The Ultrastructural Changes in Bone of Patients with Osteogenesis Imperfecta.

by

Padmini Sarathchandra

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science at the University of London, February 1998.

Institute of Orthopaedics,
University College London Medical School.
Abstract

Osteogenesis Imperfecta (OI) is a heterogeneous, heritable disorder affecting mineralised and non-mineralised connective tissues, resulting in bone fragility. Over 200 mutations in procollagen genes have now been identified in OI patients.

This study aimed to identify the morphological manifestations of collagen abnormalities in OI bone and to determine whether changes in mineral composition and collagen structure correlate with clinical types. Bone specimens from 43 OI patients and 25 normal controls were examined using a variety of techniques.

The morphological and ultrastructural alterations observed in OI bone correlate well with clinical severity, mildest forms showing the least disturbed changes with severe forms showing the greatest disorganisation.

The Ca/P ratio of OI bone mineral as measured by electron probe X-ray microanalysis was lower than normal in both resin and cryo-sections, and mirrored the severity of disease. OI type II gave the lowest ratio (Ca/P = 1.49) compared with normal age- and site-matched controls (Ca/P = 1.69).

Histomorphometric measurements revealed collagen fibrils of reduced diameter in the osteoid of all OI types. The smallest mean diameter was found in OI type II (45 nm compared with 67 nm for age- and site-matched controls). OI type II also revealed a shorter collagen D-period (45 nm); the periodicity in other OI types was comparable to normal (63 nm).

Immunogold localisation of collagen types in OI bone demonstrated type III collagen for the first time at an ultrastructural level. The labelling intensity for type I collagen was reduced when compared with that of normal controls.

Other observations include a reduced alkaline phosphatase activity in OI bone, the abnormal presence of proteoglycans in the osteocytic lacunae of type II/III bone and increased levels of proteoglycans between the collagen fibres of OI osteoid.

The abnormalities in mineral composition, collagen structure and proteoglycan distribution demonstrated in this study may be important factors contributing to the fragility of OI bone.
Contents

<table>
<thead>
<tr>
<th>Title</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Contents</td>
<td>3-6</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>7</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>8</td>
</tr>
<tr>
<td>Figures</td>
<td>9-25</td>
</tr>
<tr>
<td>Tables</td>
<td>26-27</td>
</tr>
</tbody>
</table>

Chapter 1  Introduction  28-44

1.1 General introduction  28
1.2 History of OI  28
1.3 Classification  29
1.4 Biochemical and genetic findings in OI  36
1.5 Morphological and ultrastructural findings in OI  37
1.6 Animal models  41
1.7 Therapy in OI  42
1.8 Aims of the project  43

Chapter 2  Histological and ultrastructural morphology of OI bone  45-103

2.1 Introduction  45
   Bone structure  45
   Morphological and ultrastructural abnormalities in OI bone  46
   Aims of this chapter  50
2.2 Materials and Methods  51
   Patients' details  51
   LM processing  51
## 2.3 Results

- OI type I  
- OI type II  
- OI type II/III  
- OI type III  
- OI type IV

## 2.4 Discussion

### Chapter 3  Electron probe X-ray microanalysis of OI bone by conventional and cryo-techniques  104-127

#### 3.1 Introduction
- Bone: structure and composition  
- Mechanisms of calcification  
- Electron probe X-ray microanalysis  
- Abnormalities of mineral density, composition, crystal size and distribution in OI  
- Aims of this chapter  

#### 3.2 Materials and Methods
- Patients' details  
- Conventional EM processing  
- Cryo-processing  

#### 3.3 Results

#### 3.4 Discussion

### Chapter 4  Histomorphometry of type I collagen fibrils in the osteoid of OI bone  128-153

#### 4.1 Introduction  
- Aims of this chapter  

#### 4.2 Materials and Methods

#### 4.3 Results

#### 4.4 Discussion
<table>
<thead>
<tr>
<th>Chapter 5</th>
<th>Immunogold localisation of collagen types in OI bone</th>
<th>154-175</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>Aims of this chapter</td>
<td>155</td>
</tr>
<tr>
<td>5.2</td>
<td>Materials and Methods</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Patients' details</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Tissue processing for fixed tissue</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Tissue processing for unfixed tissue by freeze substitution</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Pre-embedding immunogold localisation</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>Post-embedding immunogold localisation</td>
<td>157</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>158</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>174</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 6</th>
<th>Enzyme histochemical localisation of alkaline phosphatase activity in OI bone</th>
<th>176-190</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>Aims of this chapter</td>
<td>177</td>
</tr>
<tr>
<td>6.2</td>
<td>Materials and Methods</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>Patients' details</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td>Localisation of alkaline phosphatase</td>
<td>178</td>
</tr>
<tr>
<td>6.3</td>
<td>Results</td>
<td>179</td>
</tr>
<tr>
<td>6.4</td>
<td>Discussion</td>
<td>189</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter 7</th>
<th>Abnormal presence of proteoglycans found in OI bone</th>
<th>191-223</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Introduction</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>Aims of this chapter</td>
<td>192</td>
</tr>
<tr>
<td>7.2</td>
<td>Materials and Methods</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>Patients' details</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>Electron microscopy processing and cuprolinic blue staining</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td>Light microscopy processing</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Electron probe X-ray microanalysis (XRMA)</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Tissue processing for immunogold labelling</td>
<td>194</td>
</tr>
<tr>
<td>Page</td>
<td></td>
<td></td>
</tr>
<tr>
<td>------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunogold labelling at light microscopy level</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td>Immunogold labelling at electron microscopy level</td>
<td>195</td>
<td></td>
</tr>
</tbody>
</table>

### 7.3 Results

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light microscopy</td>
<td>197</td>
</tr>
<tr>
<td>Electron microscopy and X-ray microanalysis</td>
<td>197</td>
</tr>
<tr>
<td>Immunogold labelling (light microscopy)</td>
<td>198</td>
</tr>
<tr>
<td>Immunogold labelling (electron microscopy)</td>
<td>198</td>
</tr>
<tr>
<td>Cuprolinic blue staining to localise PGs in the osteoid</td>
<td>199</td>
</tr>
</tbody>
</table>

### 7.4 Discussion

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>220</td>
</tr>
</tbody>
</table>

### Chapter 8 General discussion and future work

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Future work</td>
<td>230</td>
</tr>
</tbody>
</table>

### References

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>232-250</td>
</tr>
</tbody>
</table>
Acknowledgments

This thesis is dedicated to the memory of my mother.
I would like to thank my supervisor Professor Yousuf Ali for his support, encouragement and advice during this study and for imparting his knowledge on calcified tissue to me. I am grateful for his patience, understanding, and providing me with the opportunity to work for a Ph.D.
I am indebted to Dr. Michael Pope, MRC Connective Tissue Genetics Group, Strangeways Laboratories in Cambridge, for the continuous supply of OI type II bone samples, providing the biochemical data with those samples, taking time off from his busy schedule to visit me at Stanmore and explaining the collagen biochemistry to me and for his constructive suggestions to this thesis.
I am grateful to Dr. Wendy Loveless, MRC Glycosciences group, NPIMR, for devoting time and effort in correcting the revised manuscript and for her invaluable contributions to this thesis.
A big thank you to Mike Kayser of the Institute of Orthopaedics for training me in cryo-ultramicrotomy and X-ray microanalysis, helping me with paraffin sectioning and for the trimming and pasting of micrographs. Without his help producing this thesis would have been an immeasurable task.
I would also like to thank the following: Dr. Patricia Fryer for the helpful suggestions in the ultrastructure and immunolocalisation chapters; Mrs. Heather Davies, EM unit at the Open University, for freeze substitution and embedding of a few bone specimens; Dr. Neelam Gurav, for her help in statistical analyses, setting up the image analysis system, and for her valuable friendship; Richard Pearson, for solving my computer problems; Dr Lucy Di Silvio, for culturing osteoblasts from OI foetal bone; and all my former colleagues at the Institute of Orthopaedics who helped me during the last three years. In addition, I would like to extend my gratitude to Mrs. Janice Upton and Dr Patricia Furr for proof reading this thesis; Dr Ketan Shah of the NPIMR for his continuous support and encouragement and James Clark for scanning schematic diagrams; my family and friends in Sri Lanka and Australia for their encouragement; my friend Parakrama who initiated the writing up and encouraged me during the first part of the thesis, but sadly departed this world before seeing the final outcome.
I am indebted to my husband Ananda without whom none of this would be possible, and finally to my daughter Nelomi for her inspirations and making some gloomier moments happier.
This work was funded by Action Research for two years and SIFTR allocation for research at RNOH in the final year.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP</td>
<td>Amorphous calcium phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CB</td>
<td>Cuprolinic blue</td>
</tr>
<tr>
<td>CPPD</td>
<td>Calcium pyrophosphate dihydrate</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomographic</td>
</tr>
<tr>
<td>CTOP</td>
<td>Calcium tetrahydrogen orthophosphate</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual energy X-ray absorptiometry</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers Danlos Syndrome</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FIO</td>
<td>Fibrogenesis Imperfecta Ossium</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GAM</td>
<td>Goat anti-mouse</td>
</tr>
<tr>
<td>GAR</td>
<td>Goat anti-rabbit</td>
</tr>
<tr>
<td>GP</td>
<td>Growth plate</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>IC</td>
<td>Iliac crest</td>
</tr>
<tr>
<td>INAA</td>
<td>Instrumental neutron activation analysis</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>OCP</td>
<td>Octacalcium phosphate</td>
</tr>
<tr>
<td>OI</td>
<td>Osteogenesis Imperfecta</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDS</td>
<td>Proteodermachondran sulphate</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TCP</td>
<td>Tricalcium phosphate</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>XRMA</td>
<td>X-ray microanalysis</td>
</tr>
<tr>
<td>Figures</td>
<td>page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>Figure 1.1</td>
<td>Typical blue sclera of OI types IA and IB.</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Translucent and discoloured teeth of Dentinogenesis Imperfecta.</td>
</tr>
<tr>
<td>Figure 1.3a</td>
<td>The earliest authenticated example of OI is an Egyptian Mummy of a child together with the skull (courtesy of the British Museum).</td>
</tr>
<tr>
<td>Figure 1.3b</td>
<td>The lower limbs of the Egyptian Mummy (Fig 1.3a) showing bowed femurs and twisted tibias (courtesy of the British Museum).</td>
</tr>
<tr>
<td>Figure 1.4a</td>
<td>Clinical appearance of a 22 week gestation OI type II/III foetus showing subtle abnormalities in arms and legs.</td>
</tr>
<tr>
<td>Figure 1.4b</td>
<td>Babygram of the foetus (22 week gestation) shown in Fig. 1.4a.</td>
</tr>
<tr>
<td>Figure 1.5a</td>
<td>Babygram of a foetus with OI sub-type IIA.</td>
</tr>
<tr>
<td>Figure 1.5b</td>
<td>Babygram of a foetus with OI sub-type IIB.</td>
</tr>
<tr>
<td>Figure 1.5c</td>
<td>Babygram of a foetus with OI sub-type IIC.</td>
</tr>
<tr>
<td>Figure 1.5d</td>
<td>Babygram of a foetus with OI type II/III.</td>
</tr>
<tr>
<td>Figure 2.1a</td>
<td>The thick osteoid from a normal juvenile control bone specimen (F.D, 10 years) showing the normal lamellar organisation of collagen bundles.</td>
</tr>
<tr>
<td>Figure 2.1b</td>
<td>A plump osteoblast from a normal control bone specimen (F.D, 10 years) showing normal ultrastructure.</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>The osteoid from an OI type I patient (B.S.A, 3 years) showing normal thick osteoid with lamellar organisation of collagen bundles.</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>The osteoid from an OI type I patient (S.D, 14 years) showing a thick osteoid with lamellar organisation of collagen bundles with gradual and normal mineralisation.</td>
</tr>
</tbody>
</table>
Figure 2.4  The osteoid from an OI type I patient (R.M, 9 years) showing a disorganised non-lamellar osteoid and osteoblasts with well developed, distended RER.

Figure 2.5  The osteoid from an OI type I patient (G.A, 10 years) showing a thick disorganised osteoid.

Figure 2.6  The osteoid from an OI type I patient (A.C, 10 years) showing a thick osteoid with lamellar organisation of collagen bundles.

Figure 2.7  The osteoid from an OI type I patient (N.L, 12 years) showing thin but organised osteoid.

Figure 2.8  The osteoid from an OI type I patient (A.A, 12 years) showing thin osteoid and osteoblasts containing well developed, distended RER.

Figure 2.9  Osteoblasts from an OI type I patient (S.D, 14 years) showing abundant glycogen deposits and mineral granules in the mitochondria.

Figure 2.10  An osteocyte from an OI type I patient (R.M, 9 years) containing spherical mitochondrial mineral nodules.

Figure 2.11  An osteoblast from an OI type I patient (S.D, 14 years) containing spherical mitochondrial mineral nodules.

Figure 2.12  The osteoid from an OI type I patient (R.M, 9 years) showing disorganised osteoid and fine fibrillar and flocculent material resembling proteoglycans.

Figure 2.13  Osteoid collagen from an OI type I patient (C.W, 42 years) showing variable diameter collagens.

Figure 2.14  Longitudinal paraffin wax section of proximal femur from a normal foetus (Foetus Gy, 19 weeks) stained with alcian blue and sirius red.

Figure 2.15  Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus F, 19 weeks) stained with alcian blue and sirius red.
Figure 2.16 Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus S, 22 weeks) stained with alcian blue and sirius red.

Figure 2.17 Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus W, 25 weeks) stained with alcian blue and sirius red.

Figure 2.18a Longitudinal paraffin wax section of proximal femur from a normal foetus (Foetus Gy, 19 weeks) stained with alcian blue and sirius red showing an intense blue colour for proteoglycan and dark red-coloured bony trabaculae.

Figure 2.18b Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus W, 25 weeks) stained with alcian blue and sirius red showing a paler blue colour for proteoglycan and an absence of red-coloured bony trabaculae.

Figure 2.19a Transverse paraffin wax section of a normal foetal mid-shaft femur (Foetus Gy, 19 weeks) stained with alcian blue and sirius red.

Figure 2.19b Transverse paraffin wax section of an OI type II foetal mid-shaft femur (Foetus F, 19 weeks) stained with alcian blue and sirius red.

Figure 2.20a Longitudinal paraffin wax section of femoral bone from an OI type II foetus (Foetus W, 25 weeks) stained with alcian blue and sirius red, showing increased numbers of osteoclasts.

Figure 2.20b Longitudinal paraffin wax section of femoral bone from an OI type II foetus (Foetus S, 22 weeks) stained with alcian blue and sirius red, showing increased numbers of osteoclasts.

Figure 2.21a Transverse paraffin wax section of a normal foetal mid-shaft femur (Foetus Gy, 19 weeks) stained with alcian blue and sirius red and viewed under polarised light, showing the regular lamellar organisation of bone.
Figure 2.21b Transverse paraffin wax section of an OI type II foetal mid-
shaft femur (Foetus F, 19 weeks) stained with alcian blue and sirius red and viewed under polarised light, showing a lack of the normal polarization pattern.

Figure 2.22 Ultra-thin araldite section of an OI type II bone (Foetus F, 19 weeks) showing thin osteoid and osteoblasts with well developed RER.

Figure 2.23 Ultra-thin araldite section of an OI type II bone (Foetus W, 25 weeks) showing thin osteoid and necrotic osteoblasts.

Figure 2.24 Ultra-thin araldite section of an OI type II bone (Foetus W, 25 weeks) showing unmineralised collagen bundles with irregular patches of mineral.

Figure 2.25 Ultra-thin araldite section of an OI type II bone (Foetus Mc, 16 weeks) showing unmineralised collagen bundles radiating from the mineral matrix.

Figure 2.26 Ultra-thin araldite section of an OI type II bone (Foetus Ki, 16 weeks) showing a gap in the femoral shaft due to incomplete mineralisation.

Figure 2.27 Ultra-thin araldite section of an OI type II bone (Foetus Ki, 16 weeks) showing thin osteoid and patchy mineralisation with mineral nodules dispersed in the osteoid.

Figure 2.28 Ultra-thin araldite section of an OI type II bone (Foetus S, 22 weeks) showing necrotic cells, a layer of disorganised thin collagens, and overlying fine fibrillar material resembling proteoglycans.

Figure 2.29 Ultra-thin araldite section of an OI type II bone (Foetus S, 22 weeks) shown at a higher magnification to show fine fibrillar and particulate material resembling proteoglycans.

Figure 2.30 Ultra-thin araldite section of an OI type II bone (Foetus F, 19 weeks) showing a necrotic osteoblast with mycoplasma-like bodies around it.
Figure 2.31 Ultra-thin araldite section of an OI type II bone (Foetus Mc, 16 weeks) showing a necrotic osteoblast with mycoplasma-like bodies around it.

Figure 2.32 Ultra-thin araldite section of an OI type II bone (Foetus S, 22 weeks) showing hyperosteoecytosis with more than one osteocyte per lacuna.

Figure 2.33 Ultra-thin araldite section of an OI type II bone (Foetus F, 19 weeks) showing hyperosteoecytosis with more than one osteocyte per lacuna.

Figure 2.34 Paraffin wax section of proximal femur from an OI type II/III foetus (Foetus Wi, 25 weeks) stained with alcian blue and sirius red, showing paucity of bone in the primary spongiosa.

Figure 2.35 Paraffin wax section of proximal femur from an OI type II/III baby (Baby V.D.K, full term) stained with alcian blue and sirius red showing paucity of bone in the primary spongiosa.

Figure 2.36 Paraffin wax section of proximal femur from an OI type II/III baby (Baby V.D.K, full term) stained with alcian blue and sirius red shown at a higher magnification than in Fig. 2.35.

Figure 2.37 Semi-thin araldite section of bone from an OI type II/III foetus (Foetus O, 18 weeks) stained with methylene blue/azur-II and basic fuchsins showing unmineralised collagen bridges between the bone spicules.

Figure 2.38 Paraffin wax section of proximal femur from an OI type II/III foetus (Foetus Wi, 25 weeks) stained with alcian blue and sirius red.

Figure 2.39 Semi-thin araldite section of mid-shaft femur from an OI type II/III foetus (Foetus Ar-II, 20 weeks) stained with Goldner stain.

Figure 2.40 Paraffin wax section of proximal femur from an OI type II/III foetus (Foetus Wi, 25 weeks) stained with alcian blue and sirius red and viewed under polarized light.
Figure 2.41 Transverse paraffin wax section of mid-shaft femur from OI type II/III baby (Baby V.D.K, full term) stained with alcian blue and sirius red and viewed under polarized light.

Figure 2.42 Electron micrograph of a section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing disorganised thick osteoid with mineral nodules and incomplete mineralisation.

Figure 2.43 Electron micrograph of a section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing an osteocyte lacuna with perilacunar unmineralised collagen band and proteoglycans around the periphery of the cell.

Figure 2.44 Electron micrograph of a section of an OI type II/III foetal rib bone (Foetus F.G, 22 weeks) showing osteoid collagen of variable diameter.

Figure 2.45 Electron micrograph of a section of an OI type II/III tibial bone (Baby G-III, full term) showing osteoid collagen of variable diameter.

Figure 2.46 Photomicrograph of a resin section of a normal bone (S.N.D, 2 years) stained with Goldner stain.

Figure 2.47 Photomicrograph of a resin section of an OI type III bone (H.S, 3 years) stained with Goldner stain.

Figure 2.48 Electron micrograph of bone osteoid from an OI type III patient (H.G, 6 years) showing unusually thick osteoid with sparsely distributed abnormal collagen fibres and patchy mineralisation.

Figure 2.49 Electron micrograph of bone osteoid from an OI type III patient (H.S, 3 years) showing unusually thick osteoid with sparsely distributed abnormal collagen fibres.

Figure 2.50 Electron micrograph of bone osteoid from an OI type III patient (B.G, 3 years) showing unusually thick osteoid with sparsely distributed abnormal collagen fibres and patchy mineralisation.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.51</td>
<td>Electron micrograph of bone osteoid from an OI type III patient (K.W, 7 years) showing disorganised osteoid with patchy mineralisation.</td>
<td>93</td>
</tr>
<tr>
<td>2.52</td>
<td>Electron micrograph of bone osteoid from an OI type III patient (B.G, 3 years) shown at a higher magnification than in Fig. 2.50.</td>
<td>94</td>
</tr>
<tr>
<td>2.53</td>
<td>Electron micrograph of bone osteoid from an OI type III patient (H.S, 3 years) shown at a higher magnification than in Fig. 2.49.</td>
<td>94</td>
</tr>
<tr>
<td>2.54</td>
<td>Electron micrograph of bone osteoid from an OI type IV patient (M.K, 25 years) showing thin osteoid with patchy mineralisation.</td>
<td>95</td>
</tr>
<tr>
<td>2.55</td>
<td>Electron micrograph of bone osteoid from an OI type IV patient (Z.A, 14 years) showing an organised osteoid and necrotic osteoblasts.</td>
<td>95</td>
</tr>
<tr>
<td>2.56</td>
<td>Electron micrograph of bone osteoid from an OI type IV patient (J.D, 9 years) showing a thick osteoid with a pre-osteocyte buried in the collagen.</td>
<td>96</td>
</tr>
<tr>
<td>2.57</td>
<td>Photomicrograph of a resin section of bone from an OI type IV patient (J.D, 9 years) stained with Miller's elastin stain.</td>
<td>96</td>
</tr>
<tr>
<td>2.58</td>
<td>Electron micrograph of a resin section of bone from an OI type IV patient (J.D, 9 years) showing abnormal aggregation of elastin in the periosteum.</td>
<td>97</td>
</tr>
<tr>
<td>2.59</td>
<td>Electron micrograph of a resin section of bone from an OI type IV patient (J.D, 9 years) shown at a higher magnification than in Fig. 2.58.</td>
<td>97</td>
</tr>
<tr>
<td>2.60a</td>
<td>Electron micrograph of bone osteoid from an OI type IV patient (K.L, 28 years) showing thin osteoid and the abnormal presence of elastin.</td>
<td>98</td>
</tr>
</tbody>
</table>
Figure 2.60b  Electron micrograph of bone osteoid from an OI type IV patient (K.L, 28 years) shown at a higher magnification than in Fig. 2.60a to show the disorganised collagen fibres and elastin.

