healing than how highly mineralized the tissue may be.
One recent longitudinal study has reported that the radiographic density of the mandible increases in women taking hormone replacement therapy (Jacobs et al 1996). This led to the conclusion that hormone replacement may have a positive influence on mandibular bone, yet close examination of this study reveals that no controls were used and hence no account was made for the fact that mandibular bone density may increase with age.

3. The mineralization and apparent densities of the mandible are significantly greater than, and show no direct relationship with, those for the fourth lumbar vertebra, the iliac crest and the femoral neck.

It is interesting that the mandible contains so much more bone per unit volume than the postcranial sites tested. Why this extra mass of bone is required in not clear. As discussed in chapter 5, the mandible does experience strains of the same order as the long bones and therefore, by definition, it must be deformed to a similar degree by the loads encountered. This would imply that the mandible must experience much greater forces in order to deform its greater mass and density to the same extent. But this study did not look at the density characteristics of the shafts of long bones, which are known to have a very much greater cortical component than the postcranial sites actually tested. Another consideration is that the mandible may share some of the protective function of calvarial bone, and that for maintenance of its volume lower strains need to be encountered.

The fact that the mandible is more highly mineralized than the calvaria which, in turn, is significantly more highly mineralized than the postcranial bones, is also interesting. The distribution of the different mineralization levels within the bone cross section have not been taken into account in this study. This is probably most important for the femoral neck, where the very highly mineralized calcified fibrocartilage exists as an incomplete shell towards the periphery of the bone, where it will have the greatest effect at increasing the stiffness of the bone.

The mineralization density has been taken as a possible indicator of the turnover of the tissue. It may be that this can only be applied reliably when comparing the same bone between individuals rather than between different bones within an individual, since different bones may have different endpoints for mineralization. But if the high levels of mineralization of the mandible do reflect a reduction in the rate of turnover, one has to question why turnover is necessary or desirable and why it may vary between sites in the skeleton.

From a biological aspect, the turnover of bone seems to be fairly expensive on materials and cells, and is under a complex system of control. Bone tends to provide the maximum structural strength for the minimum amount of material (de Aguiar et al 1968).
Superficially, if one considers the changes that occur in the mandible with aging and on tooth loss it would appear that the mechanical characteristics in the distribution of the bone tissue become worse. Yet we have seen that aging is accompanied by an increase in the mineralization density which probably increases the stiffness of the bone material. This is probably a much more efficient means of maintaining the strength of the mandible than having to maintain its size, especially as after the initial rapid remodelling following tooth loss it will not be so demanding on cellular labour.

It is very interesting that the regions in the mandible with the highest mineralization densities correspond to those regions predicted from computer models to experience the highest strains. It may be that mineralization density maps could provide another method in which strain distributions in bones could be determined. This would be particularly useful for determining the stress trajectories in bones for which the mechanical function is poorly understood. In addition, if a positive relationship was found between the level of mineralization of a bone and the stresses experienced by it, it would be of immense biological interest.

To be certain of the turnover differences between cranial and postcranial bone, it would be preferable to study a wide age range of patients with known dental, medical and social histories. This study has had to rely on cadavers where the general health, nutritional status, degree of activity, drug histories and denture wearing histories are unknown. All that can be said with any certainty is that all of the individuals were unwell enough to die, none of the deaths being accidental. Therefore, if any of the individuals were bed-ridden this may have greatly affected the pattern of bone turnover, probably more so in the postcranial than in the cranial skeleton. However, in no individual was the correlation between the cranial and postcranial sites as good as it was between the postcranial bones.

From a broader perspective, knowing whether one bone does or does not behave similarly to another is extremely important. In many cases the response to therapy is extrapolated to bones others than the one on which the particular effect is being tested. This would seem to be acceptable for the postcranial sites tested here, yet extreme caution would have to be exercised when comparing cranial with postcranial bones.

4. Osteoclasts can more readily resorb postcranial than cranial bone in vitro.
This may reflect the different roles that functional factors have upon the maintenance of bone in these sites, but may also be dependent upon the developmental origins of the bone. This may also apply on a local scale to the origins of alveolar and basal bone. The fact that alveolar bone develops in close association to the teeth is well known. In the developing tooth germs in some mammals (eg elephant, manatee), the alveolar bone may not even be attached to the basal bone, and may get its stimulus for formation from the
epithelial cell rests that surround the roots of the teeth.

5. Sharpey's fibres in mandibular bone may affect the shape of osteoclastic resorption pits. This may affect the pattern of bone loss that is seen around the teeth. It is still unknown whether bundle bone is more or less easy to resorb in vitro than other bone, and it would be interesting to study this further. It is possible that the degree of functional loading that a tooth experiences prior to extraction could affect the initial postextraction resorption rate.