Figure 2.61a  Electron micrograph of bone osteoid from an OI type IV patient (M.K, 25 years) showing thin osteoid and the abnormal presence of elastin.

Figure 2.61b  Electron micrograph of bone osteoid from an OI type IV patient (M.K, 25 years) shown at a higher magnification than in Fig. 2.61a.

Figure 3.1  Schematic diagram showing the sequence of mineral formation in the osteoid of bone.

Figure 3.2  Standard graph of the Ca/P ratios for the calcium phosphate standards as measured in the TEM and plotted against empirical ratios.

Figure 3.3a  Electron micrograph of mineral area on an unstained ultra-thin cryo-section of a normal bone (M.K, 11 years) at the site of XRMA analysis.

Figure 3.3b  Electron micrograph of mineral area on an unstained ultra-thin cryo-section of an OI type III bone (H.S, 3 years) at the site of XRMA analysis.

Figure 3.3c  Electron micrograph of mineral area on an unstained ultra-thin cryo-section of a normal foetal bone (Foetus Sy, 22 weeks) at the site of XRMA analysis, shown at a higher magnification.

Figure 3.3d  Electron micrograph of mineral area on an unstained ultra-thin cryo-section of an OI type II foetal bone (Foetus F, 19 weeks) at the site of XRMA analysis, shown at a higher magnification.

Figure 3.4a  The XRMA spectrum of a cryo-section of normal foetal bone mineral showing strong peaks for Ca and P with very low background.

Figure 3.4b  The XRMA spectrum of a cryo-section of OI type II bone mineral showing strong peaks for Ca and P with very low background.
Figure 3.4c  The XRMA spectrum generated from a cryo-section of an OI type II bone (Fig. 3.4b) was overlaid on the XRMA spectrum generated from a cryo-section of a normal foetal bone (Fig. 3.4a).

Figure 3.5a  Bar graph showing the Ca/P ratios in various clinical types of OI bone, normal bone and the hydroxyapatite (HA) standard measured on cryo-sections.

Figure 3.5b  Bar graph showing the Ca/P ratios in various clinical types of OI bone, normal bone and the hydroxyapatite (HA) standard measured on conventional resin sections.

Figure 3.6a  Bar graph showing the Ca/P ratios measured on cryo-sections of the hydroxyapatite (HA) standard, normal bone and pooled OI types.

Figure 3.6b  Bar graph showing the Ca/P ratios measured on resin sections of the hydroxyapatite (HA) standard, normal bone and pooled OI types.

Figure 3.7  Bar graph showing the Ca/P ratios of normal and OI bone measured on resin sections and compared by age groups.

Figure 4.1  Apple Macintosh Quadra Image analysis system used to measure the collagen fibres in this study.

Figure 4.2  The analogue images of the fibrils which have been outlined are converted into diameters.

Figure 4.3a  Electron micrograph of transverse collagen fibres from a section of normal juvenile bone (F.D, 10 years).

Figure 4.3b  Electron micrograph of transverse collagen fibres from a section of OI type I bone (A.C, 10 years).

Figure 4.3c  Electron micrograph of transverse collagen fibres from a section of normal foetal bone (Foetus Sy, 22 weeks).

Figure 4.3d  Electron micrograph of transverse collagen fibres from a section of OI type II bone (Foetus Mc, 16 weeks).

Figure 4.3e  Electron micrograph of transverse collagen fibres from a section of OI type III bone (H.S, 3 years).

Figure 4.3f  Electron micrograph of transverse collagen fibres from a section of OI type IV bone (J.D, 9 years).
Figure 4.4  Bar graph showing the computed mean diameter of bone osteoid collagen in OI types and normal foetal and normal young controls.

Figure 4.5  The frequency distribution of type I collagen fibres for a normal control.

Figure 4.6  The frequency distribution of type I collagen fibres for an OI type I patient.

Figure 4.7  The frequency distribution of type I collagen fibres for an OI type III patient.

Figure 4.8  The frequency distribution of type I collagen fibres for an OI type IV patient.

Figure 4.9  The frequency distribution of type I collagen fibres for a normal foetal control.

Figure 4.10  The frequency distribution of type I collagen fibres for an OI type II patient demonstrating abnormal distribution.

Figure 4.11a  Radio-labelled collagen $\alpha$ chains (from OI type II) secreted into the culture medium and separated by polyacrylamide gel electrophoresis.

Figure 4.11b  Radio-labelled collagen $\alpha$ chains (from OI types II and III) secreted into the culture medium and separated by polyacrylamide gel electrophoresis.

Figure 4.11c  Procollagen 'ladder' from severely affected OI type II foetus (Foetus W) compared with normal controls.

Figure 4.11d  Normal procollagen 'ladder' in a series of controls showing good conversion of procollagen type I to $\alpha 1$ and $\alpha 2$.

Figure 5.1a  Ultra-thin lowicryl section of normal juvenile bone (M.K, 11 years) labelled with antibody to type I collagen.

Figure 5.1b  Ultra-thin lowicryl section of OI type I bone (S,D, 14 years) labelled with antibody to type I collagen.

Figure 5.1c  Ultra-thin lowicryl section of OI type III bone (H.S, 3 years) labelled with antibody to type I collagen.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1d</td>
<td>Ultra-thin lowicryl section of normal juvenile bone (M.K, 11 years) labelled in the absence of primary antibody to type I collagen.</td>
</tr>
<tr>
<td>5.1e</td>
<td>Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled with antibody to type I collagen.</td>
</tr>
<tr>
<td>5.1f</td>
<td>Ultra-thin lowicryl section of OI type II foetal bone (Foetus F, 19 weeks) labelled with antibody to type I collagen.</td>
</tr>
<tr>
<td>5.1g</td>
<td>Ultra-thin lowicryl section of OI type II/III foetal bone (Foetus Ar-II, 20 weeks) labelled with antibody to type I collagen.</td>
</tr>
<tr>
<td>5.1h</td>
<td>Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled in the absence of primary antibody to type I collagen.</td>
</tr>
<tr>
<td>5.2a</td>
<td>Ultra-thin lowicryl section of normal juvenile bone (M.K, 11 years) labelled with antibody to type III collagen.</td>
</tr>
<tr>
<td>5.2b</td>
<td>Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled with antibody to type III collagen.</td>
</tr>
<tr>
<td>5.2c</td>
<td>Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled with antibody to type III collagen.</td>
</tr>
<tr>
<td>5.2d</td>
<td>Ultra-thin lowicryl section of OI type II foetal bone (Foetus F, 19 weeks) labelled with antibody to type III collagen.</td>
</tr>
<tr>
<td>5.2e</td>
<td>Ultra-thin lowicryl section of OI type II/III foetal bone (Foetus Ar-III, 22 weeks) labelled with antibody to type III collagen.</td>
</tr>
<tr>
<td>5.2f</td>
<td>Ultra-thin lowicryl section of OI type II foetal bone (Foetus Mc, 16 weeks) labelled in the absence of primary antibody to type III collagen.</td>
</tr>
<tr>
<td>5.3a</td>
<td>Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled with antibody to type IV collagen.</td>
</tr>
<tr>
<td>5.3b</td>
<td>Ultra-thin lowicryl section of OI type II/III bone (Foetus Ar-II, 20 weeks) labelled with antibody to type IV collagen.</td>
</tr>
<tr>
<td>5.3c</td>
<td>Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled with antibody to type IV collagen.</td>
</tr>
</tbody>
</table>
Figure 5.3d  Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled in the absence of primary antibody to type IV collagen.

Figure 5.4a  Unfixed freeze-substituted lowicryl-embedded normal juvenile bone (M.K, 11 years) ultra-thin section labelled with antibody to type I collagen.

Figure 5.4b  Unfixed freeze-substituted lowicryl-embedded normal juvenile bone (M.K, 11 years) ultra-thin section; negative control for type I collagen labelling.

Figure 5.4c  Unfixed freeze-substituted lowicryl-embedded OI type III bone (H.S, 3 years) ultra-thin section labelled with antibody to type I collagen.

Figure 5.4d  Unfixed freeze-substituted lowicryl-embedded OI type III bone (H.S, 3 years) ultra-thin section; negative control for type I collagen labelling.

Figure 5.5a  Unfixed freeze-substituted lowicryl-embedded normal juvenile bone (M.K, 11 years) ultra-thin section labelled with antibody to type III collagen.

Figure 5.5b  Unfixed freeze-substituted lowicryl-embedded OI type III bone (H.S, 3 years) ultra-thin section labelled with antibody to type III collagen.

Figure 5.6a  Ultra-thin section of normal juvenile bone (A.H.A, 12 years) showing pre-embedding immunogold labelling for type I collagen.

Figure 5.6b  Ultra-thin section of OI type II bone (Foetus Mc, 16 weeks) showing pre-embedding immunogold labelling for type I collagen.

Figure 6.1a  Normal foetal bone (Foetus Gy, 19 weeks) demonstrating alkaline phosphatase activity around the osteoblasts and the mineral front.

Figure 6.1b  Normal foetal bone (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor showing no reaction product on the osteoblasts.

Figure 6.2a  OI type II foetal bone (Foetus F, 19 weeks) showing low alkaline phosphatase activity around the osteoblasts.
Figure 6.2b  OI type II foetal bone (Foetus F, 19 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor.

Figure 6.3a  OI type II foetal bone (Foetus W, 25 weeks) stained for alkaline phosphatase activity.

Figure 6.3b  OI type II foetal bone (Foetus W, 25 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor.

Figure 6.4a  Normal juvenile bone (A.H.A, 12 years) stained for alkaline phosphatase activity.

Figure 6.4b  Normal juvenile bone (A.H.A, 12 years) stained for alkaline phosphatase activity in the presence of levamisole inhibitor.

Figure 6.5a  OI type III bone (H.S, 3 years) stained for alkaline phosphatase activity.

Figure 6.5b  OI type III bone (H.S, 3 years) stained for alkaline phosphatase activity in the presence of levamisole inhibitor.

Figure 6.6a  Normal foetal growth plate (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity showing an intense reaction product around the chondrocytes.

Figure 6.6b  Normal foetal growth plate (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity and shown at a higher magnification than in Fig. 6.6a.

Figure 6.7a  OI type II foetal growth plate (Foetus F, 19 weeks) stained for alkaline phosphatase activity.

Figure 6.7b  OI type II foetal growth plate (Foetus F, 19 weeks) stained for alkaline phosphatase activity and shown at a higher magnification than in Fig. 6.7a.

Figure 6.8a  OI type II foetal growth plate (Foetus Mc, 16 weeks) stained for alkaline phosphatase activity.

Figure 6.8b  OI type II foetal growth plate (Foetus Mc, 16 weeks) stained for alkaline phosphatase activity and shown at a higher magnification than in Fig. 6.8a.
Figure 6.9a Normal foetal growth plate (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor.

Figure 6.9b OI type II foetal growth plate (Foetus F, 19 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor.

Figure 7.1a Araldite section (1μm) of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks) stained with methylene blue/azur II and basic fuchsin, showing the porotic nature of the bone.

Figure 7.1b Araldite section of a normal foetal femoral bone (Foetus M, 22 weeks) stained with methylene blue/azur II and basic fuchsin as in Fig. 7.1a.

Figure 7.1c Araldite section (1μm) of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks) stained with toluidine blue.

Figure 7.1d Araldite section of a normal foetal femoral bone (Foetus M, 22 weeks) stained with toluidine blue.

Figure 7.1e Paraffin wax section of an OI type II/III foetal rib bone (Foetus Ar-III, 22 weeks) stained with alcian blue and counter stained with Van Gieson stain.

Figure 7.1f Paraffin wax section of a normal foetal rib bone (Foetus M, 22 weeks) stained in similar manner to that of Fig 7.1e.

Figure 7.2a1 Ultra-thin araldite section of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks), showing unmineralised osteoid collagen in the periphery of the lacunae.

Figure 7.2a2 Higher magnification of the osteocytic lacuna shown in Fig.7.2a1

Figure 7.2b1 Ultra-thin araldite section of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks), showing perilacunar proteoglycan-like material.

Figure 7.2b2 Higher magnification of the osteocytic lacuna shown in Fig.7.2b1
Figure 7.3a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) immunogold labelled for type I collagen.

Figure 7.3b  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) immunogold labelled for type II collagen.

Figure 7.3c  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) immunogold labelled in the absence of primary antibody.

Figure 7.4a  The XRMA spectrum from an osteocyte lacuna area in an unstained and non-osmicated araldite section of an OI type II/III femoral foetal bone (Foetus Ar-III, 22 weeks).

Figure 7.4b  The XRMA spectrum from an osteocyte lacuna area in an unstained and non-osmicated araldite section of a normal foetal femoral bone (Foetus M, 22 weeks).

Figure 7.5a  Immunogold labelled and silver enhanced lowicryl section (1µm) of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) stained with antibodies to proteoglycans.

Figure 7.5b  Immunogold labelled, silver enhanced lowicryl section (1µm) of a normal foetal femoral bone (Foetus M, 22 weeks) showing no staining for proteoglycans.

Figure 7.5c  Serial section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing no staining for proteoglycans when the primary antibodies were replaced by non-immune mouse serum in the staining protocol.

Figure 7.6a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) osteocytic lacuna immunogold labelled for proteoglycans.

Figure 7.6b  Higher magnification of the osteocytic lacuna shown in Fig. 7.6a.

Figure 7.7  Ultra-thin lowicryl section of a normal foetal femoral bone (Foetus M, 22 weeks) labelled for proteoglycans.
Figure 7.8  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) osteocytic lacuna labelled in the absence of primary antibodies for proteoglycans.

Figure 7.9a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-keratan sulphate antibody (5D4) followed by GAM IgG gold.

Figure 7.9b  Higher magnification of the osteocytic lacuna shown in Fig. 7.9a.

Figure 7.10a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-chondroitin 4-sulphate antibody (2B6) followed by GAM IgG gold.

Figure 7.10b  Higher magnification of the osteocytic lacuna shown in Fig. 7.10a.

Figure 7.11a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-chondroitin 6-sulphate antibody (3B3) followed by GAM IgG gold.

Figure 7.11b  Higher magnification of the osteocytic lacuna shown in Fig. 7.11a.

Figure 7.12  Ultra-thin lowicryl section of an osteocytic lacuna from an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-decorin antibody (LF30) followed by GAR IgG gold.

Figure 7.13  Ultra-thin lowicryl section of an osteocytic lacuna from an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-biglycan antibody (LF15) followed by GAR IgG gold.

Figure 7.14a  Ultra-thin lowicryl section of an osteocytic lacuna from an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled in the absence of primary antibodies to proteoglycans.

Figure 7.14b  Higher magnification of the osteocytic lacuna shown in Fig. 7.14a.

Figure 7.15a  Ultra-thin araldite section of an OI type II/III bone (Foetus Ar-II, 20 weeks) en-bloc stained with cuprolinic blue (CB).
Figure 7.15b  Higher magnification of the osteocytic lacuna shown in Fig. 7.15a.

Figure 7.16  Ultra-thin araldite section of osteoid collagen from a normal foetal bone (Foetus Gy, 19 weeks) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.17  Ultra-thin araldite section of osteoid collagen from an OI type II foetal bone (Foetus W, 25 weeks) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.18  Ultra-thin araldite section of osteoid collagen from a normal foetal bone (Foetus Sy, 22 weeks) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.19  Ultra-thin araldite section of osteoid collagen from an OI type II foetal bone (Foetus F, 19 weeks) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.20  Ultra-thin araldite section of osteoid collagen from a normal foetal bone (Foetus L, 24 weeks) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.21  Ultra-thin araldite section of osteoid collagen from an OI type II/III bone (Baby V.D.K, full term) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.22  Ultra-thin araldite section of osteoid collagen from a normal juvenile bone (M.K, 11 years) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.23  Ultra-thin araldite section of osteoid collagen from an OI type II/III bone (Baby G-III, full term) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.24  Ultra-thin araldite section of osteoid collagen from a normal juvenile bone (N.A, 13 years) *en-bloc* stained with cuprolinic blue (CB).

Figure 7.25  Ultra-thin araldite section of osteoid collagen from an OI type III bone (H.S, 3 years) *en-bloc* stained with cuprolinic blue (CB).
| Table 1.1 | Sillence classification of OI showing the sub-types. | 31 |
| Table 2.1 | Morphological and ultrastructural abnormalities reported in the literature for OI. | 46 |
| Table 2.2 | OI Type I microscopic findings and collagen abnormalities. | 56 |
| Table 2.3 | OI Type II microscopic findings and collagen abnormalities. | 58 |
| Table 2.4 | OI Type II/III microscopic findings and collagen abnormalities. | 61 |
| Table 2.5 | OI Type III microscopic findings and collagen abnormalities. | 63 |
| Table 2.6 | OI Type IV microscopic findings and collagen abnormalities. | 64 |
| Table 2.7 | Summary of morphological and ultrastructural observations according to type of OI. | 65 |
| Table 3.1 | Empirical and observed Ca/P ratios for known standards. | 113 |
| Table 3.2.1 | Details of normal foetal bone and the Ca/P ratios. | 117 |
| Table 3.2.2 | Details of normal bone and the Ca/P ratios. | 118 |
| Table 3.3.1 | Details of OI type I bone and the Ca/P ratios. | 119 |
| Table 3.3.2 | Details of OI type II bone and the Ca/P ratios. | 120 |
| Table 3.3.3 | Details of OI type II/III bone and the Ca/P ratios. | 121 |
| Table 3.3.4 | Details of OI type III bone and the Ca/P ratios. | 122 |
| Table 3.3.5 | Details of OI type IV bone and the Ca/P ratios. | 122 |
| Table 4.1 | Reproducibility test for collagen fibril measurements. | 137 |
| Table 4.2.1 | Results of normal foetal control bone collagen fibril diameter measurements and other sample details. | 138 |
| Table 4.2.2 | Results of normal juvenile/adult control bone collagen fibril diameter measurements and other sample details. | 139 |
Table 4.3 Results of OI type I bone collagen diameter measurements and other sample details. 140

Table 4.4 Results of OI type II bone collagen diameter measurements and other sample details. 141

Table 4.5 Results of OI type II/III bone collagen diameter measurements and other sample details. 142

Table 4.6 Results of OI type III bone collagen diameter measurements and other sample details. 143

Table 4.7 Results of OI type IV bone collagen diameter measurement and other sample details. 144

Table 4.8 Measurements of bone collagen fibre periodicity. 149

Table 5.1.1 Immunogold staining intensities of different collagen types in normal bone. 159

Table 5.1.2 Immunogold staining intensities of different collagen types in OI bone. 160

Table 7.1 Patients' details and the techniques used to confirm the presence of proteoglycans in the osteocytic lacunae. 200
Chapter 1

Introduction

1.1 General introduction

Osteogenesis Imperfecta (OI) is a heterogeneous heritable connective tissue disorder frequently caused by the mutations of two genes (COL1A1 and COL1A2) encoding for type I collagen. The most striking manifestation of the disease is the ease and frequency of fractures. Other clinically variable features are short stature, blue sclera (Fig. 1.1), hearing loss, osteopenia, dental abnormalities (Fig. 1.2), cardiac abnormalities and joint laxity (Byers 1993). All these characteristics clearly point to a generalised connective tissue disorder. OI is a relatively rare disease affecting about 2,000 patients in the UK. The worldwide distribution is about 1:15,000 to 1:20,000 (Byers 1993; Smith 1995) and it does not show any geographical, ethnic or racial distribution. There may be some forms of OI that are more common in isolated regions, possibly because of the accumulation of a rare recessive allele (Beighton and Versfeld 1985). Nearly all cases of OI are inherited as an autosomal dominant trait (Sillence et al 1979; Rowe 1992), the recessive cases could be due to mosaicism.

1.2 History of OI

The earliest cases of OI recorded are represented by an Egyptian Mummy with deforming features (Figs. 1.3a & 1.3b) dating from about 1000 B.C. (Gray 1969), and by a partial skeleton from the seventh century found in Britain (Wells 1965). At the beginning of the 18th century abnormal bone brittleness and multiple fractures were reported by several authors (Hartsoecker 1712; Amand 1715; Bordenave 1763). The first description of autosomal dominant inheritance was described by Ekman (1788) in his doctoral thesis where he described four generations who had bony malformations. That OI affects other organs apart from bones was first noted by Axmann (1831), who himself along with two brothers had fragile bones, blue sclera and hypermobile joints. Lobstein (1835) described abnormal brittleness of the bone particularly affecting children and adults. He reported three children from one family who had this disease and called it Osteopsathyrosis Idiopathica. The lethal form of OI was first recognised by Vrolik in 1849 and he named the disease Osteogenesis Imperfecta. Osteopsathyrosis Idiopathica and Osteogenesis Imperfecta were thought to be two different diseases until Schmidt (1897) showed the similarities between the two. His findings were validated by Looser (1906) who gave conclusive evidence that the microscopic picture was similar in both diseases. Blue sclera and a tendency to fracture were mentioned by Spurway
Chapter 1

(1896) and by Eddowes (1900). Hearing loss associated with brittle bones and blue sclera was first identified by Adair-Dighton in 1912; he reported that it was usually caused by otosclerosis. This was again observed by Bronson (1917) and Van der Hoeve and de Kleyn (1917) in the early part of this century. In 1940 Bauer carried out a thorough investigation of OI cases and reported the involvement of dental tissue, cartilage, skin and blood vessels and summarised that OI was a generalised mesenchymal disorder.

1.3 Classification

The first classification of OI was proposed by Looser in 1906. He suggested two groups based on the time at which the first fracture was discovered. These were OI "congenita" which was the lethal form present at birth, and OI "tarda" the mild form which became evident at a later stage. In 1928 Bell, after studying large numbers of OI families in Britain, recognised the additional heterogeneity within these groups. Seedorf (1949) recognised that there were two milder forms of the disease; one with normal height, little deformity and blue sclera and the other with marked deformity. With the increase in the number of cases and increasingly sophisticated diagnostic techniques this classification became out dated. Bauze et al (1975) classified OI into three different groups based on the extent of deformity; mild, moderate and severe. The clinical phenotype varies from normal looking children with occasional fractures beginning in infancy to severely crippled short-limbed dwarfs dying either in utero or perinatally. In 1979 Sillence et al, having recognised this variability, introduced a classification which has proved popular and is in current use. According to the Sillence classification, OI can be separated into four main groups based on radiographic, genetic and clinical criteria. Types I and IV are the milder forms with an autosomal dominant mode of inheritance. Type II is the lethal type of OI usually resulting in perinatal death, while type III is the severe form with crippling deformities. The mode of inheritance for types II and III can be either autosomal dominant or recessive. Sillence updated his classification in 1981 by introducing sub-types into the four main types of OI. According to Sillence OI type II has three sub-types; type IIA, IIB, IIC (Table 1.1 after Byers 1993). This classification has been used throughout this study to categorise patients into clinical types of OI. Sillence type II babies with severe OI die either in utero or perinatally from a variety of causes; the most common are cerebral haemorrhage, strangulated hernias and chest infections. Some occasionally have severe congenital heart disease but the majority die from chest infections secondary to severely fractured ribs. In 1990 Cole et al, having studied two type IIB babies with known collagen mutations, proposed that type IIB can be further sub-classified into two groups, the first with helical mutations and both normal and mutant type I collagen in the tissues, and the second with carboxy terminal
propeptide mutations and a severe type I collagen deficiency, but without mutant collagen in the tissues. OI type II and type III sometimes overlap with one another, so do types III and IV. In this study there are eight foetal bone specimens which have been categorised under type II/III. These samples could have been categorised under type II because they were from aborted foetuses; however if the foetuses had been born they could have survived and would be considered as severe type III with progressive deformities. This type II/III is actually an infantile form of type III as shown in Figs. 1.4a and 1.4b.
Table 1.1 Sillence Classification of OI showing the sub-types.

<table>
<thead>
<tr>
<th>OI type</th>
<th>Characteristics</th>
<th>Mode of inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>type IA</td>
<td>mild form, normal stature, little or no deformity, blue sclera, presenile hearing loss.</td>
<td>autosomal dominant</td>
</tr>
<tr>
<td>type IB</td>
<td>type IA characteristics plus Dentinogenesis Imperfecta.</td>
<td>autosomal dominant</td>
</tr>
<tr>
<td>type IIA</td>
<td>lethal OI, multiple intrauterine fractures, broad short femora, continuous beading of ribs, poorly mineralised calvarium.</td>
<td>autosomal dominant, new mutations</td>
</tr>
<tr>
<td>(Fig. 1.5a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>type IIB</td>
<td>as in IIA but thin ribs without continuous beading and better mineralised calvarium.</td>
<td>autosomal recessive</td>
</tr>
<tr>
<td>(Fig. 1.5b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>type IIC</td>
<td>less frequent than A or B, much more severely twisted thin limbs.</td>
<td>mostly autosomal dominant, some autosomal recessive</td>
</tr>
<tr>
<td>(Fig. 1.5c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>type III</td>
<td>severe OI with multiple fractures, marked growth retardation, severe scoliosis, blue or white sclera.</td>
<td>autosomal dominant (75%), autosomal recessive (25%)</td>
</tr>
<tr>
<td>type IVA</td>
<td>moderately severe, white sclera, frequent fractures with skeletal deformity.</td>
<td>autosomal dominant</td>
</tr>
<tr>
<td>type IVB</td>
<td>features as in type IVA plus Dentinogenesis Imperfecta.</td>
<td>autosomal dominant</td>
</tr>
</tbody>
</table>
Fig. 1.1  Typical blue sclera of OI types IA and IB.

Fig. 1.2  Translucent and discoloured teeth of Dentinogenesis Imperfecta.
Fig. 1.3a The earliest authenticated example of OI is an Egyptian Mummy of a child together with the skull (courtesy of the British Museum).

Fig. 1.3b The lower limbs of the Egyptian Mummy (Fig 1.3a) showing bowed femurs and twisted tibias (courtesy of the British Museum).
Fig. 1.4a Clinical appearance of a 22 week gestation OI type II/III foetus showing subtle abnormalities in arms and legs. The clinical features in this type II/III are not as severe as in OI type II.

Fig. 1.4b Babygram of the foetus (22 week gestation) shown in Fig. 1.4a. The gracile limbs, slender ribs (not beaded) and bowed femurs are different from the babygrams of typical OI type II (Figs. 1.5 a,b,c).
Fig. 1.5a  Babygram of a foetus with OI sub-type IIA. The ribs are broadened and beaded due to callus formation. The long bones are severely deformed and crumpled.

Fig. 1.5b  Babygram of a foetus with OI sub-type IIB. The ribs are thin without continuous beading. The long bones are severely deformed.

Fig. 1.5c  Babygram of a foetus with OI sub-type IIC. Here the thin ribs and thin crumpled long bones are evident.

Fig. 1.5d  Babygram of a foetus with OI type II/III. Note the normal ribs (not broadened or beaded) and well modelled long bones.
1.4 Biochemical and genetic findings in OI

Collagen is the most abundant protein in the body and it is the major structural protein of the extracellular matrix. Although there are more than 16 genetically distinct collagen types encoded by more than 28 collagen genes (Byers 1995), bone matrix consists largely of type I collagen. The type I collagen molecule is a heterotrimer composed of two identical α1 chains and one α2 chain; each contains 1000 amino acids which form a 300 nm long and 1.4 nm wide semiflexible rod that spontaneously assembles into microfibrils. The molecules overlap by a quarter of their lengths, so producing the characteristic 67 nm periodicity of type I collagen fibrils (Miller 1976). The triple helix is formed from the uninterrupted sequence of 338 Gly-X-Y triplets in which X is frequently proline and Y is frequently hydroxyproline (Cole 1994). The presence of glycine, the smallest amino acid with no side chains, in the first position allows the chain to maintain a tightly coiled state and substitution of any other amino acid for glycine will disrupt the highly ordered geometry of the helix. The most common biochemical and genetic abnormality in OI is the substitution of glycyl residues in the triple helical domain in α1(1) and α2(1) chains in all OI types except type I. In this type, glycine substitutions are comparatively rare and the commonest abnormality is "nonfunctional" or "null allele" (Willing et al 1992, 1994). The null allele prematurely terminates the message for half of the type I collagen synthesized while the remaining collagen is structurally normal. Other errors include splice-junction point mutations (Weatherall 1991), large and small in-frame deletions (Superti-Forga et al 1989), frame shift mutations (Nicholls et al 1984), and helical point mutations (Byers 1990; Kuivaniemi et al 1991). To date over 200 mutations have now been documented in type I collagen genes in OI (Cole and Dalgleish 1995; Dalgleish 1997). The location and type of substitution is very important as it reflects the severity of the disease. In general, glycine substitutions in the α1(1) chain are more deleterious than those occurring at equivalent positions in the α2(1) chain (Byers 1990; Kuivaniemi et al 1991); also carboxy terminal mutations produce more severe phenotypes than amino terminal mutations (Vogel et al 1988). Other factors which influence the effect of a glycine substitution in the type I collagen phenotype are the size of the substituting amino acid, the amount exported from the cell, the amount deposited in the extracellular matrix, the amount of normal non-mutant collagen and the type of cell and tissue expression (Smith 1994). Somatic and gonadal mosaicism occurs in some families in which the parents are clinically normal or one parent is minimally affected by OI. Direct evidence of mosaicism has been found in the sperm and somatic cells of some fathers and in the somatic cells of some mothers. These parents carry the mutations although they are clinically normal. All these mutations influence the stability, the rate of secretion and the mechanical properties of collagen thus causing impairment of normal collagen function.
1.5 Morphological and ultrastructural findings in OI

To date there are numerous reports on morphological and ultrastructural abnormalities in OI but there are many discrepancies in these findings. Most of the studies have concentrated on a small number of OI specimens from different clinical types and different anatomical sites and tissues other than bone. Although the primary manifestation of OI is bone, information on OI bone is scanty. Abnormalities in collagen, mineral, bone turnover rates and the extracellular matrix proteins have been implicated in OI.

Falvo and Bullough (1973) reviewed previous histological reports and examined iliac crest biopsies from sixteen OI patients in various clinical groups. The most consistent finding in their review was the increased number of osteocytes (hyperosteoecytosis). They suggested that the increased osteocytes, large osteoblasts and increase in the number of osteoid seam surfaces were indicative of increased bone formation. Jowsey (1977) pointed out that the presence of woven bone is responsible for large osteocyte lacunae and the excess of osteocytes is probably apparent rather than real because of the reduction of bone matrix. Baron et al (1979) studied iliac crest biopsies after double fluorescent labelling with tetracycline from nine OI tarda children and compared these with five unlabelled biopsies from normal children. The results indicated that the OI children had low trabecular volumes associated with an increase in bone turnover rates. They suggested that the bone formation is increased at tissue level despite the decrease in activity of individual osteoblasts, and postulated that the original defect in OI could be the rate of matrix synthesis and inability to accumulate bone. Bullough et al (1981) studied different tissues from different anatomical sites from two OI patients. On the basis of their light microscopic study, they suggested increased bone turnover in OI. They also reported that the growth plates were abnormal and electron microscopy of rib periosteum showed thinner collagen fibrils with cross sectional diameters ranging from 40 nm - 80 nm. The skin fibroblasts had dilated rough endoplasmic reticulum (RER) and excessive amounts of microfibrillar material. Recently Glorieux and Travers (1994) studied iliac crest biopsies of 39 patients of OI types I, III and IV, and 35 normal control specimens at light microscopy level. They reported that the cancellous bone volume, cortical width and trabecular thickness were decreased in all OI patients. A mild mineralisation defect was also present in all OI patients but it was only statistically evident in OI type IV patients. In OI type I, cancellous bone consisted of lamellar bone and cortical bone with few Haversian systems and no fibrous tissue in the marrow space. In OI type III, cancellous bone was a mixture of lamellar and woven bone and did not have a mature Haversian system. Marrow fibrosis was also evident. In OI type IV, there was more pronounced osteopenia and thinner trabeculae, lamellae were present but not organised, Haversian systems were rare and fibrosis was present.
Marion et al (1993) carried out a histological study on skeletal tissue of OI type II foetuses and reported abnormalities such as growth plates without any bony spongiosa, persistence of calcified cartilage in the diaphyses, micro-fractures, failure of formation of intramembranous cortical diaphyseal bone and scanty perichondrial rings with poorly organised fibrous tissue.

Few histological studies have been carried out recently on OI patients with known molecular defects (Cole et al 1992; Cohen-Solal et al 1994; Sztrolovics et al 1994). Cole et al (1992) studied 3 babies of OI type II, all with substitution of glycine by valine in the triple helix of procollagen. The histological findings were that the trabecular bone was sparse in the long bones and vertebrae, trabeculae contained cartilage cores with overlying layer of woven bone or osteoid, and the diaphyses lacked cortical bone. Sztrolovics et al (1994) reported similar findings in OI type II and III patients with point mutations converting glycine into arginine in the triple helix.

Haebara et al (1969) conducted histochemical and ultrastructural studies on a case of OI type II and reported that bone and skin contained pathologically immature collagen which had small, fine fibrils with short cross-striation periodicity. Furthermore there was an increase in chondroitin sulphate in epiphyseal cartilage and in the central cores of metaphyseal calcified matrix. They suggested that the presence of abnormal, pathologically immature collagen and increased chondroitin sulphate may cause the abnormal osteoid tissue in this disease.

Riley et al (1973) in a study of iliac crest biopsies from 11 patients with mild OI found normal ultrastructure of osteocytes, bone collagen and scleral collagen but reduced corneal collagen diameter. Teitelbaum et al (1974) carried out a scanning electron microscope study on iliac crest biopsies from three children with severe OI, and reported that the fine collagen fibres did not undergo extracellular aggregation into bundles as in normal bone and suggested that the inherited defect was one of fibrillogenesis after the post-translational stage of collagen synthesis. Cassella et al (1996) recently reported some ultrastructural abnormalities which have not been previously documented. They carried out light and electron microscopic studies (LM and EM) on bone tissue from 13 OI patients and 18 normal control specimens and found that all the OI bone specimens showed poor lamellar pattern with increased vascular channels but there was no increase in osteoblast or osteoclast numbers. At EM level many patients demonstrated both spherical and needle-shaped mitochondrial inclusions with a Ca/P ratio of 1.100 compared with the normal hydroxyapatite ratio of 1.602; there were fibrous bundles which ran parallel to type I collagen fibrils, and in some patients lobulated collagen fibres and microfibrils were also seen. In some patients an amorphous stromal calcification surrounding the calcified cartilage septae was evident.
Doty and Mathews (1971) reported alkaline phosphatase activity and acid phosphatase activity in OI bone. Alkaline phosphatase, which was localised along the osteoblast cell membrane, had 2-3 times lower activity in OI than in normal bone. They also reported an increased number of osteocytes, although osteoblast and osteoclast numbers were the same as in normals. However osteoblasts contained abundant glycogen to the extent that some normal subcellular structures were absent. There also seemed to be a spillover of the glycogen into the extracellular spaces.

There are reports of abnormalities in the mineral composition in OI. Engfeldt et al (1954) stated that the alignment of the mineral salts in the matrix is disordered presumably due to abnormal organic compounds in OI bone. Solomons and Styner (1969) and Hatherway et al (1972) reported an abnormality in pyrophosphate metabolism in OI resulting in an increased accumulation of organic pyrophosphate, an inhibitor of hydroxyapatite formation. A previous study of OI in this laboratory showed a lower Ca/P ratio in OI bone when compared with normal bone on resin-embedded sections (Cassella and Ali 1992; Cassella et al 1995). A transgenic mouse model also revealed a lower Ca/P ratio than in the normal litter mates (Cassella et al 1994b), and it was postulated that this could be due to ion substitution in the hydroxyapatite lattice. Bucsi et al (1994) studied three bone biopsies from OI type I patients and reported that the collagen fibril diameter was more variable than that of controls, the structure of newly formed apatite crystals was smaller in size but the Ca/P ratio was the same as in normal controls. The smaller size of apatite crystals in OI type II has been previously reported by several authors. Vetter et al (1991a) studied apatite crystal size using X-ray diffraction and found that it was markedly reduced in OI type II. Traub et al (1994) carried out a scanning electron microscopy (SEM) and a transmission electron microscopy (TEM) study of 15 OI patients and 7 normal controls and concluded that there was a good deal of normal lamellar bone structure even in severe OI, and suggested that it could be due to the heterozygous nature of mutations of the gene products producing normal extracellular matrix components. Furthermore in OI type III (and probably in types II and IV) there was abnormal collagen in loose fibrous unmineralised regions, apatite crystals were generally smaller and not plate-like as in normal bone and these crystals were poorly organised in relation to the collagen fibres. This last observation was in agreement with the findings of Vetter et al (1991a) and Cohen-Solal et al (1994). The latter presented biochemical and ultrastructural findings of skin and bone from a type II patient with a substitution of glycine to aspartic acid in the α2 (1) chain. They found that the collagen matrix of both tissues was dramatically decreased and abnormal collagen mostly remained in the fibroblasts and osteoblasts. In addition there were spheritic aggregates of mineral unrelated to the scarce and thin collagen fibrils.
There are discrepancies in the reports regarding collagen fibril diameter. Jones et al (1984) studied three patients with OI type I and reported that in the OI bone collagen diameter was smaller than in normal controls. Stoss and Freisinger (1993) reported a similar finding, whereas Cassella et al (1994a), in a histomorphometric analysis of 23 OI bone specimens and 20 normal specimens, found that bone collagen fibril diameter was larger than normal.

de Matteis and Bonnucci (1968) reported that the periodicity of collagen was 340 Å while Haebera et al (1969) reported it as 200-400 Å in OI type II. Cassella (1993) could not find any difference between the normal and OI bone collagen periodicity.

There are few reports on biochemically abnormal collagens in OI. Bleckmann et al (1971) reported that there was an increased hydroxylysine : hydroxyproline ratio in OI bone, moreover others have reported that the hydroxylysine : lysine ratio was also increased in OI bone (Eastoe et al 1973; Fujii and Tanzer 1977; Trelstad et al 1977). The abnormal presence of type III and type V collagens in OI bone has been documented by several authors (Müller et al 1977; Cole 1980; Pope et al 1980).

Proteoglycan (PG) abnormalities have also been suspected in OI; for example both Bleckmann et al (1971) and Engfeldt and Hjerpe (1976) found increased glycosaminoglycans (GAGs) in OI type II bone biopsies (old classification-OI congenita: new-type II), when compared with normal bone. Brown et al (1975) also observed abnormal distributions of GAGs in OI bone. Goldberg (1978) reported that urinary PGs in OI patients were abnormal and especially high in younger patients.

There are several reports of changes in non-collagenous proteins in OI bone. Dickson et al (1975) observed higher than normal levels of non-collagenous proteins and α2HS-glycoprotein in OI bone. Termine et al (1984) reported decreased levels of osteonectin and bone PG but bone sialoprotein levels were not affected in bovine OI. Vetter et al (1991b) studied the levels of non-collagenous proteins of bone in three type I, two type II, eight type III and thirteen type IV OI patients compared with seven normal controls. They found reduced levels of osteonectin, high levels of bone sialoproteins, osteocalcin and α2HS-glycoproteins in all OI patients. There were no alterations in decorin levels in OI patients when compared with the normal controls. Fedarko et al (1995) have recently reported an elevated level of matrix hyaluronan, fibronectin and thrombospondin while osteonectin and three PGs were reduced in OI bone cells. Furthermore when they grouped the OI cell strains by clinical types and plotted the average of steady state levels of matrix components, chondroitin sulphate PG showed a higher level in OI type II.
There are numerous reports on morphological abnormalities in skin, periosteum, growth plates, heart valves, aorta and on cultured cell lines of OI.

In the skin, Follis (1953) found argyrophilic reticulin-like fibres in an OI congenita (type II) patient. Stadil (1961) examined skin biopsies from 17 members of an OI family which had eight unaffected members and nine with mild OI. The OI skin biopsies showed increased amounts of argyrophilic and elastic fibres. These elastic fibres showed a variable degree of degeneration. Furness and White (1973) and Riley et al (1973) reported loosely textured thin collagen fibrils in the thin dermis. Pasquali-Ronchetti et al (1986) studied skin and aortic samples from two patients with OI type II and reported that no significant abnormalities were seen in the dermis apart from a small difference in the diameter of the reticular dermis, but aortic collagen diameter was significantly smaller in OI patients than in the normal controls. Furthermore, elastin lamellae were deeply altered and consisted of roundish aggregates of elastin permeated by cytochemically recognisable glycosaminoglycans. They suggested that there could be severe lysyl oxidase deficiency in these patients besides the defective type I collagen.

In the periosteum of OI bone, Nogami et al (1993) found a defective microvascular system. They reported that the vascular walls of arterioles and capillaries were thick and the lumina of the vessels were narrowed or occluded by hypertrophic endothelial cells and multiplication of smooth muscle layers. They concluded that a circulatory deficiency in the OI periosteum could be regarded as one of the causes of slender osteopenic diaphyseal bone in OI.

Despite the identification of mutations in a large number of OI patients, relatively few of these cases have been studied at the level of tissue organisation. The heterogeneity of OI at genetic level, as well as tissue level, makes such studies very difficult and the presumption that identification of the collagen gene mutations in OI would completely explain the phenotype is unjustified. All these findings implicate the panoramic heterogeneity of OI at the level of tissue organisation.