6. Dead bone is resorbed more readily than live bone in vitro.
The presence of extensive areas of dead bone within the aging mandible would seem to reflect a decrease in the turnover of the tissue. However, it was shown in chapter 7 that dead bone is more readily resorbed than live bone in vitro. The effects of the vasculature have to be included when considering the events that may be occurring in vivo. It is possible that surgical procedures may expose these areas of dead bone to osteoclastic resorption by increasing the local blood flow to such areas during healing. This might be another reason that bone with lower overall mineralization density may be more favourable for osseointegration.

Concluding Remarks
The reasons for the interindividual differences in rates of mandibular residual ridge resorption are unknown, but it has been suggested that, if they could be attributed to systemic factors, systemic therapies would be the most appropriate mode of prevention. This thesis failed to show any correlation between the bone structure and turnover of the mandible and those of the postcranial skeletal sites tested. This would tend to suggest that attention should continue to be paid to the local factors. Obviously, prevention of tooth loss would be the ideal form of prevention of residual ridge resorption, but further studies should be done on increasing the use of the edentulous mandible. This could be through the provision of dentures which the patient can use with confidence and comfort. But above all, forms of exercise therapy which would help to maintain the overall stimulus to the bone and to the blood supply should be investigated. If osteocytes do release inhibitory factors to prevent the resorption of the surrounding bone, it could be that encouragement of some intracanalicular flow would help to stimulate their release and to distribute the message to the appropriate receptor cell.

Not only is it apparent that bone may adapt to its functional demands by altering its internal architecture and external form, but it may be that variations in the levels of mineralization may also enhance the mechanical properties. The determination of this by such an accurate method has not been tested previously and it would be very interesting and exciting to test this hypothesis further.
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216


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APPENDIX 1

LISTS OF SPECIMENS

Table A.1 List of ages and causes of death for individuals with multiple sites studied

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Table A.4 List of ages of individuals for which is and calvarial material was retrieved

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*=Afro-Caribbean
APPENDIX 2

PUBLICATIONS ARISING FROM THIS THESIS

Paper

Abstracts


This thesis was examined by Dr DA Luke from the Division of Anatomy and Cell Biology, United Medical and Dental Schools, London, and Dr DK Whittaker from the Department of Oral Biology, University College of Medicine, Cardiff on 23rd July 1996.

VIRGINIA KINGSMILL, ALAN BOYDE, POOPATHY KATHIRGAMANATHAN*
Department of Anatomy and Developmental Biology, *Department of Chemistry, University College London, London, UK

Summary: Specimens with complicated, intricate three-dimensional structures, which are otherwise difficult to coat adequately by conventional means (sputter or evaporative coating) for scanning electron microscopy, can quickly and simply be rendered conductive by electroless plating. The technique can be recommended only when studying specimens at low magnification because fine detail may be lost.

Key words: scanning electron microscopy, charging, coating, cancellous bone, electroless plating

Introduction

With somewhat rare exceptions, nonconductive samples for scanning electron microscopy (SEM) have to be given a conductive coating of evaporated or sputtered carbon or metal or of an organic antistatic agent that can be deposited from solution. In our laboratories, a principal interest is in the structural organisation of cancellous bone. The bone must first be cleaned of all overlying cellular, soft-tissue elements—usually achieved with a combination of mechanical washing, enzymes, and other solvent treatments prior to drying from a volatile solvent (Boyde and Jones 1974, Boyde 1984).

Because of the complex porous structure of this material, it is exceptionally difficult to apply continuous metallic-surface conductive coatings either by evaporation or by sputtering. Front surface facets are successfully coated, but remain as insulated islands.

In this report, we describe successful attempts at applying metallic coatings by electroless plating in wet solution. The procedure may have applications to other porous insulating materials in which the advantages of the depth of field of low magnification SEM could be felt.

Materials and Methods

Electroless plating (so called because it does not require the use of electrodes) is a process by which nonconductive specimens can be plated with metal (Canning 1953, Reidel 1991). Since the specimen is completely immersed for this procedure, all aspects of the sample are coated (including areas that might not be penetrated satisfactorily using sputter or evaporative coating techniques); the specimen need not be dehydrated first. Once the tissue is rendered conductive by this process, it can be viewed in secondary electron mode. Alternatively, conventional electroplating can be used to completely embed the specimen in metal, thus conferring all the attendant advantages of embedding—viz, provision of support, polishability, etc.—and can then be viewed in back-scattered electron mode, probably after the application of a carbon coating.

Nickel Coating

The nickel-coating procedure was based on a technique described by Reidel (1991). Bone specimens, which had been prepared by a combination of mechanical (waterpik) and biochemical (Terg-A-Zyme, Alconox Inc, New York, N.Y.) means were sonicated in solution A for 5–10 s then immediately washed in distilled water. After removal of excess moisture, the specimens were then suspended in solution B, which had been heated to 40°C in a waterbath. Small amounts
(a few ml) of solution C were added slowly until effervescence occurred; the samples were removed after various time intervals between 5 s and 30 min and sonicated in distilled water. Specimens that had been selected for complete embedding in nickel were left for 4 h (during which time the solutions were refreshed to maintain effervescence) and were then coated electrolytically in solution D, with a current density of 0.33 mA/mm\(^2\) at approximately 50°C (a current density greater than this was found to cause a dendritic deposition).