1.6 Animal Models

A congenital disease similar to OI has been found in Holstein-Friesian cattle, and there are two naturally occurring populations of this bovine Osteogenesis Imperfecta; BOI-A is the Australian variant while BOI-T is the Texas variant of OI. Both these syndromes apparently result from spontaneous mutations and are transmitted as autosomal dominant traits (Denholm and Cole 1983). Transgenic experiments in animals can also produce animal models resembling OI. Insertion of an engineered mutant collagen gene containing a substitution of glycine 859 by cysteine into mice produces a lethal disorder
similar to OI type II (Stacey et al 1988). Another lethal phenotype is produced in transgenic mice bearing an in-frame deletion in the human COL 1A1 gene (Khillan et al 1991). The MOV-13 strain of mice show many features of OI type I and the omi strain of mice have a mutation in the gene encoding the α2 chain of type I procollagen which prevents proper assembly of the α2 chains. The homozygous omi/omi strain shows moderate to severe OI (Saban et al 1996). These animal models will be helpful for future research associated with antisense oligonucleotides to inhibit the expression of mutated procollagen genes (Prockop et al 1993), gene therapy and drug trials.

1.7 Therapy in OI

The management of OI patients includes medical and surgical treatment. Since the primary manifestation of OI is bone fragility, all attempts to treat the disease have been directed at correcting the generalised osteoporosis. The main objective of the medical therapeutic agent in OI is to reduce fracture rate and to improve bone density. A variety of hormone and mineral supplements (fluorides, ascorbic acid, magnesium oxide, catechine, growth hormone, salmon calcitonin, vitamin D) have been tried on OI patients but none seems to be effective mainly because none of these therapeutic agents attack the primary defect in OI, that is, failure to produce normal type I collagen. The use of fluorides is based on the observation that when sodium fluoride was given to osteoporosis patients for a long time it showed beneficial effects such as skeletal deposition due to increased bone formation. However, in a double blind placebo-controlled randomised study conducted by Whyte et al (1996) fluoride was found to be ineffective for the treatment of OI. The use of ascorbic acid increases the hydroxylation of proline to hydroxyproline, which is a major component of collagen. Ascorbic acid is needed as a co-factor for the enzyme prolyl hydroxylase (Winterfeldt et al 1970). Treatment with magnesium oxide is based on defective pyrophosphate metabolism as claimed by Solomons and Styner (1969). Cetta et al (1977) and Stoss (1990) claimed that catechine (a flavonoid) is effective and that it improved the quality of osteoblasts and collagen. Marini et al (1993) treated patients with growth hormone and claimed that some patients have experienced a doubling in their pre-treatment growth rate. The recent study conducted by Marini and Gerber (1997) showed increases in the linear growth rate and positive changes in bone formation on histomorphometrics in a substantial group of OI type IV children. Salmon calcitonin also been used to treat OI patients. The major action of calcitonin is to inhibit bone resorption thus improving the bone mineralisation. Recently Astrom and Soderhall (1998) reported the beneficial value of bisphosphonate (APD) in treating three girls with severe OI; bisphosphonates inhibit osteoclast activity (Boonekamp et al, 1986), which might increase the bone mass in OI patients. At the moment there is no effective hormonal, mineral or vitamin
therapy for any type of OI, but in the future gene therapy will probably be the ultimate answer for treating patients with OI.

At present surgery is a more reliable treatment for OI patients to reduce deformity and promote normal function. Usually realignment of a deformed limb is achieved by inserting either a pin or rod. The use of an extendible intermedullary rod with separate bone fragments threaded onto the rod will allow bone elongation to proceed normally. Once rodded many children are able to ambulate, sometimes walking for the first time. The patient eventually has less deformity.

A study of 79 patients in the UK by McAllion and Patterson (1996) showed that life expectancy in mild forms of OI was normal. The early mortality in those with OI type III was found to be due to respiratory illness, injury and basilar invagination.

1.8 Aims of the project

Morphological analysis of normal and diseased tissue can provide information about the structure of the tissue and its components, the relative distribution of these components (fibres, fibrils, molecules) and their biochemical identity, and sometimes the manner in which the molecules react. A number of technological advances have made such studies possible. Histochemical and immunohistochemical staining both at LM and EM level can be used to localise proteins and identify the biochemical composition of a specific structural entity (i.e. collagen fibrils as type I or III). The elemental composition of matrix components can be determined using electron probe microanalysis combined with scanning electron microscopy (SEM) or transmission electron microscopy (TEM). Other morphological information can be quantitated using morphometric techniques (i.e. collagen fibril diameter). Thus these modern techniques of tissue analysis are bridging the gap that existed between biochemistry and morphology. To fully understand this crippling disease one has to correlate the biochemical findings and clinical features with ultrastructural and pathomorphological findings.

Previous studies in this laboratory used TEM to identify ultrastructural abnormalities of OI bone (Cassella et al 1996), electron probe microanalysis on resin-embedded sections to measure Ca/P ratio in OI bone mineral (Cassella et al 1995), and histomorphometry to measure collagen fibril diameter of the osteoid collagen (Cassella et al 1994a). The number of specimens used was limited to 15 for the electron probe microanalytical study and 23 for histomorphometry. In addition to these methods, the present study has used immunogold localisation to identify collagen types and proteoglycans found in OI bone. Having analysed bone mineral on ultra-thin cryosections without any aqueous contact to minimise possible artefactual demineralisation,
one can quantify the mineral composition with some degree of accuracy and precision. Over 40 OI bone specimens and 25 normal age- and site-matched control bone specimens have been studied by correlating old methods with new methods and this has given new insights into OI. Furthermore all the quantitative results presented here are statistically more significant than those presented previously, and this is the first report to correlate these data with the four clinical types of OI as described by Sillence et al (1979).

The main objective of this study of OI bone was to correlate our morphological and ultrastructural findings with available data on biochemical and mutational abnormalities of collagen, and with the disease severity.

The aims of this thesis were:

(1). To investigate the morphological and ultrastructural abnormalities of OI bone and compare these abnormalities with normal age- and site-matched control specimens.

(2). To measure the collagen fibril diameter and the periodicity of collagen fibres in the osteoid of OI and normal bone using histomorphometric analysis.

(3). To establish the Ca/P ratio of OI and normal bone using electron probe X-ray microanalysis in the transmission electron microscope on ultra-thin cryo-sections to minimise artefactual demineralisation and compare these with conventionally prepared ultra-thin resin sections.

(4). To identify at EM level other types of collagen (apart from the major type I collagen) which have previously been shown to be present in OI bone using biochemical methods, and study their relative distribution using immunogold labelling.

(5). To label alkaline phosphatase at EM level on OI and normal age- and site-matched control bone specimens to obtain information on osteoblast function.

(6). To correlate all these findings with the clinical severity of Osteogenesis Imperfecta.
Chapter 2

Histological and Ultrastructural Morphology of OI bone

2.1 Introduction

Bone structure

Bone is composed of three main cell types, collagenous osteoid and mineralised matrix. The three bone cell types are osteoblasts, osteocytes and osteoclasts. The osteoblasts are responsible for the synthesis of organic components of bone matrix which consists mainly of collagen (95%). The newly synthesized collagenous matrix adjacent to osteoblasts is called osteoid. The osteoid undergoes many changes before mineral is laid down to form the mineralised matrix (Fig. 2.1a).

Osteoblasts are polar cells located at the surface of bone tissue lining the matrix. When active, they have a cuboid shape and contain well developed rough endoplasmic reticulum (RER; Fig. 2.1b) and Golgi apparatus. When the matrix synthesizing activity of the osteoblasts declines, they become flattened and form thin lining cells.

Osteocytes are osteoblasts that have become surrounded by mineral matrix. Although trapped in a lacuna, they have contacts with osteoblasts and other osteocytes through a network of long, slender cytoplasmic processes. Their function is not well defined but they are believed to be involved in the maintenance of the bone matrix and regulation of bone remodeling (Mullender et al 1996).

Osteoclasts are multinucleated large cells responsible for bone resorption. In normal bone there is a fine balance between bone formation and resorption.

Histologically there are two varieties of bone which can be distinguished using polarizing light microscopy; immature woven bone and mature lamellar bone. In the woven bone, collagen bundles are arranged in a disorganised random fashion and lack structural orientation. In this way bone can be laid down quickly and resorption may also be facilitated. This type of bone is present in foetal tissue but little normally remains in human long bones after infancy. In lamellar bone, collagen bundles are arranged in parallel layers running in different directions at approximately 90° to each other (Fig. 2.1a).
Morphological and ultrastructural abnormalities in OI bone

Despite the significant and rapid advances in molecular biology and biochemistry which have identified over 200 different mutations in procollagen genes in OI (Cole and Dalgleish 1995; Dalgleish 1997), it is often not possible to fully explain the variable phenotypes in OI (Smith 1994). This may be because relatively few cases have been examined in detail at tissue level. In addition, the description of ultrastructural abnormalities in OI is conflicting and often controversial. This may be due to the marked heterogeneity of OI which makes it difficult to unify the varied observations. The important morphological and ultrastructural findings in OI are presented in Table 2.1.

Table 2.1 Morphological and ultrastructural abnormalities reported in the literature for OI.

<table>
<thead>
<tr>
<th>Authors</th>
<th>OI type &amp; specimens used</th>
<th>LM or EM findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doty &amp; Mathews (1971)</td>
<td>Tarda (type I), long bones &amp; iliac crest biopsies from 4 patients.</td>
<td>TEM: osteoblasts contained abundant glycogen and dense material in the Golgi; increased number of osteocytes; reduced alkaline phosphatase activity in OI compared with normal; gross lamellar pattern within the bone matrix abnormal.</td>
</tr>
<tr>
<td>Falvo &amp; Bullough (1973)</td>
<td>Tarda &amp; Congenita (types I &amp; II), iliac crest biopsies from 16 patients.</td>
<td>LM: increased number of osteocytes; large osteoblasts; increased number of osteoid seam surfaces.</td>
</tr>
<tr>
<td>Riley et al (1973)</td>
<td>probably type II, iliac crest biopsies from 11 patients.</td>
<td>TEM: decreased cortical thickness; decreased osteoid width; number of osteons reduced; bone surface activity lower than normal; bone collagen diameter normal but corneal collagen fibril diameter thinner than normal.</td>
</tr>
<tr>
<td>Baron et al (1979)</td>
<td>type I, iliac crest biopsies from 9 patients.</td>
<td>LM: reduced bone density; reduced mean trabecular width; reduced absolute osteoid volume; increased relative osteoid volume; reduced mean osteoid thickness; increased osteoblastic surface; increased osteoclastic surface; increased number of osteoclasts.</td>
</tr>
<tr>
<td>Authors</td>
<td>OI type &amp; specimens used</td>
<td>LM or EM findings</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bullough et al (1981)</td>
<td>OI Congenita (type II), different tissues from 2 patients.</td>
<td><strong>LM:</strong> in growth plate, disorganisation of proliferation &amp; hypertrophic zones; permeation of the cartilage by metaphyseal blood vessels; thinner calcified zone of growth plate; scanty primary spongiosa; thin cortices in long bones; marked osteoporosis; increased amounts of woven osseous tissue; more osteocytes per unit volume of matrix; cartilaginous fracture callus. <strong>EM:</strong> in rib, periosteal collagen thinner cross sectional diameter ranging from 40-80 nm; fibroblasts with dilated RER; excessive amounts of microfibrillar material.</td>
</tr>
<tr>
<td>Pontz et al (1985)</td>
<td>type I, iliac crest biopsies from 3 patients.</td>
<td><strong>TEM:</strong> irregular and patchy mineralisation; osteoblasts with swollen mitochondria; dilated RER; enlarged Golgi with distended vesicles; osteoid narrowed to varying degrees.</td>
</tr>
<tr>
<td>Cole et al (1992)</td>
<td>type II, long bones from 3 patients.</td>
<td><strong>LM:</strong> grossly defective intramembranous ossification; trabecular bone sparse in long bones &amp; vertebrae; cartilage core in the trabeculae; overlying layer of woven bone.</td>
</tr>
<tr>
<td>Marion et al (1993)</td>
<td>type II, long bones from 2 patients.</td>
<td><strong>LM:</strong> abnormal growth plate tissue due to failure of formation of bony spongiosa; persistence of calcified cartilage in the diaphyses; metaphyseal microfractures; presence of cartilage fracture callus; absence of bony callus; failure of formation of intramembranous cortical diaphyseal bone.</td>
</tr>
<tr>
<td>Nogami et al (1993)</td>
<td>types III &amp; IV, periosteum from 5 patients.</td>
<td><strong>TEM:</strong> thickened periosteum with defective microvascular systems; vascular walls thickened &amp; lumina narrowed or occluded by hypertrophic endothelial cells.</td>
</tr>
<tr>
<td>Authors</td>
<td>OI type &amp; specimens used</td>
<td>LM or EM findings</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bucsi <em>et al</em> (1994)</td>
<td>type I, bone biopsies from 3 patients.</td>
<td><strong>LM &amp; TEM</strong>: osteoid rim; osteoid surface covered by greater numbers of osteoblasts than normal; relative quantity of osteoid less than normal; uneven mineralisation front; smaller hydroxyapatite crystals but Ca/P ratio similar to normal; collagen fibres of variable diameter.</td>
</tr>
<tr>
<td>Cohen-Solal <em>et al</em> (1994)</td>
<td>type II with known mutations, skin and bone from one patient.</td>
<td><strong>TEM</strong>: reduced collagen matrix; abnormal collagen retained in fibroblasts and osteoblasts; presence of spheritic aggregates of mineral unrelated to scarce and thin collagen fibrils in bone.</td>
</tr>
</tbody>
</table>
| Glorieux and Travers (1994) | all OI types except type II, iliac crest biopsies from 29 patients. | **LM**: increased cancellous bone volume; decreased cortical width & trabecular thickness; mild mineralisation defect but only statistically evident in type IV.  
  type I: in cancellous bone, hyperosteocytosis & lamellar matrix;  
in cortical bone, few mature Haversian systems; no fibrous tissue in marrow space.  
  type III: in cancellous bone, mixture of woven & lamellar structures;  
in cortical bone, immature Haversian systems & marrow fibrosis.  
  type IV: lamellation present but not as organised as in type I; Haversian systems rare; marrow fibrosis. |
| Sztrolovis *et al* (1994) | type II & type III with known mutations, iliac crest or femur from 2 patients. | **LM**: severe osteopenia due to paucity of mineralised structures in cortical bone; thin osteoid; woven bone. |
### Table 2.1 continued

<table>
<thead>
<tr>
<th>Authors</th>
<th>OI type &amp; specimens used</th>
<th>LM or EM findings</th>
</tr>
</thead>
</table>
| Traub et al (1994) | all OI types, femoral bone biopsies from 15 patients. | **SEM:** type I bone structure similar to normal (rotated plywood); type II spongy appearance & crumbly to touch; types III & IV normal looking lamellar structures but many more open spaces than normal; in some areas loose unorientated fibrous texture and abnormal deposition of mineral.  
**TEM:** bone fragments with layers of orientated crystals; clusters of apatite crystals apparently unassociated with collagen matrix; unmineralised collagen fibrils. |
| Cassella et al (1996) | all OI types, long bone biopsies from 13 patients. | **LM:** poor lamellar pattern with increased vascular channels; no increase in osteoblasts and osteoclasts.  
**TEM:** granular and needle-shaped mitochondrial mineral; fibrous bundles parallel to type I collagen fibrils; in some patients lobulated collagen fibres & microfibrils; in some patients amorphous stromal calcification surrounding cartilage septae. |
| McCarthy et al (1997) | type IA, iliac crest biopsies from 8 patients. | **LM:** lamellar bone with mature cortical Haversian systems; reduced eroded surfaces and osteoid surfaces; slightly reduced bone formation rate; decreased cortical thickness and bone volume. |

The common findings in the bone tissue of OI were an increased number of osteocytes (hyperosteoctytosis), thin cortical width, reduced osteoid thickness and increased amounts of woven bone. The thin osteoid has been observed particularly in OI type II bone with known collagen mutations (Cohen-Solal et al 1994; Sztrolovis et al 1994). There is no agreement on the number of osteoblasts or osteoclasts in OI bone.

Common observations in the growth plate of OI were scanty primary spongiosia, a thinner calcified zone, persistence of calcified cartilage in diaphyses and the presence of cartilage fracture calluses.
Aims of this chapter

As the primary manifestation of OI is bone fragility, it is important to perform a detailed histological and ultrastructural study of bone in order to understand this disease properly. In this study 36 OI bone samples representing all OI types and 20 normal age- and site-matched control samples were studied at LM and EM level. Furthermore an attempt has been made to correlate the corresponding pathomorphological findings with the clinically different types of OI and with the biochemical abnormalities of collagen type I (in collaboration with Dr. Michael Pope of the MRC Connective Tissue Genetics Laboratory, Cambridge). Some illustrations of abnormal collagen chemistry are presented in chapter 4.
2.2 Materials and Methods

Patients' details

The clinical classification of OI types II and II/III was carried out by Dr. Michael Pope of the MRC Connective Tissue Genetics Laboratory, Cambridge, and was based on clinical signs, babygram X-rays and collagen biochemistry from cultured skin fibroblasts.

Bone samples from twelve OI type I patients ages ranging from 3 to 42 years, eight type II foetuses ages ranging from 16 weeks of gestation to full term, eight OI type II/III foetuses ages ranging from 18 weeks of gestation to full term, four type III patients ages ranging from 3 to 7 years and four type IV patients ages ranging from 9 to 28 years were used in this study. Patients' details are shown in Tables 2.2 to 2.6.

In addition six normal foetal bone specimens ages ranging from 19 weeks gestation to full term and fourteen normal bone specimens ages ranging from 2 to 25 years were studied.

LM processing

Bone specimens mainly from mid-shaft femur and growth plate area from several OI type II patients were fixed in 4% paraformaldehyde for 48 hours at room temperature. Samples were transferred into neutral ethylene diamine tetraacetic acid (EDTA) solution at room temperature to decalcify, and were X-rayed periodically using a 'Faxitron' X-ray machine to monitor decalcification. When the specimens no longer showed the presence of opaque mineral, they were dehydrated through a graded series of methanol (30-100%), cleared in xylene, and infiltrated with paraffin wax before embedding in wax at 60°C.

Histological sections were cut on a Leitz sledge microtome and 5μm sections floated onto a 50°C water bath. Sections were collected on albumin-coated glass slides and stained with either Erlich's haematoxylin and eosin or alcian blue and sirius red (Junqueira et al 1979). The alcian blue/ sirius red stain shows red-coloured bone collagen or osteoid collagen and blue-coloured cartilage areas.

All LM sections were viewed and relevant areas photographed on an Olympus BH-2 photomicroscope using Fuji Reala 100 ASA colour film or Ilford Pan F 50 ASA black and white film.
Chapter 2

**EM processing**

Bone tissue from OI and normal patients (mostly from mid-shaft femur) was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Agar Scientific Ltd., Essex, England) for 2 hours. During the first hour of fixation, specimens were sub-divided into 1 mm$^3$ pieces. After two buffer washes, the secondary fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer was carried out for one hour at room temperature. The specimens were then dehydrated through an increasing ethanol series, and transferred to propylene oxide (Agar Scientific Ltd.,) prior to infiltration with 1:1 propylene oxide : araldite CY212 resin overnight in the specimen rotator. After two changes of fresh resin, under vacuum (150 mbarr, Anglia Scientific Ltd., Cambridge, England), for a minimum of three hours each, samples were embedded in araldite CY212 resin and blocks were polymerised at 60 °C for 18 hours.

Semi-thin (1μm) sections were cut on a Reichert-Jung Ultracut E ultramicrotome and floated onto distilled water and collected on glass slides. Sections were stained with either 1% toluidine blue in borax or methylene blue and acid fuchsin (Humphrey and Pittman 1974) in order to select suitable areas for TEM. On two occasions semi-thin araldite sections were stained with either Goldner stain (Bancroft and Stevens 1990) to show osteoid collagen or modified Miller's elastin stain to show the abnormal presence of elastin at LM level. Ultra-thin sections were cut using a Diatome diamond knife, floated onto distilled water, collected on formvar-coated copper grids and stained with 2% uranyl acetate and lead citrate for 10 minutes in each solution. The stained sections were viewed on a Philips CM12 electron microscope.
2.3 Results

Morphological and ultrastructural abnormalities of OI bone observed in this study are detailed in Tables 2.2 to 2.6. A summary of these observations is given in Table 2.7 and detailed below.

**OI type I**

At EM level most of the OI type I bone specimens showed fairly well organised osteoid (Figs. 2.2 and 2.3), similar to the normal bone organisation (Fig. 2.1a). Two samples (R.M & G.A) had a disorganised osteoid with collagen fibres of variable diameter (Figs. 2.4 & 2.5). Many patients showed thick organised osteoid with more than two lamellae (Fig. 2.6) while three patients out of twelve had thin osteoid with less than two lamellae (Figs. 2.7 & 2.8). All OI type I osteoblasts contained glycogen (Fig. 2.9), and well developed, distended RER. Two patients demonstrated mitochondrial mineral in the osteoblasts and osteocytes (Figs. 2.10 & 2.11). One patient (R.M) demonstrated fine fibrillar and flocculent material resembling proteoglycans (Fig. 2.12); another patient (C.W) showed collagens of variable diameter (Fig. 2.13). Osteocytes were normal in structure and distribution. Most OI type I samples had lamellar bone.

The collagen biochemistry was tested only in one patient (N.L) who had overmodified α1 and α2 chains of type I collagen. Ultrastructurally these collagen fibres demonstrated a reduced diameter and were contained within a thin osteoid (Fig. 2.7).

**OI type II**

There were a few distinctive features in OI type II bone which were very different from normal age- and site-matched controls.

The most striking feature visible by light microscopy was the abnormal bone formation of the calcified cartilage septae beneath the growth plate. In the normal age- and site-matched controls, bone formation started in the calcified cartilage septae of the primary spongiosa (Fig. 2.14); in contrast, in all the OI type II samples, calcification at this site was absent, and instead started further down the femur (Figs. 2.15 - 2.17). Furthermore there appeared to be defective calcification in the cartilage septae. In normal bone calcified cartilage cores stained deep blue with alcian blue, indicating the presence of proteoglycans (Fig. 2.18a), but in all the OI type II samples this area did not show such a reaction (Fig. 2.18b). Most of the OI type II growth plates showed disorganisation of
cell columns with proteoglycan depletion as judged by the colour reaction to alcian blue. Staining of sections with similar thickness of normal and OI growth plates was carried out on a single slide to enable direct comparisons to be made and to exclude any possible staining artefacts (Figs. 2.18 a & b). All the OI type II femurs showed defective cortical bone formation (Fig. 2.19b) when compared with normals (Fig. 2.19a), and in some samples there was an increased number of osteoclasts (Figs. 2.20a & b). In contrast to normal controls (Fig. 2.21a), all OI type II specimens examined contained immature woven bone as seen under polarized light (Fig. 2.21b).

At EM level, most OI type II samples showed abnormally thinned osteoid with normal mineralisation (Figs. 2.22 & 2.23). The few samples with thicker osteoid were always poorly mineralised. The unmineralised collagen bridges radiating from the mineral matrix suggest uneven mineralisation (Figs. 2.24 & 2.25); these collagen bundles display some similarities to Sharpey's fibres but are unlikely to be Sharpey's fibres since they did not originate from the periosteum and did not show any continuity with ligamentous structures. Patchy mineralisation and mineralised globules unrelated to collagen fibrils were also dispersed in the osteoid (Figs. 2.26 & 2.27). Exceptionally, one specimen (Foetus S) showed proteoglycan-like material in the osteoid (Figs. 2.28 & 2.29), while two specimens (Foetus F and Foetus Me) showed mycoplasma-like bodies around the osteoblasts (Figs. 2.30 & 2.31). Hyperosteocytosis was universal and often there was more than one osteocyte per lacuna (Figs. 2.32 & 2.33). Hyperosteocytosis and conjoined lacunae are features of woven bone.

Six individuals from this OI type II group had abnormal collagen chains, which were either overmodified α1 and α2 chains or double α1 chains. Ultrastructurally collagen fibres were thinner and were sparsely distributed in thin osteoid (Figs. 2.22 - 2.27). In addition, one exceptional individual, whose collagen chains were overmodified with retained pN propeptides, showed a shortened D-periodicity suggesting a major disorganisation of fibril assembly (see chapter 4).

OI type II/III

Paraffin sections of two OI type II/III specimens showed abnormalities similar to those observed in OI type II. The delayed onset of calcification (Figs. 2.34 & 2.35) was similar to that observed in type II samples (Figs. 2.15, 2.16, 2.17) but the cortical bone was much broader than in the type II femurs. With one exception, all the OI type II/III specimens showed many vascular channels and hyperosteocytosis (Figs. 2.38 & 2.39). A mixture of lamellar and woven bone was common (Figs. 2.40 & 2.41).
At EM level, all OI type II/III specimens had thick osteoid (Fig. 2.42), with a peripheral perilacunar unmineralised collagen band and proteoglycans around the osteocytes (Fig. 2.43). This finding is detailed in chapter 7. Some specimens showed irregular collagen fibres of variable diameter (Figs. 2.44 & 2.45).

Collagen biochemistry was available for five patients from this group. Two affected siblings (Ar-II and Ar-III) with normal \( \alpha_1 \) and \( \alpha_2 \) chains had normal collagen fibres. In contrast, two patients with overmodified \( \alpha_1 \) and \( \alpha_2 \) collagen chains showed collagen fibrils of variable diameter (Figs. 2.44 & 2.45). The fifth patient had double \( \alpha_1 \) chains.

**OI type III**

Three out of four OI type III bone specimens showed very similar but striking abnormalities of collagen fibril morphology. All had very thick and disorganised osteoid (Figs. 2.47 - 2.50), which in one case (H.S) resembled Osteomalacia and Fibrogenesis Imperfecta Ossium (FIO). Polarized light microscopy excluded the typical lamellar pattern of Osteomalacia (Ralph *et al* 1985). This individual, who like his cousin has OI type III, may also have FIO. Two other OI type III siblings (H.G and B.G) also had similar collagen fibril disorganisation. The collagen fibres from these patients were spiralled and sparsely distributed throughout a very thick osteoid (Figs. 2.48 & 2.50). In addition the normal appearance of compact longitudinal bundles and cylindrical transverse bundles was altered, and the collagen fibres appeared unravelled in longitudinal sections and irregular, non-cylindrical in transverse sections (Figs. 2.52 - 2.53). One specimen (K.W) showed disorganised osteoid (Fig. 2.51) with fine fibril bundles between the collagen bundles. Some osteoblast mitochondria contained needle-shaped mineral crystals (not shown).

The type I collagen biochemistry was normal in these exceptional patients, although the morphological changes of collagen strongly suggest an abnormality in collagen fibril organisation and assembly.

**OI type IV**

This group of OI patients showed variable thin or thick osteoid, and the osteoblasts contained myelin figures and vacuoles (Figs. 2.54 - 2.56). Three patients demonstrated the abnormal presence of elastin in the osteoid and periosteum (Figs. 2.57 - 2.61).

None of the OI type IV patients was tested for collagen abnormalities.
### Table 2.2  OI Type I microscopic findings and collagen abnormalities.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>LM / EM finding</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. S. A</td>
<td>3 years, male</td>
<td>femur</td>
<td><strong>EM</strong>: fairly thick organised osteoid (Fig. 2.2); lamellar bone; osteoblasts: plump with well developed RER; osteocytes: normal in structure &amp; distribution; osteoclasts: increased.</td>
<td>not tested</td>
</tr>
<tr>
<td>S. V</td>
<td>8 years, male</td>
<td>tibia</td>
<td><strong>EM</strong>: thin but organised (lamellar) osteoid with thin collagen fibres; lamellar bone; osteoblasts: thin lining cells; osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
<tr>
<td>R. M</td>
<td>9 years, male</td>
<td>femur</td>
<td><strong>EM</strong>: fairly disorganised osteoid with variable diameter collagen fibres (Fig. 2.4); woven bone; osteoblasts: plump with well developed RER; granular mitochondrial mineral; glycogen and fine fibrillar bundles; above osteoblast layer, fine fibrillar and flocculent material associated with fibroblasts (Fig. 2.12); osteocytes: large &amp; numerous with dilated RER and mitochondrial mineral (Fig. 2.10); some conjoined lacunae; perilacunar osteoid band of collagen and flocculent material.</td>
<td>not tested</td>
</tr>
<tr>
<td>G. A</td>
<td>10 years, female</td>
<td>tibia</td>
<td><strong>EM</strong>: fairly thick disorganised osteoid (Fig. 2.5); osteoblasts: well developed, distended RER; osteocytes: normal.</td>
<td>not tested</td>
</tr>
<tr>
<td>A. C</td>
<td>10 years, male</td>
<td>iliac crest</td>
<td><strong>EM</strong>: fairly thick organised osteoid (Fig. 2.6); lamellar bone; osteoblasts: thin lining cells; osteocytes: normal.</td>
<td>not tested</td>
</tr>
<tr>
<td>H. M</td>
<td>10 years, female</td>
<td>iliac crest</td>
<td><strong>EM</strong>: normal organised osteoid; lamellar bone; osteoblasts: well developed, distended RER; osteocytes: normal.</td>
<td>not tested</td>
</tr>
<tr>
<td>N. L</td>
<td>12 years, male</td>
<td>iliac crest</td>
<td><strong>EM</strong>: thin but organised osteoid (Fig. 2.7); lamellar bone; osteoblasts: well developed RER; disrupted mitochondria; osteocytes: normal in structure &amp; distribution.</td>
<td>overmodified α1 &amp; α2 chains</td>
</tr>
</tbody>
</table>
### Table 2.2 continued.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>LM / EM finding</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. A</td>
<td>12 years, male</td>
<td>tibia</td>
<td><strong>EM</strong>: fairly thick organised osteoid; lamellar bone; osteoblasts: well developed RER and dense bodies (Fig. 2.8); osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
<tr>
<td>S. D</td>
<td>14 years, female</td>
<td>tibia</td>
<td><strong>EM</strong>: fairly thick organised osteoid (Fig. 2.3); lamellar bone; osteoblasts: thin continuous layer with abundant glycogen deposits (Fig. 2.9) and granular mitochondrial mineral (Fig. 2.11); osteocytes: granular mitochondrial mineral.</td>
<td>not tested</td>
</tr>
<tr>
<td>A. M</td>
<td>16 years, male</td>
<td>iliac crest</td>
<td><strong>EM</strong>: normal organised osteoid; lamellar bone; osteoblasts: plump with well developed, distended RER; osteocytes: normal.</td>
<td>not tested</td>
</tr>
<tr>
<td>C. W</td>
<td>42 years, male</td>
<td>iliac crest</td>
<td><strong>EM</strong>: fairly thick organised osteoid with variable diameter collagen fibres (Fig. 2.13); lamellar bone; osteoblasts: thin lining cells; osteocytes: normal.</td>
<td>not tested</td>
</tr>
<tr>
<td>N. N</td>
<td>not known</td>
<td>not known</td>
<td><strong>EM</strong>: thin but organised osteoid; osteoblasts: abundant distended RER and glycogen; osteocytes: normal in numbers &amp; ultrastructure.</td>
<td>not tested</td>
</tr>
</tbody>
</table>
## Table 2.3 OI Type II microscopic findings and collagen abnormalities.

<table>
<thead>
<tr>
<th>Name &amp; OI sub-type</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>LM / EM finding</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus Mc, type IIA</td>
<td>16 weeks, male</td>
<td>femur &amp; growth plate</td>
<td><strong>LM</strong>: normal growth plate area; paucity of bone in primary spongiosa; <strong>EM</strong>: thin osteoid with thin collagen fibres; unmineralised collagen bundles (Fig. 2.25); <strong>osteoblasts</strong>: well developed, distended RER; mycoplasma-like bodies around the osteoblasts (Fig. 2.31); <strong>osteocytes</strong>: many but normal in structure.</td>
<td>overmodified $\alpha_1$ and $\alpha_2$ chains</td>
</tr>
<tr>
<td>Baby Ko, type IIA</td>
<td>perinatal, male</td>
<td>femur &amp; growth plate</td>
<td><strong>LM</strong>: normal growth plate area; paucity of bone in primary spongiosa; calcified cartilage septae look normal; <strong>EM</strong>: disorganised osteoid with thin collagen fibres; <strong>osteoblasts</strong>: well developed, distended RER; <strong>osteocytes</strong>: normal.</td>
<td>overmodified $\alpha_1$ and $\alpha_2$ chains</td>
</tr>
<tr>
<td>Foetus F, type IIB</td>
<td>19 weeks, female</td>
<td>femur &amp; growth plate</td>
<td><strong>LM</strong>: stunted growth plate area; paucity of bone in primary spongiosa (Fig. 2.15); cartilaginous fracture callus in the femoral shaft; broader femoral shaft; thinner cortical bone due to incomplete mineralisation (Fig. 2.19b); persistence of calcified cartilage in the diaphysis; woven bone (Fig. 2.21b); <strong>EM</strong>: femur, thin osteoid with thinner collagen fibres (Fig. 2.22); <strong>osteoblasts</strong>: highly vacuolated with distended RER; mycoplasma-like bodies around the cells (Fig. 2.30); <strong>osteocytes</strong>: increased in number (Fig 2.33) with distended Golgi and RER; <strong>osteoclasts</strong>: increased.</td>
<td>overmodified $\alpha_1$ chains</td>
</tr>
<tr>
<td>Name &amp; OI sub-type</td>
<td>Age &amp; sex</td>
<td>Site of biopsy</td>
<td>LM / EM finding</td>
<td>Collagen abnormality</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------</td>
<td>----------------</td>
<td>-----------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Foetus Ki, type IIc</td>
<td>16 weeks, female</td>
<td>rib, femur &amp; growth plate</td>
<td>LM: growth plate, two cartilaginous fracture calluses in the femoral shaft; thinner femoral shaft; persistence of cartilage in the diaphysis; gaps in mineralisation in femoral shaft; woven bone; EM: thin osteoid with thin collagen fibres; patchy mineralisation (Figs. 2.26 &amp; 2.27); osteoblasts: plump with well developed RER and swollen mitochondria (Fig 2.27); osteocytes: normal; osteoclasts: increased.</td>
<td>double α₁ and α₂ chains</td>
</tr>
<tr>
<td>Foetus S, type IIc</td>
<td>22 weeks, male</td>
<td>femur &amp; growth plate</td>
<td>LM: defective columnar organisation of growth plate area; paucity of bone in the metaphyses (Fig. 2.16); narrow femoral shaft; cartilaginous fracture calluses in the femoral shaft; increased number of large osteocytes; woven bone; EM: osteoid with thin collagen fibres; PG-like material in the osteoid (Fig. 2.28); osteoblasts: vacuolated with needle-shaped mitochondrial mineral (Fig. 2.29). osteocytes: increased in number (Fig. 2.32) with distended RER; osteoclasts: increased (Figs. 2.20 b).</td>
<td>not tested</td>
</tr>
<tr>
<td>Foetus T-VI, type IIc</td>
<td>23 weeks, male</td>
<td>femur</td>
<td>EM: disorganised osteoid with thin collagen fibres; patchy mineralisation; osteoblasts: well developed, distended RER; osteocytes: normal in structure but increased in number.</td>
<td>modified α₁ and α₂ chains</td>
</tr>
<tr>
<td>Name &amp; OI sub-type</td>
<td>Age &amp; sex</td>
<td>Site of biopsy</td>
<td>LM / EM finding</td>
<td>Collagen abnormality</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------</td>
<td>----------------</td>
<td>------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Foetus D, sub-type not known</td>
<td>22 weeks, not stated</td>
<td>EM; thin osteoid with thin collagen fibres; osteoblasts: well developed RER and glycogen; osteocytes: normal in structure.</td>
<td>not tested</td>
<td></td>
</tr>
<tr>
<td>Foetus W, sub-type not known</td>
<td>25 weeks, female</td>
<td>femur &amp; growth plate</td>
<td>LM: shorter growth plate area; paucity of bone in the metaphyses (Fig. 2.17 &amp; Fig.18b); EM: patchy mineralisation; unmineralised collagen bridges radiating from mineralised matrix (Fig. 2.24); osteoblasts: highly vacuolated &amp; necrotic (Fig. 2.23); osteocytes: highly vacuolated &amp; increased in number. osteoclasts: increased (Fig. 2.20a).</td>
<td>overmodified α1 and α2 chains, retained pN propeptides.</td>
</tr>
<tr>
<td>Name</td>
<td>Age &amp; sex</td>
<td>Site of biopsy</td>
<td>LM / EM finding</td>
<td>Collagen abnormality</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>----------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Foetus O</td>
<td>18 weeks, male</td>
<td>LM: femur &amp; growth plate; EM: rib</td>
<td>LM: normal growth plate area; paucity of bone in primary spongiosa; gaps in mineralisation in femoral shaft. EM: patchy mineralisation with abnormal unmineralised collagen bundles radiating from the mineralised matrix (Fig. 2.37); woven bone; osteoblasts: well developed, distended RER and Golgi; osteocytes: hyperostocytosis; conjoined lacunae (3-4 osteocytes in one lacuna); some with well developed RER and Golgi.</td>
<td>not tested</td>
</tr>
<tr>
<td>Foetus Ar-II</td>
<td>20 weeks, male</td>
<td>femur or tibia</td>
<td>LM: many vascular channels and osteocytes (Fig. 2.39). EM: fairly thick osteoid (Fig. 2.42); osteoblasts: well developed RER; osteocytes: many osteocytes with perilacunar band of osteoid-like collagen and PG-like material in the lacunae (Fig. 2.43).</td>
<td>normal α1 and α2 chains</td>
</tr>
<tr>
<td>Foetus Ar-III</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>LM: many vascular channels and osteocytes; EM: fairly thick osteoid; osteoblasts: well developed, distended RER; osteocytes: many with perilacunar band of osteoid-like collagen and PG-like material in the lacunae.</td>
<td>normal α1 and α2 chains</td>
</tr>
<tr>
<td>Foetus F. G, sibling of G-III (below)</td>
<td>22 weeks, female</td>
<td>rib</td>
<td>EM: fairly thick osteoid with collagen of variable diameter (Fig. 2.44); osteoblasts: well developed RER; osteocytes: perilacunar band of osteoid-like collagen and PG-like material in the lacunae.</td>
<td>overmodified α1 and α2 chains</td>
</tr>
</tbody>
</table>
Table 2.4 continued.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>LM / EM finding</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus Gr</td>
<td>22 weeks, male</td>
<td>rib</td>
<td><strong>EM</strong>: fairly thick osteoid; osteoblasts: glycogen and distended RER; osteocytes: surrounded by perilacunar unmineralised band of collagen &amp; PG-like material in some lacunae.</td>
<td>not tested</td>
</tr>
<tr>
<td>Foetus Wi</td>
<td>25 weeks, female</td>
<td>femur &amp; growth plate</td>
<td><strong>LM</strong>: shorter growth plate area; paucity of bone in the metaphyses; four cartilaginous fracture calluses in the femoral shaft (Fig. 2.34); thinner femoral shaft &amp; periosteum; persistence of cartilage in the diaphysis; mixture of lamellar &amp; woven bone (Fig. 2.40); osteoblasts: normal; osteocytes: increased (Fig. 2.38); osteoclasts: increased.</td>
<td>double α1 chains</td>
</tr>
<tr>
<td>Baby G-III, sibling of F.G (above)</td>
<td>full term, female</td>
<td>tibia</td>
<td><strong>EM</strong>: fairly thick unorganised osteoid with variable diameter collagen fibres (Fig. 2.45); patchy mineralisation with unmineralised collagen bundles radiating from the mineralised matrix; osteoblasts: well developed RER; osteocytes: large; sometimes two in one lacuna.</td>
<td>over modified α1 and α2 chains</td>
</tr>
<tr>
<td>Baby V. D. K</td>
<td>full term, male</td>
<td>femur &amp; growth plate</td>
<td><strong>LM</strong>: shorter growth plate area; paucity of bone in the metaphyses; thinner femoral shaft &amp; periosteum (Figs. 2.35 &amp; 2.36); persistence of cartilage in the diaphysis; mixture of lamellar &amp; woven bone (Fig. 2.41); osteoblasts: normal; osteocytes: increased; osteoclasts: increased.</td>
<td>not tested</td>
</tr>
<tr>
<td>Name</td>
<td>Age &amp; sex</td>
<td>Site of biopsy</td>
<td>LM / EM finding</td>
<td>Collagen abnormality</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>----------------</td>
<td>---------------------------------------------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>H. S</td>
<td>3 years, male</td>
<td>femur</td>
<td>EM: very thick osteoid (Fig. 2.47) with abnormal collagen fibres sparsely distributed; occasional normal clusters of collagen; majority of collagen fibres spiralled (Figs. 2.49 &amp; 2.53); osteoblasts: thin continuous layer; abundant glycogen; osteocytes: normal in structure and distribution.</td>
<td>normal α1 and α2 chains</td>
</tr>
<tr>
<td>B. G, sibling of H.G (below)</td>
<td>3 years, male</td>
<td>not known</td>
<td>EM: thick osteoid with abnormal collagen fibres sparsely distributed; spiralled collagen fibres (Figs. 2.50 &amp; 2.52); flocculent material between the collagen fibres; patchy mineralisation; osteoblasts: thin continuous layer; abundant glycogen and RER; osteocytes: normal in structure and distribution.</td>
<td>normal α1 and α2 chains</td>
</tr>
<tr>
<td>H. G, sibling of B.G (above)</td>
<td>6 years, female</td>
<td>femur</td>
<td>EM: thick osteoid with abnormal collagen fibres sparsely distributed (Fig. 2.48); majority of collagen fibres spiralled; occasional normal clusters of collagen; osteoblasts: thin continuous layer; abundant glycogen; osteocytes: normal in structure &amp; distribution.</td>
<td>normal α1 and α2 chains</td>
</tr>
<tr>
<td>K. W</td>
<td>7 years, female</td>
<td>femur</td>
<td>EM: thick; unorganised osteoid; osteoblasts: abundant glycogen &amp; well developed RER (Fig. 2.51); needle-shaped mitochondrial mineral; osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
</tbody>
</table>
### Table 2.6  OI Type IV microscopic findings and collagen abnormalities.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>LM / EM finding</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>J. D</td>
<td>9 years, male</td>
<td>femur</td>
<td>EM: thick organised osteoid (Fig. 2.56); abundant elastin in clusters in the periosteum (Figs. 2.57 - 2.59); lamellar bone; osteoblasts: well developed RER (Fig. 2.56); osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
<tr>
<td>Z. A</td>
<td>14 years, female</td>
<td>iliac crest</td>
<td>EM: organised osteoid (Fig. 2.55); lamellar bone; osteoblasts: distended RER; swollen mitochondria and vesicles (Fig. 2.55); osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
<tr>
<td>M. K</td>
<td>25 years, female</td>
<td>tibia</td>
<td>EM: thin osteoid (Fig. 2.54) with a mixture of collagen and elastin (Figs. 2.61 a &amp; b); osteoblasts: long &amp; thin in some areas; osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
<tr>
<td>K. L</td>
<td>28 years, male</td>
<td>not known</td>
<td>EM: thin and unorganised osteoid with collagen and elastin (Figs. 2.60 a &amp; b); osteoblasts: degenerative with myelin figures and vacuoles (Fig. 2.60a); osteocytes: normal in structure &amp; distribution.</td>
<td>not tested</td>
</tr>
</tbody>
</table>
Table 2.7  Summary of morphological and ultrastructural observations according to type of OI.