**Composition of Solutions**

The makeup of the four solutions follows:

A: an acidified solution of palladium chloride (Aldrich Chemical Co Ltd, Gillingham, Dorset, UK), containing 10–50 mg of palladium chloride dissolved in 100 ml 0.1 M hydrochloric acid.

B: an aqueous solution containing 0.23 M nickel (II) chloride (Aldrich) and 0.28 M citric acid (Aldrich), adjusted to a pH of 7 with ammonia.

C: a 0.84 M solution of dimethyl aminoborane (Caendorchemie GmbH, Bochum, Germany) dissolved in ethanol.

D: a mixture of 0.92 M nickel (II) sulphate (Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire, UK), 0.13 M nickel (II) chloride and 0.49 M boric acid (Fisons).

**Copper Coating**

A similar procedure was used as above, but, after palladium chloride treatment, the bones were suspended in an aqueous solution containing 0.15 M copper (II) sulphate (BDH Laboratory Supplies, Poole, UK), 0.66 M potassium sodium tartrate (Aldrich), and 2.07 M sodium hydroxide at room temperature; the reductant in this instance was 38% (m/v) formaldehyde (Canning 1953).

Upon removal from the plating solutions, the specimens were rinsed in distilled water or in acidified palladium chloride solution to remove any loose surface debris. They were then dried in an oven or vacuum, after which the surface resistivity of each specimen was calculated (with a four-probe test apparatus) prior to viewing in an SEM.

**Results**

All specimens, irrespective of treatment time, appeared to have a continuous adherent surface coating that covered all aspects of the bone, including fine trabeculae at the centres of the largest samples tested (ca. 10 × 20 × 30 mm). The coating was black in those specimens which had been treated for less than 30 min or silver-coloured, thus providing good contrast for optical microscopic applications.

Specimens treated for less than 30 min had surface resistivities at 13–23 ohms, which may be compared with a mounting medium—plastic conductive carbon cement (“Leit C Plast”, Neubauer Chemikalien, Münster, Germany)—value of 136 ohms. The specimens coated for 4 h or more had surface resistivities of less than 0.1 ohms.

Up to instrumental magnification values of 100x, the microscopic surface texture was not unduly perturbed by the metallic crystalline structure of the applied coating. The appearance was acceptable (Fig. 1), and charging effects were dramatically reduced when compared with conventionally coated samples of the same nature.

The specimen should be thoroughly rinsed upon removal from the plating solution, or loosely adherent surface debris, which is prone to charging, may remain.

At higher resolution, the metal totally obscured the fine structure of the bone. Details such as the collagen fibre arrangement and resorption pits could no longer be readily identified (Fig. 2).

The thickness of the metal coating of the heavily coated samples could be measured directly in the SEM after one surface of the bone had been polished to expose the profile of the coating. Usually, bones treated for longer than 10 min had coating thicknesses of 10–20 \(\mu\)m.

During electroplating, approximately 1 gm/100 mm\(^2\) nickel was deposited on a specimen in 24 h. Once one surface had been polished to expose the bone, the
surface resistivity of the bone on that side was found, predictably, to have increased markedly: from $<0.05$ ohms prior to polishing to $>1 \times 10^7$ ohms afterwards. The larger pore spaces within these specimens had been penetrated (Fig. 3).

Discussion

The procedure we have outlined provides a satisfactory solution to coating awkward three-dimensional structures, such as trabecular bone, and may be tested as an alternative to established procedures. These procedures may have special advantages for low-magnification, wide-field, SEM when the fine structure of the sample surface is not important.

Alternative procedures for dealing with intractable charging problems in porous composites include (a) soaking in a solution of an organic antistatic, which may also obscure surface detail but has the advantage of optical transparency for cathodoluminescence applications (Boyd 1984); (b) using a low accelerating voltage, for example, 1–2 kV, and videorate scanning and frame averaging, which would usually be limited to 512 line resolution; (c) employing backscattered electrons and an applied positive surface voltage to inhibit low-energy electron emission (Boyd and Cowham 1980); and (d) using uncoated samples, careful selection of the accelerating voltage (in our case, to ca. 1.24 kV), and a multichannel plate detector, which are currently very expensive and require exceptionally careful operation. In particular, the vacuum conditions required (better than $5 \times 10^{-6}$Torr) are rather stringent.

Sputter coating is very convenient, and if surface detail can be sacrificed, charging problems can be eliminated by applying thick coatings. However, most laboratories are equipped with coaters with precious metal targets, so that cost becomes an important element. In comparison, the time cost of preparing the solutions used here will contrast favourably with the labour involved in cleaning either a sputter coater or an evaporative coater after heavy use.

Acknowledgments

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References