<table>
<thead>
<tr>
<th>OI type</th>
<th>LM</th>
<th>EM</th>
<th>osteoblasts</th>
<th>osteoclasts</th>
<th>osteocytes</th>
<th>osteoid</th>
<th>PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>type I</td>
<td>normal lamellar bone</td>
<td>RER-distended; Mit. swollen; 2/12- Mit. mineral &amp; glycogen</td>
<td>normal</td>
<td>increased</td>
<td>10/12-organised, 2/12-disorganised; 9/12- thick; 3/12- thin; 1/12-variable diameter collagens</td>
<td>1/12</td>
<td>+ve</td>
</tr>
<tr>
<td>type II</td>
<td>woven bone; calcification of GP delayed; cartilage fracture calluses; discontinuous trabecular bone; thin cortical bone</td>
<td>vacuolated; some necrotic; dilated RER</td>
<td>increased</td>
<td>increased</td>
<td>thin and disorganised; patchy mineralisation</td>
<td>1/8</td>
<td>+ve</td>
</tr>
<tr>
<td>type II/III</td>
<td>mixture of woven and lamellar bone</td>
<td>RER-distended; Mit. swollen</td>
<td>increased</td>
<td>increased, perilacunar unmineralised band</td>
<td>thick and disorganised; 2/8 - variable diameter collagens</td>
<td>5/8</td>
<td>+ve</td>
</tr>
<tr>
<td>type III</td>
<td>mixture of woven and lamellar bone</td>
<td>RER-distended; Mit. swollen 1/4-Mit. mineral &amp; glycogen</td>
<td>normal</td>
<td>increased</td>
<td>thick and disorganised; 3/4 - abnormal structure &amp; distribution of collagen; patchy mineralisation</td>
<td>-ve</td>
<td></td>
</tr>
<tr>
<td>type IV</td>
<td>lamellar bone</td>
<td>necrotic with vesicles, vacuoles &amp; myelin figures</td>
<td>normal</td>
<td>normal</td>
<td>3/4 - elastin present</td>
<td>-ve</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: GP = growth plate; Mit. = mitochondria; PG = proteoglycan; RER = rough endoplasmic reticulum.
Fig. 2.1a  The thick osteoid from a normal juvenile control bone specimen (F.D, 10 years) showing the normal lamellar organisation of collagen bundles which are arranged in alternating longitudinal and transverse layers.
L.C- longitudinal collagen bundles, MM- mineralised matrix,
T.C-transverse collagen bundles.
Bar = 1.75 μm

Fig. 2.1b  A plump osteoblast from a normal control bone specimen (F.D, 10 years) showing mitochondria, nucleus and well developed RER which is not distended.
Bar = 1 μm
Fig. 2.2  The osteoid from an OI type I patient (B.S.A, 3 years) showing normal thick osteoid with lamellar organisation of collagen bundles, plump osteoblasts with swollen mitochondria and well developed RER. Note the normal, gradual, dynamic mineralisation front.
Bar = 1.75 µm

Fig. 2.3  The osteoid from an OI type I patient (S.D, 14 years) showing a thick osteoid with lamellar organisation of collagen bundles with gradual and normal mineralisation. The osteoblasts show swollen mitochondria and well developed, distended RER. Note some RER vesicles contain flocculent material.
Bar = 1.52 µm
Fig. 2.4  The osteoid from an OI type I patient (R.M, 9 years) showing a disorganised non-lamellar osteoid and osteoblasts with well developed, distended RER. Bar = 1.75 μm

Fig. 2.5  The osteoid from an OI type I patient (G.A, 10 years) showing a thick disorganised osteoid and a pre-osteocyte buried in the osteoid collagen. Bar = 1.75 μm
Fig. 2.6 The osteoid from an OI type I patient (A.C, 10 years) showing a thick osteoid with lamellar organisation of collagen bundles. Long and thin osteoblasts line the osteoid. Note the static mineralisation front which may be due to inactive osteoblasts with very few cell processes and matrix vesicles.
Bar = 1.38 μm

Fig. 2.7 The osteoid from an OI type I patient (N.L, 12 years) showing thin but organised osteoid. Osteoblasts have well developed RER and disrupted mitochondria; osteocytes also have disrupted mitochondria.
Bar = 1.75 μm
Fig. 2.8 The osteoid from an OI type I patient (A.A, 12 years) showing thin osteoid and osteoblasts containing well developed, distended RER. Note the dense bodies in the cisternae of the RER. 
Bar = 1.05 μm

Fig. 2.9 Osteoblasts from an OI type I patient (S.D, 14 years) showing abundant glycogen deposits and mineral granules in the mitochondria. 
Bar = 1.75 μm
Fig. 2.10  An osteocyte from an OI type I patient (R.M, 9 years) containing spherical mitochondrial mineral nodules.  
Bar = 1.05 μm

Fig. 2.11  An osteoblast from an OI type I patient (S.D, 14 years) containing spherical mitochondrial mineral nodules.  
Bar = 830 nm
Fig. 2.12 The osteoid from an OI type I patient (R.M, 9 years) showing disorganised osteoid and fine fibrillar and flocculent material resembling proteoglycans. Bar = 1.38 µm

Fig. 2.13 Osteoid collagen from an OI type I patient (C.W, 42 years) showing variable diameter collagens. Bar = 110 nm
Fig. 2.14 Longitudinal paraffin wax section of proximal femur from a normal foetus (Foetus Gy, 19 weeks) stained with alcian blue and sirius red showing epiphyseal cartilage with solid calcified cartilage septae and bony trabeculae in the primary spongiosa.

x 132

Fig. 2.15 Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus F, 19 weeks) stained with alcian blue and sirius red showing epiphyseal cartilage with thin calcified cartilage septae and the absence of bony trabeculae.

x 132
Fig. 2.16  Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus S, 22 weeks) stained with alcian blue and sirius red showing irregular cell columns, thin calcified cartilage septae and the absence of bone trabaculae when compared with Fig. 2.14.

x 132

Fig. 2.17  Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus W, 25 weeks) stained with alcian blue and sirius red showing irregular cell columns, thin calcified cartilage septae and the absence of bone trabeculae.

x 132
Fig. 2.18a  Longitudinal paraffin wax section of proximal femur from a normal foetus (Foetus Gy, 19 weeks) stained with alcian blue and sirius red showing an intense blue colour for proteoglycans and dark red-coloured bony trabeculae. (This section was mounted and stained on a glass slide along with the section shown in Fig. 2.18b).  
\[x\times 53\]

Fig. 2.18b  Longitudinal paraffin wax section of proximal femur from an OI type II foetus (Foetus W, 25 weeks) stained with alcian blue and sirius red showing a paler blue colour for proteoglycans and an absence of red-coloured bony trabeculae compared with normal bone stained simultaneously (to rule out any artefacts of staining) and shown above.  
\[x\times 53\]
Chapter 2

2.18 a

2.18 b
Fig. 2.19a  Transverse paraffin wax section of a normal foetal mid-shaft femur (Foetus Gy, 19 weeks) stained with alcian blue and sirius red showing thick cortical bone and continuous trabecular bone. 

x 53

Fig. 2.19b  Transverse paraffin wax section of an OI type II foetal mid-shaft femur (Foetus F, 19 weeks) stained with alcian blue and sirius red showing thin discontinuous cortical bone and trabecular bone. 

x 53
Chapter 2

2.19 a

2.19 b
Fig. 2.20a  Longitudinal paraffin wax section of femoral bone from an OI type II foetus (Foetus W, 25 weeks) stained with alcian blue and sirius red. Note that there are at least 12 blue-stained multinucleated osteoclasts in this field indicating rapid bone resorption.

x 264

Fig. 2.20b  Longitudinal paraffin wax section of femoral bone from an OI type II foetus (Foetus S, 22 weeks) stained with alcian blue and sirius red, showing increased numbers of osteoclasts in this field.

x 264
Fig. 2.21a Transverse paraffin wax section of a normal foetal mid-shaft femur (Foetus Gy, 19 weeks) stained with alcian blue and sirius red and viewed under polarized light, showing the regular lamellar organisation of bone.

x 132

Fig. 2.21b Transverse paraffin wax section of an OI type II foetal mid-shaft femur (Foetus F, 19 weeks) stained with alcian blue and sirius red and viewed under polarized light, showing a lack of the normal polarization pattern and indicating the presence of woven bone.

x 132
Chapter 2

2.21a

2.21b
Fig. 2.22 Ultra-thin araldite section of an OI type II bone (Foetus F, 19 weeks) showing thin osteoid and osteoblasts with well developed RER. Note the static mineralisation front.
Bar = 1.75 μm

Fig. 2.23 Ultra-thin araldite section of an OI type II bone (Foetus W, 25 weeks) showing thin osteoid and necrotic osteoblasts. Note the static mineralisation front.
Bar = 1.38 μm
Fig. 2.24 Ultra-thin araldite section of an OI type II bone (Foetus W, 25 weeks) showing unmineralised collagen bundles with irregular patches of mineral.
Bar = 1.75 μm

Fig. 2.25 Ultra-thin araldite section of an OI type II bone (Foetus Mc, 16 weeks) showing unmineralised collagen bundles radiating from the mineral matrix. These bundles are unlikely to be Sharpey's fibres because there is no evidence of continuous ligamentous structures containing fibrous collagen even at LM level (see Fig. 2.37).
Bar = 5.2 μm
Fig. 2.26 Ultra-thin araldite section of an OI type II bone (Foetus Ki, 16 weeks) showing a gap in the femoral shaft due to incomplete mineralisation. There appears to be a failure of mineral nodules to grow and mineralise the collagenous matrix. Bar = 1.75 μm

Fig. 2.27 Ultra-thin araldite section of an OI type II bone (Foetus Ki, 16 weeks) showing thin osteoid and patchy mineralisation with mineral nodules dispersed in the osteoid. The osteoblasts have swollen mitochondria and RER. Bar = 1.75 μm
Fig. 2.28 Ultra-thin araldite section of an OI type II bone (Foetus S, 22 weeks) showing necrotic cells, a layer of thin collagens, and overlying fine fibrillar material resembling proteoglycans.
Bar = 1.75 μm

Fig. 2.29 Ultra-thin araldite section of an OI type II bone (Foetus S, 22 weeks) shown at a higher magnification than in Fig. 2.28. Note the fine fibrillar and particulate material resembling proteoglycans. The necrotic osteoblast contains needle-shaped mineral crystals and distended RER.
Bar = 500 nm
Fig. 2.30  Ultra-thin araldite section of an OI type II bone (Foetus F, 19 weeks) showing a necrotic osteoblast with mycoplasma-like bodies around it.  
Bar = 1.75 μm  

Fig. 2.31  Ultra-thin araldite section of an OI type II bone (Foetus Mc, 16 weeks) showing a necrotic osteoblast with mycoplasma-like bodies around it.  
Bar = 1 μm
Fig. 2.32 Ultra-thin araldite section of an OI type II bone (Foetus S, 22 weeks) showing hyperosteocytosis with more than one osteocyte per lacuna. 
Bar = 5.2 μm

Fig. 2.33 Ultra-thin araldite section of an OI type II bone (Foetus F, 19 weeks) showing hyperosteocytosis with more than one osteocyte per lacuna. 
Bar = 3.8 μm
Fig. 2.34  Paraffin wax section of proximal femur from an OI type II/III foetus (Foetus Wi, 25 weeks) stained with alcian blue and sirius red showing paucity of bone in the primary spongiosa and thin femoral shaft. Note the disorganised bone trabaculae and small islands of cartilage-type material which are stained blue and may be fracture calluses.

x 13.2

Fig. 2.35  Paraffin wax section of proximal femur from an OI type II/III baby (Baby V.D.K, full term) stained with alcian blue and sirius red showing paucity of bone in the primary spongiosa, scanty thin trabeculae and thin femoral shaft.

x 13.2
Fig. 2.36  Paraffin wax section of proximal femur from an OI type II/III baby (Baby V.D.K, full term) stained with alcian blue and sirius red shown at a higher magnification than in Fig. 2.35. Note the irregular cell columns, paucity of bone in the primary spongiosa and scanty thin trabeculae.  
X 53

Fig. 2.37  Semi-thin araldite section of bone from an OI type II/III foetus (Foetus O, 18 weeks) stained with methylene blue/azur II and basic fuchsin showing unmineralised collagen bridges between the bone spicules (arrowed). These do not appear to be Sharpey's fibres because there is no evidence of continuous ligamentous structures with fibrous collagen.  
X 500
Fig. 2.38  Paraffin wax section of proximal femur from an OI type II/III foetus (Foetus Wi, 25 weeks) stained with alcian blue and sirius red showing many vascular channels and hyperosteocytosis.

x 152

Fig. 2.39  Semi-thin araldite section of mid-shaft femur from an OI type II/III foetus (Foetus Ar-II, 20 weeks) stained with Goldner stain showing hyperosteocytosis and many vascular channels. Note the red-coloured collagen in the periphery of the lacunae and around the vascular channels, which was subsequently found to be an unmineralised band of collagen bundles.

x 152
Fig. 2.40 Paraffin wax section of proximal femur from an OI type II/III foetus (Foetus Wi, 25 weeks) stained with alcian blue and sirius red and viewed under polarized light, showing a mixture of woven and lamellar bone.

x 132

Fig. 2.41 Transverse paraffin wax section of mid-shaft femur from an OI type II/III baby (Baby V.D.K, full term) stained with alcian blue and sirius red and viewed under polarized light, showing a mixture of woven and lamellar bone.

x 132
Fig. 2.42  Electron micrograph of a section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing disorganised thick osteoid with mineral nodules and incomplete mineralisation.
Bar = 1.75 μm

Fig. 2.43  Electron micrograph of a section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing an osteocyte lacuna with perilacunar, unmineralised collagen band and proteoglycans around the periphery of the cell.
CO = collagen, MM = mineral matrix, OC = osteocyte, PG = proteoglycan
Bar = 830 nm
Fig. 2.44  Electron micrograph of a section of an OI type II/III foetal rib bone (Foetus F.G, 22 weeks) showing osteoid collagen of variable diameter.
Bar = 110 nm

Fig. 2.45  Electron micrograph of a section of an OI type II/III tibial bone (Baby G-III, full term) showing osteoid collagen of variable diameter.
Bar = 110 nm
Fig. 2.46 Photomicrograph of a resin section of a normal bone (S.N.D, 2 years) stained with Goldner stain showing thin red-coloured osteoid rim around the vascular channels, and lamellar organisation.

x300

Fig. 2.47 Photomicrograph of a resin section of an OI type III bone (H.S, 3 years) stained with Goldner stain showing very thick osteoid.

x300
Fig. 2.48  Electron micrograph of bone osteoid from an OI type III patient (H.G, 6 years) showing unusually thick osteoid with sparsely distributed abnormal collagen fibres and patchy mineralisation. Thin and long osteoblasts line the osteoid.  
Bar = 1.75 μm

Fig. 2.49  Electron micrograph of bone osteoid from an OI type III patient (H.S, 3 years) showing unusually thick osteoid with sparsely distributed abnormal collagen fibres.  
Bar = 1.05 μm
Fig. 2.50  Electron micrograph of bone osteoid from an OI type III patient (B.G, 3 years) showing unusually thick osteoid with sparsely distributed abnormal collagen fibres and patchy mineralisation.
Bar = 1.05 μm

Fig. 2.51  Electron micrograph of bone osteoid from an OI type III patient (K.W, 7 years) showing disorganised osteoid with patchy mineralisation and an osteoblast with distended RER.
Bar = 1.75 μm
Fig. 2.52  Electron micrograph of bone osteoid from an OI type III patient (B.G, 3 years) shown at a higher magnification than in Fig. 2.50. Note the spiralled collagen with irregular edges (arrowed). Bar = 380 nm

Fig. 2.53  Electron micrograph of bone osteoid from an OI type III patient (H.S, 3 years) shown at a higher magnification than in Fig. 2.49. Note the spiralled collagen with irregular edges (arrowed). Bar = 300 nm
Fig. 2.54  Electron micrograph of bone osteoid from an OI type IV patient (M.K, 25 years) showing thin osteoid with patchy mineralisation. The osteoblasts contain well developed RER and myelin figures.
Bar = 1.75 μm

Fig. 2.55  Electron micrograph of bone osteoid from an OI type IV patient (Z.A, 14 years) showing an organised osteoid and necrotic osteoblasts with well developed, distended RER and vacuoles (arrowed).
Bar = 1.75 μm
Fig. 2.56  Electron micrograph of bone osteoid from an OI type IV patient (J.D, 9 years) showing a thick osteoid with a pre-osteocyte buried in the collagen. Osteoblasts have well developed, distended RER and vacuoles.  
Bar = 1.38 μm

Fig. 2.57  Photomicrograph of a resin section of bone from an OI type IV patient (J.D, 9 years) stained with Miller's elastin stain showing abnormal aggregation of elastin in the periosteum.  
x 53
Fig. 2.58  Electron micrograph of a resin section of bone from an OI type IV patient (J.D, 9 years) showing abnormal aggregation of elastin (arrowed) in the periosteum.  
Bar = 5.2 µm

Fig. 2.59  Electron micrograph of a resin section of bone from an OI type IV patient (J.D, 9 years). The arrowed area in Fig. 2.58 is enlarged to show the ultrastructural appearance of elastin.  
Bar = 520 nm
Fig. 2.60a  Electron micrograph of bone osteoid from an OI type IV patient (K.L, 28 years) showing thin osteoid. The osteoblasts show degenerative changes such as disrupted mitochondria and myelin figures, and the abnormal presence of elastin between collagen fibres.
Bar = 1.6 μm

Fig. 2.60b  Electron micrograph of bone osteoid from an OI type IV patient (K.L, 28 years) shown at a higher magnification than in Fig. 2.60a to show the disorganised collagen fibres and elastin.
Bar = 380 nm
Fig. 2.61a  Electron micrograph of bone osteoid from an OI type IV patient (M.K., 25 years) showing thin osteoid and the abnormal presence of elastin in the osteoid between sparsely distributed collagen fibres.
Bar = 1.75 μm

Fig. 2.61b  Electron micrograph of bone osteoid from an OI type IV patient (M.K., 25 years) shown at a higher magnification than in Fig. 2.61a to show the ultrastructural appearance of elastin.
Bar = 790 nm
2.4 Discussion

In this kind of investigative electron microscopy, one needs to be extremely cautious in interpreting the significance of various observations. For example, the microscopic appearance will vary with anatomical site and artefactual distortions may be introduced during specimen preparation. To minimise these possibilities, a uniform biopsy site was used whenever possible, and all biopsies were treated identically under the stringent requirements necessary to maintain the cellular and extracellular structures in a state as close to the \textit{in vivo} situation as possible. Age- and site-matched normal controls were also obtained for comparison.

Morphological and ultrastructural features of thirty six patients of all OI types are presented here. There are clear correlations of structural morphology with clinical phenotype. Thus, OI type I, the mildest form of OI, showed the fewest abnormalities in bone ultrastructure, with normal lamellar bone, normally organised osteoid with normal mineralisation pattern and fairly normal bone cells. OI type IV closely resembled type I with only minor abnormalities in the bone cells and osteoid. OI type III showed abnormalities in the three dimensional organisation and distribution of collagen, which plays an important role in bone structure. Not surprisingly OI type II and II/III, which are either lethal or severely crippling, revealed many varied abnormalities. OI type II had thin bone osteoid with collagen fibres sparsely distributed and thin in diameter, defective bone formation in the growth plate, and paucity of bone with patchy mineralisation.

OI osteoblasts from all groups showed certain common characteristics such as swollen mitochondria, distended RER and Golgi, sometimes with many vesicles and dense bodies in the RER. This is consistent with reported abnormalities with ER retention of both mutant and over modified type I collagen (Byers 1990; Rowe and Shapiro 1990). Barsh and Byers (1981) suggested that interruption of the triple helix decreases the rate of secretion of abnormal molecules from the cell, leading to dilation of the RER. Some osteoblasts contained large amounts of glycogen which displaced other normal cellular organelles. Similar changes have previously been reported by Doty and Mathews (1971). The osteoblasts' size varied from specimen to specimen; in some they were plump whilst in others they were long and thin. In a few patients granular and needle-shaped mitochondrial mineral nodules were observed in the osteoblasts. This finding was previously reported by Kjaer and Matthiessen (1975) and Cassella \textit{et al} (1996). Furthermore Cassella \textit{et al} (1996) analysed these crystals by electron probe X-ray microanalysis and found the ratio of Ca/P was 1.100 when compared with the normal hydroxyapatite ratio of 1.602. They suggested that the presence of mitochondrial mineral was an indication of metabolic imbalance in these cells.
In the present study the number of osteoblasts appeared to be diminished in OI type II; the cells did not form a continuous uniform layer covering the osteoid surfaces whereas in other OI types there was a continuous layer of either thin or plump osteoblasts covering the osteoid surfaces. To date there is no agreement as to the relative osteoblast or osteoclast numbers in OI bone. According to some authors osteoblast numbers are decreased (Riley et al. 1973), but according to others they are increased (Falvo and Bullough 1973; Ornoy and Kim 1977; Baron et al. 1979). The diminished number of osteoblasts observed in this study may partially explain the impairment of osteoid formation in OI type II. The increased number of osteoclasts observed in most of the type II bone samples (especially type IIC - thin bone type) implies there is increased bone resorption; alternatively the number of osteoclasts may be normal but there is less bone to resorb, which gives the artificial impression of increased numbers. Our observations imply that there are relatively higher numbers of osteoclasts per unit surface area of bone in OI type II.

There is general agreement that osteocytes are either normal or increased in OI bone (Doty and Mathews 1971; Falvo and Bullough 1973). Hyperosteoctytosis may be caused by matrix depletion rather than by increased cellular proliferation. Mullender et al. (1996) reported significantly higher lacuna and osteocyte numbers in osteoporotics compared with controls, and speculated that osteoblasts produce less bone per cell. In the present study there was consistent hyperosteoctytosis in OI type II and type II/III bone. Osteocytes were enlarged, sometimes containing two to three cells in one lacuna. This could be due to the lack of mineralised matrix around the individual osteocytes. This suggestion is supported by the observation that some specimens contained unmineralised septae between the osteocytes.

In this study all OI type II foetal bone samples showed excessive immature woven bone whilst normal age-matched controls had mature lamellar bone organisation. Type II/III and type III OI samples showed mixtures of woven and lamellar bone but type I bone always showed normal lamellar bone organisation. Glorieux and Travers (1994) have described a similar pattern in OI types I, III and IV.

The patchy mineralisation observed by various authors in OI type II (Cohen-Solal et al. 1994, Traub et al. 1994) was a consistent feature of this study. Most OI type II femoral shafts showed patchy mineralised matrix and sparse cortical bone formation. Furthermore bone osteoid contained isolated mineral nodules. Landis (1995) proposed a hypothesis on mineralisation of bone in the oim/oim mouse, which resembles moderately severe OI. In the oim/oim mouse, collagen assembly in parts of the tissue was shown to be disorganised, many collagen fibrils were twisted and kinked, and the characteristic 67 nm D-period was out of register across some adjacent fibrils. Landis suggested that, in normal calcified tissue, collagen fibrils are arranged in a strictly ordered manner to
generate extensive channels and grooves. The nucleation of mineral occurs initially in the
hole zones and extended mineral sheets (developing in length and parallel to one another
in neighbouring collagen layers) eventually fuse together in the same or adjacent collagen
layers to form a thicker and broader collagen-mineral composite. In pathological
examples such as OI, the assembly of collagen molecules is disturbed resulting in
irregular hole and overlap zones, which are out of register, and consequently there are
limited channel spaces for the crystal growth. The resultant crystals are irregular in size,
shape, alignment and orientation within the collagen layers. This type of collagen-mineral
composite will be sparse, weak and fragile.

The histological findings of OI type II bone samples presented here were always
different from normal age- and site-matched controls. The failure of bone formation in
primary spongiosa and the persistence of calcified cartilage in the diaphyses observed in
this study has been previously reported by Bullough et al (1981), Cole et al (1992) and
Marion et al (1993). The defective calcification in the cartilage septae and depletion of
proteoglycans in the proliferating regions in the growth plates, as judged by the colour
reaction of alcian blue, are new observations. As alcian blue shows an intense blue colour
in the presence of salts, future work should clarify whether the paler colour observed for
OI type II specimens is due to the absence of mineral salts or represents an actual
depletion of proteoglycans.

The abnormal collagen fibres consistently observed here in a subset of OI type III bone
are also important as paradoxically these patients did not have any detectable
biochemical abnormalities in collagen type I, and yet their bone collagen was
ultrastructurally abnormal. In other type III children, from whom bone samples were
unavailable for EM studies, collagen chemistry is often abnormal (Dr.Michael Pope -
personal communication). In the OI type III bone specimens studied here, collagen fibres
had irregular edges and in most no clear periodicity was observed. The fibres were also
spiralled in transverse section whilst in longitudinal section they appeared unravelled and
had lost their normal compactness. Furthermore they were sparsely distributed
throughout a very thick osteoid. A similar disorganisation is characteristic of certain
Ehlers Danlos Syndrome (EDS) subsets (Vogel et al 1979) and of Fibrogenesis
Imperfecta Ossium (Ralphs et al 1985), and also occurs in the skin of cattle and sheep
with dermatoparaxis (Fjolsted and Helle 1974). Although biochemically these collagens
contain apparently normal \( \alpha 1 \) and \( \alpha 2 \) chains, the abnormality could be due to a defective
aggregation of collagen filaments to form compact fibrils, or due to the dissociation,
disaggregation or fraying of previously normal fibres. This implies that either there is an
abnormality in collagen processing or, as in EDS I/II, that other collagens, or even
extracellular matrix components, are disrupting type I collagen fibrillogenesis. Whatever
the reason, these structurally abnormal collagens disturb the sites of scaffolding and
nucleation essential for normal mineralisation; hence the thick unmineralised osteoid and patchy mineralisation observed in these specimens.

In the present study OI type IV bone demonstrated abnormalities in the osteoblasts, the osteoid and the periosteum. The osteoblasts showed degenerative changes; this has been previously reported by Stoss (1990). The elastin found in the osteoid and in the periosteum of three specimens confirms previous observation by Cassella et al (1996), who described the presence of "fibrous bodies". The typical ultrastructural appearance and the positive colour reaction of Miller's elastin stain confirms that elastin was present in these specimens. The abnormal presence of elastin could well be a characteristic feature in OI type IV but more specimens would have to be examined in order to clarify this in future. However similar increases in elastin have also been reported in other inherited diseases of connective tissue, such as EDS IV; this has been suggested to be an induced secondary response to collagen depletion (Pope et al 1996). Depletion of collagen and the presence of elastin observed in this study probably reflects the poor nucleation and lack of scaffolding sites for mineral deposition in OI type IV bone.

This study therefore confirms that there is a heterogeneous variability between OI clinical phenotype, collagen chemistry and fibril morphology, proteoglycan distribution and ultrastructural morphology. In general the mildest forms of OI have the least disturbed changes whilst the more severe OI patterns show the greatest disorganisation.
Chapter 3

Electron Probe X-ray microanalysis of OI bone by conventional and cryo-techniques

3.1 Introduction

Bone: structure and composition

Bone structure is discussed in detail in chapter 2 and in this chapter bone formation and mineralisation theories are discussed.

Bone is not only a supportive tissue which is essential for maintaining the shape of the animal, it also plays an important role in calcium and phosphorus homeostasis. Bone is a reservoir for calcium, inorganic orthophosphates, magnesium, sodium, carbonate and other ions. Bone is composed of organic fibrils (20-30 % by weight), 10% water and the remainder is inorganic mineral, mainly calcium phosphate salts such as hydroxyapatite. De Jong (1926) was the first to document the similarity between X-ray diffraction patterns of bone powder and hydroxyapatite. It is now believed that the bone mineral is a calcium-deficient (about 5%) carbonate containing a poorly crystalline analogue of the naturally occurring mineral hydroxyapatite with a calcium to phosphorus molar ratio of 1.67 (Armstrong and Singer 1965; Landis and Glimcher 1978; Ali 1992). The organic component of bone consists mainly of type I collagen (95%) with small amounts (5%) of non-collagenous proteins such as osteocalcin, osteonectin, osteopontin, sialoproteins, phospholipids and proteoglycans.

Mechanisms of calcification

There are two primary types of bone formation. The first is intramembranous ossification as in the skull, and the second is endochondral ossification as in the long bones of the body. The sequence of bone formation in endochondral ossification has been worked out in detail. The new bone is formed on a core of mineralised cartilage by bone forming cells, the osteoblasts. These cells form a continuous monolayer and start to produce a collagenous matrix which becomes mineralised. Tissue mineralisation involves a complex series of sequential events. During the first step of the sequence, soluble calcium and phosphate ion concentrations reach a level high enough to form a complex and produce an insoluble solid. These small solid particles grow or reorganise to form apatite crystals, which increase in number to saturate the matrix of the tissue. This is followed by further reorganisation of mineralised matrix to form a stable, organised and structurally sound calcified tissue (Posner 1969; Ali 1992). To prevent
spontaneous *de novo* apatite crystal formation in connective tissues, it has been shown that the calcium and phosphorus ion levels in serum and in the tissue fluids are not saturated (Vaughan 1981), although it is possible that total calcium and phosphorus levels are over saturated in terms of bone mineralisation but crystal formation is prevented in the presence of ion-binding proteins and specific inhibitors. In general there are three factors that are thought to facilitate the initial calcification process (Posner 1978). Firstly raising the ionic concentrations of calcium and phosphorus to supersaturation levels may initiate spontaneous precipitation. Secondly the mineralisation process is believed to be triggered by a "nucleating agent", and candidates for this include mitochondria (Martin and Mathews 1969), matrix vesicles in epiphyseal growth plates (Anderson 1967; Bonucci 1967; Ali et al 1970) and in bone (Bernard and Pease 1969; Ali 1992), and distinct sites on collagen fibrils (Glimcher 1985; Traub et al 1992; Landis 1995). Thirdly the removal or neutralisation of inhibitors such as pyrophosphate (Fleisch and Neuman 1961; Meyer 1984) and aggregating proteoglycans (Chen et al 1984; Chen and Boskey 1985, 1986) may allow bone mineralisation to occur.

Matrix vesicles appear to qualify for all of the above mentioned factors of calcification. The alkaline phosphatase enzyme contained within the vesicles is able to cleave phosphate containing substrates and release phosphorus ions to raise the concentration of calcium phosphate to a saturated level, thus initiating calcification. Matrix vesicles can also act as nucleating agents, and have been shown to contain the first apatite microcrystals (Bonucci 1967; Anderson 1976; Ali 1983; Anderson 1995); in addition, matrix vesicles can hydrolyse pyrophosphate, thus removing the inhibitors of calcification.

Collagen as a nucleating agent has been supported by many workers (Solomons and Neuman 1960; Glimcher 1976). On the basis of electron microscopic observations and the proposal of a staggered array model for collagen fibril organisation, the gap region was recognised as a possible initial site of mineralisation (Weiner 1986). Glimcher (1989) pointed out in his paper on mechanisms of calcification that the matrix vesicles cannot directly cause new crystals to be initiated on collagen fibrils because of the spatial separation of these two components. Soares et al (1992) concluded from the evidence of lanthanum tracer and freeze fracture studies that the compartmentalisation of bone matrix is an important requirement for initial mineralisation. They suggested that the matrix vesicles act as a micro-compartment which initiates mineralisation. Once the matrix compartmentalises, mineralisation proceeds without vesicles. Recently Kohler et al (1994), based on the evidence of a three dimensional analysis of mineralising turkey leg, suggested that a temporal sequence of calcification starts in matrix vesicles and progresses to nearby collagen fibrils, spreading within the fibrils. This finding is consistent with the concept proposed by Ali (1976, 1983, 1992) that the initial mineralisation starts in matrix vesicles, then mineral nodules of calcospherite
form followed by the impregnation and alignment of crystals with the collagenous matrix (Figure 3.1). Recently Höhling et al (1995) concluded that initially calcium phosphate is composed of nanometer size particles (dots and islands) which coalesce rapidly to form needles. In collagen mineralisation the distances between the dots in the mineral chains represent the distance between nucleating sites (active sites) which bind calcium primarily for subsequent nucleation.

Fig. 3.1 Schematic diagram showing the sequence of mineral formation in the osteoid of bone. For visual clarity collagen fibrils in the osteoid (stage 1 and 2) have been omitted (from Ali 1992).

**Electron probe X-ray microanalysis**

Electron probe X-ray microanalysis is a semi-quantitative method used in conjunction with the transmission electron microscope for the measurement of elemental composition. This procedure allows the conversion of measured X-ray intensities into an expression of the relative or absolute concentration of elements in biological specimens. There are two commonly used procedures for thin biological specimens. First is the ratio method, which determines the relative amounts of two or more elements, and the second is the Hall method, which can be used to determine the mass fraction of a single element. In this study the ratio method was used to determine the relative Ca/P ratio in ultra-thin sections of OI and normal bone. The use of electron probe X-ray microanalysis (XRMA) has been applied to the study of biological mineralisation in both pathological and normal conditions (Ali 1976; Ali et al 1977; Landis 1979). This technique gives focal information about the Ca/P ratio and provides more valuable information when combined with electron diffraction studies. Akesson et
(1994) evaluated the accuracy of bone mineral composition by electron probe microanalysis (EDAX) when compared with instrumental neutron activation analysis (INAA) and chemical analysis (ICPES). He concluded that EDAX provides a useful tool for the simultaneous elemental quantification of regular bone biopsy material and is a useful technique for microstructural evaluation of the degree of mineralisation.

Abnormalities of mineral density, composition, crystal size and distribution in OI

There are reports on bone mineral density of OI patients determined using non-invasive methods. Paterson (1978) measured the metacarpal medullary width in adults with OI type I and found that, although the hand bones were slender, the cortical bone widths were normal. Pedersen et al (1979) reported reduced mineral content in the forearm bones measured by photon absorptiometry. Kurtz et al (1985) measured the vertebral bone mineral content in OI patients by computed tomographic scan and reported that all OI subjects have reduced bone mineral density for their age and it was roughly proportional to the severity of disease. Recently Zionts et al (1995) demonstrated reduced mineral density in OI type I using dual energy X-ray absorptiometry (DEXA).

A previous study in this laboratory showed a reduced calcium to phosphorus ratio in OI bone when compared with normal bone using the semi-quantitative analytical technique of electron probe microanalysis in the transmission electron microscope (Cassella & Ali 1992; Cassella et al 1995). The previous study was carried out using conventional preparative techniques modified to avoid possible artefactual demineralisation for electron microscopy. Bucsi et al (1994) studied three bone biopsies from OI type I patients and reported that the structure of newly formed apatite crystals was smaller in size but the Ca/P ratio was the same as that of normal controls.

Several workers reported a reduction in crystal lengths in OI type II patients (Vetter et al 1991a; Traub et al 1994) and in a bovine model of OI (Fisher et al 1987). Furthermore Traub and co-workers (1994) reported that not only are the apatite crystals in OI smaller than normal crystals, but they may not be confined to the normal plate-like shapes. Cohen-Solal et al (1994) reported isolated mineralized globules, unrelated to the collagen fibrils, from OI type II patients. Brenner et al (1993) reported elevated apatite crystal size in a boy belonging to OI type IV. Culbert et al (1995) studied two infants with lethal OI type II and reported that the number of crystallites of hydroxyapatite in collagen fibrils was markedly less (less than 5% of collagen fibres encrusted with mineral crystallites) than that found in the collagen fibrils of normal age-matched controls (about 70% of fibrils were encrusted with crystallites). Furthermore the c-axes
of the hydroxyapatite were sometimes poorly aligned with the long axes of the collagen fibrils.

Transgenic mice which serve as a model for human OI have been used in several studies and show a low Ca/P ratio (Cassella et al 1994b), with abnormalities in hydroxyapatite crystal size and distribution (Landis 1995). Landis proposed a model for mineralisation for the oim/oim mouse model that resembles moderately severe OI, and this will be discussed in the later part of this chapter. Fratzl and co-workers (1996) studied the cortical bone of oim/oim by small angle X-ray scattering and reported that the mineral crystals are thinner and their alignments more variable than in normal litter mates. All these findings suggest that there are abnormalities in crystal size and distribution in OI bone, and that these abnormalities are more pronounced in OI type II, which is the severest type of OI.

Aims of this Chapter

This chapter had two aims. The first was to compare conventional preparative techniques with a more reliable cryo-sectioning technique that can avoid artefactual changes in the mineral composition of bone that may occur in aqueous media. For this part of the study, freshly dissected bone was rapidly frozen and sectioned dry in order to avoid contact with any solvent prior to an examination by electron microscopy. Many workers (Thorogood and Gray 1975; Ali et al 1977 & 1978; Nicholson et al 1977; Landis 1979) have implied that artefactual demineralization may possibly occur during fixation, dehydration and embedding in resin. In addition, ultramicrotomy could remove bone crystals from the sections (Boothroyd 1964). Hall and Höhling (1969) concluded from their work that ultra-thin frozen sections are preferable for such analytical work. Furthermore it is known that the solid phase of amorphous calcium phosphate (ACP) in vitro is very labile and rapidly converts to poorly crystalline hydroxyapatite when exposed to water (Termine 1972). Thus it is likely that aqueous solvents used in tissue preparation may induce mineral transformations especially in newly formed bone where ACP is thought to be abundant (Termine 1972). Cryo-ultramicrotomy avoids aqueous contact altogether, thus offering better opportunities for accurate and precise electron probe microanalysis.

Secondly, by carrying out the analysis on thirty five OI bone specimens and twenty five age- and site-matched normal bone specimens, this study planned to differentiate the results according to the four clinical types of OI as described by Sillence et al (1979) and express the findings separately for the first time.
3.2 Materials and Methods

Patients' details

Foetal bone specimens mostly from femoral diaphyses were obtained from autopsies. Bone from other OI types was obtained during intermedullary rodding treatment of these patients.

In this study bone specimens from 9 normal foetuses, 9 OI type II foetuses, 6 type II/III foetuses, and from 14 OI type I, 4 OI type III, 2 OI type IV and 16 normal adults were analysed (specimen details, age and site of biopsy have been detailed in Tables 3.2 and 3.3). In this study it was not possible to carry out cryo-ultramicrotomy on all the above specimens as it was difficult to obtain fresh bone samples. The calcium phosphate standards used were calcium pyrophosphate dihydrate (CPPD), calcium tetrahydrogen orthophosphate (CTOP; BDH, Merck Ltd., Poole, Dorset, UK) tricalcium phosphate (TCP) and hydroxyapatite (HA). The TCP and HA standards were provided by Dr. C. Klein and were well characterised by X-ray diffraction (Klein et al 1988). The bone specimens and the standards were treated similarly using the same buffer, fixative and resin mixtures throughout the processing of all samples.

Conventional EM processing

Bone tissue from OI patients and normals (mostly from femur) was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Agar Scientific Ltd., Essex, England) for 2 hours. During the first hour of fixation, specimens were sub-divided into pieces of 1 mm³. The secondary fixation with osmium tetroxide was omitted to avoid interference with the kα peak of phosphorus. After fixation, specimens were processed and embedded in araldite CY212 resin as described in chapter 2. The calcium phosphate standards were powdered and processed in Eppendorf centrifuge tubes, and dehydrated, infiltrated and embedded in araldite CY212 resin.

Ultra-thin sections (100nm) were cut on a Reichert-Jung Ultracut E ultramicrotome using distilled water at pH 7.4 in the boat, and quickly collected on to 200 mesh formvar-coated nickel grids. Specimens were viewed uncontrasted in a Philips CM 12 transmission microscope in the TEM mode and analysed with an EDAX 9800 microanalysis system. To minimise the TEM column and detector defects, a beryllium low dose specimen holder was used and the parameters used for analysis were as follows: the specimen eucentric height was set using a goniometer stage, the accelerating voltage was 80 KV, magnification was 8000, the spot size was four, the tilt angle was 20° and the take off angle was 40°. The PV 9800 system was set to measure 10 eV per...
channel with a preset count time of 200 live seconds. The TEM was regularly realigned and the EDAX system was calibrated at each sitting using a commercial aluminum and copper standard (Agar Scientific Ltd. Essex, England). The standard file for hydroxyapatite was created by analysing ten areas of the HA standard with the EDAX PV9800 computer programmed to create one cumulative spectrum from the ten analyses. This procedure was repeated for the CPPD, CTOP and TCP standards. Once the standard files were created, the Ca/P ratio observed in TEM was plotted against the known empirical Ca/P ratio (Table 3.1 and Fig. 3.2). Ultra-thin bone sections were viewed in TEM mode until an area of dense mineral was observed. Ten areas were counted randomly over the dense mineral of the cortical bone of each specimen under identical conditions. All analyses gave strong peaks for calcium and phosphorus with a very low background. For semi-quantitation of the Ca/P ratio, the EDAX microanalysis system utilised the Cliff-Lorimer ratio method, which makes use of a relationship of the measured X-ray intensities of two elements to the ratio of their respective concentrations.

Cryo-processing

This was adapted from the method of Ali et al (1977).

Minute (about 0.5 mm³) pieces of cortical bone mostly from femur were individually fixed on to the groove of a silver cryo-pin with a drop of 2.3 M sucrose in 0.1 M sodium cacodylate buffer. The pin containing the bone was then frozen by immersion in liquid nitrogen and was transferred to the cryo-chamber of the Reichert-Jung Ultracut E Ultramicrotome in liquid nitrogen. Sectioning was performed using a cryo-diamond knife at -90 °C and a cutting speed of 0.1 mm/second. Sections were picked up dry using an eye lash probe directly on to 200 mesh formvar-coated nickel grids. Specimens were analysed and quantified as described above.
3.3 Results

In this study the Ca/P ratio of the bone mineral in OI and normal bone was determined using electron probe X-ray microanalysis in the TEM on conventional resin-embedded sections and cryo-sections.

In order to establish the accuracy of the methodology used to quantitate the bone mineral ratios and to establish the linear calibration of the EDAX microanalysis system, four chemically prepared calcium phosphate standards of known structure and composition, namely HA, TCP, CPPD and CTOP, were used. The observed Ca/P ratio for the four standards when plotted against theoretical values (Table 3.1) on a standard graph (Fig 3.2) gave a linear response and a good correlation (R=1.000) with the theoretical values. This demonstrated that the methodology used here was accurate enough to study the mineral composition of normal and OI bone.

Figs. 3.3a, 3.3b, 3.3c and 3.3d show unstained, ultra-thin cryo-sections demonstrating the nature of mineral in normal and OI bone. The normal mineral crystals appeared more mature and showed proper alignment in the collagen fibres (Figs. 3.3a and 3.3c), whereas the OI bone contained thin crystals (Figs. 3.3b and 3.3d) which were not properly aligned.

Figs. 3.4a and 3.4b show the EDAX spectra generated from cryo-sections of normal bone and OI type II bone. Both spectra showed prominent peaks for calcium at 3.71 KeV and for phosphorus at 2.0 KeV with low background signals. However the peak height for phosphorus was higher in OI bone and this is shown in Fig. 3.4c in which an EDAX spectrum generated from a cryo-section of an OI type II bone (Fig. 3.4b) was overlaid on an EDAX spectrum generated from a cryo-section of a normal specimen (Fig. 3.4a). The peak heights for calcium were similar in OI and normal bone mineral. These results suggest that the change in the Ca/P ratio in OI type II bone is likely to be due to a higher level of phosphorus compared with that of normal bone.

The Ca/P ratios for normal controls by conventional and cryo-preparative methods are presented in the Tables 3.2.1 and 3.2.2 and those for OI groups are presented in Tables 3.3.1 to 3.3.5. All groups were tested for parametric distribution and subjected to analysis of variance (ANOVA). Comparison of group means after ANOVA was performed by the Tukey-Kramer HSD (Honestly Significant Difference) test. The results indicate that there was no significant difference in the Ca/P ratio when hydroxyapatite standard sections were prepared by conventional preparative methods and compared with cryo-ultramicrotomy methods (Figs 3.5a &3.5b). Furthermore the Ca/P ratio of normal human bone was the same when these two techniques were compared, yielding a Ca/P ratio of 1.68 - 1.70 which is similar to that of
hydroxyapatite. Although there was no statistically significant difference between cryo- and resin sections in OI type II for this study (p > 0.05 by unrelated student's T test), a difference was observed (1.49 for cryo-sections and 1.55 for resin sections) and a greater number of specimens will have to be examined in order to clarify this in the future.

When all the OI groups data was pooled there was a significant difference (p<0.05) between the Ca/P ratio in OI and normal bone mineral in both cryo- and resin sections (Figs 3.6a & 3.6b) with OI bone showing a reduced Ca/P ratio. This study has gone one step further by dividing OI samples into clinical types and comparing these ratios with normal foetal and adult bone mineral Ca/P ratios. A highly significant difference was seen between type II (Ca/P ratio 1.49 - 1.55) and type II/III (Ca/P ratio 1.57 - 1.59) with normal foetal bone (Ca/P ratio 1.68 - 1.69) in both cryo- and resin sections (p< 0.01). Furthermore the reduced Ca/P ratios appeared to reflect the severity of this disease (Figs. 3.5a & 3.5b). In OI type I, III and IV Ca/P ratio values were slightly lower (OI type I = 1.63, type III = 1.65, type IV = 1.62) than normal bone (1.70) and the HA standard (1.66) measured in resin sections, but there was no statistical difference between these OI types and normal bone.

When the mean Ca/P ratios of normal and OI bone mineral were compared in age groups, OI bone always showed a lower ratio than the corresponding normal control (Fig. 3.7). However, with the exception of normal foetal and OI foetal bone, this difference in Ca/P ratio was not statistically significant.
Table 3.1 Empirical and observed Ca/P ratios for known standards.

<table>
<thead>
<tr>
<th>Standard</th>
<th>Empirical Ca/P ratio</th>
<th>Observed Ca/P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>1.67</td>
<td>1.66 +/- 0.06</td>
</tr>
<tr>
<td>TCP</td>
<td>1.50</td>
<td>1.52 +/- 0.05</td>
</tr>
<tr>
<td>CPPD</td>
<td>1.00</td>
<td>1.00 +/- 0.01</td>
</tr>
<tr>
<td>CTOP</td>
<td>0.50</td>
<td>0.51 +/- 0.01</td>
</tr>
</tbody>
</table>

Fig. 3.2 Standard graph of the Ca/P ratio for the calcium phosphate standards as measured in the TEM and plotted against empirical ratios. The range of values obtained for normal bone and type II bone sections is also shown.
Fig. 3.3a  Electron micrograph of mineral area on an unstained ultra-thin cryosection of a normal bone (M.K, 11 years) at the site of XRMA analysis. Note the alignment of solid mineral needles in the collagen fibres.
Bar = 260 nm

Fig. 3.3b  Electron micrograph of mineral area on an unstained ultra-thin cryosection of an OI type III bone (H.S, 3 years) at the site of XRMA analysis. Note the thin needles of mineral which are not properly aligned.
Bar = 260 nm

Fig. 3.3c  Electron micrograph of mineral area on an unstained ultra-thin cryo-section of a normal foetal bone (Foetus Sy, 22 weeks) at the site of XRMA analysis, shown at a higher magnification.
Bar = 66 nm

Fig. 3.3d  Electron micrograph of mineral area on an unstained ultra-thin cryo-section of an OI type II foetal bone (Foetus F, 19 weeks) at the site of XRMA analysis, shown at a higher magnification.
Bar = 66 nm
Fig. 3.4a The XRMA spectrum of a cryo-section of normal foetal bone mineral showing strong peaks for Ca and P with very low background.

Fig. 3.4b The XRMA spectrum of a cryo-section of OI type II bone mineral showing strong peaks for Ca and P with very low background.
Fig. 3.4c  The XRMA spectrum generated from a cryo-section of an OI type II bone (Fig. 3.4b) was overlaid on the XRMA spectrum generated from a cryo-section of a normal foetal bone (Fig. 3.4a). Note the Ca peaks are matched in height whereas the peak height for phosphorus is increased in the OI type II bone.
Chapter 3

3.4 c
### Table 3.2.1 Details of normal foetal bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P-cryo</th>
<th>Relative Ca/P-resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus D</td>
<td>20 weeks, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.77</td>
</tr>
<tr>
<td>Foetus M</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.60</td>
</tr>
<tr>
<td>Foetus Sy</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>1.65</td>
<td>1.69</td>
</tr>
<tr>
<td>Foetus C</td>
<td>22-24 weeks,</td>
<td>femur</td>
<td>1.70</td>
<td>1.70</td>
</tr>
<tr>
<td>Foetus T</td>
<td>23 weeks, male</td>
<td>not known</td>
<td>1.70</td>
<td>1.67</td>
</tr>
<tr>
<td>Foetus L</td>
<td>24 weeks, male</td>
<td>femur</td>
<td>1.64</td>
<td>1.69</td>
</tr>
<tr>
<td>Foetus G</td>
<td>24 weeks, male</td>
<td>not known</td>
<td>1.66</td>
<td>1.72</td>
</tr>
<tr>
<td>Foetus Ta</td>
<td>not known</td>
<td>not known</td>
<td>1.69</td>
<td>1.70</td>
</tr>
<tr>
<td>Foetus H</td>
<td>not known</td>
<td>not known</td>
<td>1.69</td>
<td>1.64</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>1.68</strong></td>
<td><strong>1.69</strong></td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td></td>
<td></td>
<td><strong>0.03</strong></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td></td>
<td></td>
<td><strong>0.01</strong></td>
<td><strong>0.01</strong></td>
</tr>
</tbody>
</table>
Table 3.2.2 Details of normal bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P-cryo</th>
<th>Relative Ca/P-resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. N. D</td>
<td>2 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.62</td>
</tr>
<tr>
<td>M. D</td>
<td>4 years, male</td>
<td>not known</td>
<td>no cryo</td>
<td>1.63</td>
</tr>
<tr>
<td>M. M</td>
<td>5 years, female</td>
<td>femur</td>
<td>no cryo</td>
<td>1.69</td>
</tr>
<tr>
<td>A. F</td>
<td>9 years, male</td>
<td>not known</td>
<td>no cryo</td>
<td>1.68</td>
</tr>
<tr>
<td>R. M</td>
<td>9 years, male</td>
<td>not known</td>
<td>no cryo</td>
<td>1.64</td>
</tr>
<tr>
<td>F. D</td>
<td>10 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.65</td>
</tr>
<tr>
<td>M. K</td>
<td>11 years, male</td>
<td>femur</td>
<td>1.70</td>
<td>1.68</td>
</tr>
<tr>
<td>R. A. M</td>
<td>12 years, male</td>
<td>femur</td>
<td>1.70</td>
<td>1.71</td>
</tr>
<tr>
<td>A. H. A</td>
<td>12 years, male</td>
<td>tibia</td>
<td>1.74</td>
<td>1.72</td>
</tr>
<tr>
<td>P. K</td>
<td>16 years, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.66</td>
</tr>
<tr>
<td>P. C</td>
<td>21 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.80</td>
</tr>
<tr>
<td>N. G</td>
<td>25 years, female</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.75</td>
</tr>
<tr>
<td>D. St</td>
<td>27 years, male</td>
<td>tibia</td>
<td>no cryo</td>
<td>1.76</td>
</tr>
<tr>
<td>J. D</td>
<td>31 years, male</td>
<td>tibia</td>
<td>no cryo</td>
<td>1.66</td>
</tr>
<tr>
<td>J. P</td>
<td>71 years, male</td>
<td>femur</td>
<td>1.67</td>
<td>1.69</td>
</tr>
<tr>
<td>L. C</td>
<td>71 years, female</td>
<td>femur</td>
<td>1.67</td>
<td>1.76</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>1.69</td>
<td>1.70</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td></td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Standard Error</td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Table 3.3.1  Details of OI type I bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P- cryo</th>
<th>Relative Ca/P- resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. S. A</td>
<td>type I</td>
<td>3 years, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.66</td>
</tr>
<tr>
<td>S. V</td>
<td>type I</td>
<td>8 years, male</td>
<td>tibia</td>
<td>1.69</td>
<td>1.63</td>
</tr>
<tr>
<td>R. M</td>
<td>type I</td>
<td>9 years, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.57</td>
</tr>
<tr>
<td>M. G</td>
<td>type I</td>
<td>9 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.64</td>
</tr>
<tr>
<td>H. M</td>
<td>type I</td>
<td>10 years, female</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.55</td>
</tr>
<tr>
<td>A. C</td>
<td>type I</td>
<td>10 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.68</td>
</tr>
<tr>
<td>G. A</td>
<td>type I</td>
<td>10 years, female</td>
<td>tibia</td>
<td>no cryo</td>
<td>1.68</td>
</tr>
<tr>
<td>A. A</td>
<td>type I</td>
<td>12 years, male</td>
<td>tibia</td>
<td>no cryo</td>
<td>1.63</td>
</tr>
<tr>
<td>A. Mc</td>
<td>type I</td>
<td>14 years, male</td>
<td>not known</td>
<td>1.62</td>
<td>1.58</td>
</tr>
<tr>
<td>S. D</td>
<td>type I</td>
<td>14 years, female</td>
<td>tibia</td>
<td>no cryo</td>
<td>1.65</td>
</tr>
<tr>
<td>A. M</td>
<td>type I</td>
<td>16 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.63</td>
</tr>
<tr>
<td>J. W</td>
<td>type I</td>
<td>30 years, female</td>
<td>ulna</td>
<td>1.56</td>
<td>1.66</td>
</tr>
<tr>
<td>C. W</td>
<td>type I</td>
<td>42 years, male</td>
<td>iliac crest</td>
<td>no cryo</td>
<td>1.68</td>
</tr>
<tr>
<td>N. N</td>
<td>probably type I</td>
<td>not known</td>
<td>not known</td>
<td>no cryo</td>
<td>1.60</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>1.62</td>
<td>1.63</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Standard Error</td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>
### Table 3.3.2 Details of OI type II bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P-cryo</th>
<th>Relative Ca/P-resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus Mc</td>
<td>type II</td>
<td>16 weeks, male</td>
<td>femur</td>
<td>1.50</td>
<td>1.51</td>
</tr>
<tr>
<td>Foetus Ki</td>
<td>type II</td>
<td>16 weeks, female</td>
<td>rib</td>
<td>no cryo</td>
<td>1.55</td>
</tr>
<tr>
<td>Foetus F</td>
<td>type II</td>
<td>19 weeks, female</td>
<td>femur</td>
<td>1.45</td>
<td>1.52</td>
</tr>
<tr>
<td>Foetus S</td>
<td>type II</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.54</td>
</tr>
<tr>
<td>Foetus T-VI</td>
<td>type II</td>
<td>23 weeks, male</td>
<td>femur</td>
<td>1.57</td>
<td>1.62</td>
</tr>
<tr>
<td>Foetus W</td>
<td>type II</td>
<td>25 weeks, female</td>
<td>femur</td>
<td>1.40</td>
<td>1.45</td>
</tr>
<tr>
<td>Foetus Mo</td>
<td>type II</td>
<td>22 weeks, femur or tibia</td>
<td>no cryo</td>
<td>1.56</td>
<td></td>
</tr>
<tr>
<td>Baby K</td>
<td>type II</td>
<td>perinatal, male</td>
<td>femur</td>
<td>1.46</td>
<td>1.56</td>
</tr>
<tr>
<td>Baby A</td>
<td>type II</td>
<td>perinatal, female</td>
<td>femur</td>
<td>1.53</td>
<td>1.61</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>1.49</strong></td>
<td><strong>1.55</strong></td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.06</strong></td>
<td><strong>0.05</strong></td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>0.02</strong></td>
<td><strong>0.02</strong></td>
</tr>
</tbody>
</table>
Table 3.3.3  Details of OI type II/III bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P-</th>
<th>Relative Ca/P-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cryo</td>
<td>resin</td>
</tr>
<tr>
<td>Foetus O</td>
<td>type II/III</td>
<td>18 weeks, male</td>
<td>rib</td>
<td>no cryo</td>
<td>1.60</td>
</tr>
<tr>
<td>Foetus Ar-II</td>
<td>type II/III</td>
<td>20 weeks, male</td>
<td>femur or tibia</td>
<td>1.57</td>
<td>1.62</td>
</tr>
<tr>
<td>Foetus Ar-III</td>
<td>type II/III</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>no cryo</td>
<td>1.49</td>
</tr>
<tr>
<td>Foetus F.G</td>
<td>type II/III</td>
<td>22 weeks, female</td>
<td>rib</td>
<td>1.58</td>
<td>1.59</td>
</tr>
<tr>
<td>Baby G-III</td>
<td>type II/III</td>
<td>perinatal, female</td>
<td>tibia</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>Baby Ak-II</td>
<td>type II/III</td>
<td>not known</td>
<td>femur</td>
<td>1.55</td>
<td>1.65</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.57</td>
</tr>
<tr>
<td><strong>Standard Deviation</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Table 3.3.4  Details of OI type III bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P-</th>
<th>Relative Ca/P-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cryo</td>
<td>resin</td>
</tr>
<tr>
<td>B. S</td>
<td>type III</td>
<td>still birth, female</td>
<td>femur</td>
<td>1.67</td>
<td>1.64</td>
</tr>
<tr>
<td>B. G</td>
<td>type III</td>
<td>3 years, male</td>
<td>not known</td>
<td>no cryo</td>
<td>1.62</td>
</tr>
<tr>
<td>H. S</td>
<td>type III</td>
<td>3 years, male</td>
<td>femur</td>
<td>1.62</td>
<td>1.65</td>
</tr>
<tr>
<td>K. W</td>
<td>type III</td>
<td>7 years, female</td>
<td>femur</td>
<td>1.65</td>
<td>1.67</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>1.65</td>
<td>1.65</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Standard Error</td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 3.3.5  Details of OI type IV bone and the Ca/P ratios.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; Sex</th>
<th>Site of biopsy</th>
<th>Relative Ca/P-</th>
<th>Relative Ca/P-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cryo</td>
<td>resin</td>
</tr>
<tr>
<td>M. K</td>
<td>type IV</td>
<td>25 years, female</td>
<td>tibia</td>
<td>no cryo</td>
<td>1.62</td>
</tr>
<tr>
<td>K. L</td>
<td>type IV</td>
<td>28 years, male</td>
<td>not known</td>
<td>no cryo</td>
<td>1.62</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>Standard Deviation</td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Standard Error</td>
<td></td>
<td></td>
<td></td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.5a Bar graph showing the mean Ca/P ratios in various clinical types of OI bone, normal bone and the hydroxyapatite (HA) standard measured on cryo-sections. Each error bar represents the standard error of the mean.

Fig. 3.5b Bar graph showing the mean Ca/P ratios in various clinical types of OI bone, normal bone and the hydroxyapatite (HA) standard measured on conventional resin sections. Each error bar represents the standard error of the mean.
Chapter 3

Relative Ca/P ratio of bone mineral in cryo sections of normal & OI bone

3.5 a

Relative Ca/P ratio of bone mineral in resin sections of normal & OI bone

3.5 b
Fig. 3.6a Bar graph showing the mean Ca/P ratios measured on cryo-sections of the hydroxyapatite (HA) standard, normal bone, and pooled OI types. Each error bar represents the standard error of the mean.

Fig. 3.6b Bar graph showing the mean Ca/P ratios measured on resin sections of the hydroxyapatite (HA) standard, normal bone, and pooled OI types. Each error bar represents the standard error of the mean.
Fig. 3.7 Bar graph showing the mean Ca/P ratios of normal and OI bone measured on resin sections and compared by age groups. Each error bar represents the standard error of the mean.
3.4 Discussion

After careful standardization and calibration in the electron microscope, the Ca/P ratio of OI bone mineral demonstrated lower values (1.58) than the normal controls (1.69). When the results were divided into clinical types of OI they appeared to reflect the severity of disease with OI type II bone sections giving the lowest value (1.55). The results of the cryo-sections were even more emphatic (for OI type II, Ca/P ratio =1.49). Other OI types demonstrated slightly lower Ca/P ratio values (OI type I = 1.63, type III = 1.65, type IV = 1.62) than normal bone (1.70) and the hydroxyapatite (HA) standard (1.66), but showed no significant statistical difference.

This study confirms the findings of Cassella and Ali (1992) and Cassella et al (1995) that the OI bone Ca/P ratio is lower than normal bone. Furthermore this answers all the criticisms of artefactual demineralisation during tissue processing. This is the first report on Ca/P ratios determined on cryo-sections of OI and normal bone, and the first one to express them separately according to clinical types. The OI type II bone mineral shows the lowest Ca/P ratio, and clearly shows a significant difference in Ca/P ratios from the normal foetal and adult bone mineral and also from the other OI types. The lower bone mineral composition in severe forms of OI could be due to several factors.

As mentioned in the introduction, HA is the main inorganic crystalline constituent of bone and dentine. HA is a more or less ideal crystalline component with a Ca/P ratio of 1.66. Materials deviating from ideal HA composition are referred to as non-stoichiometric apatite (Brown 1966). There are several proposals to account for non-stoichiometric apatite but the most widely accepted ones are as follows:

a) calcium deficiency in the HA lattice.

b) substitution in the HA lattice.

c) intracrystalline mixtures of HA and octacalcium phosphate (OCP) and tetracalcium phosphate (TCP).

d) increase in phosphate ions.

Any of the above mentioned factors can be responsible for making the HA crystals non-stoichiometric. According to Brown (1966) intracrystalline mixtures of HA and OCP and TCP are the major cause of stoichiometric variability in apatite. There is evidence that OCP initially precipitates and then hydrolyses to HA. Nancollas et al (1989) demonstrated the overgrowth of OCP on HA by using high resolution TEM at 2.5 Å resolution. Posner and Perloff (1957) gave two explanations for lower Ca/P ratio in non-stoichiometric apatite. The first is the adsorption of excess phosphate ions onto the surface of the HA crystal; the second is the statistical absence of calcium ions from the interior lattice sites. However they discount the surface adsorption theory because surface areas of some synthetic apatites are too low to account for their non-
stoichiometry. As it was not possible to test any of the theories that account for non-stoichiometry in this study, the low Ca/P ratio in OI type II and type II/III could be due to any of the above mentioned factors. The growing body of evidence that OI type II contains shorter crystals favours the lattice substitution theory as a possible explanation for the lower Ca/P ratio observed in the type II and II/III OI bone mineral. This reduction in crystal size may separate the crystallites from one another, and make them more soluble. Hence the calcium ions can be easily substituted by sodium ions or potassium ions or replaced by H$_2$O or H$_3$O$^+$ ions thus making the HA crystals non-stoichiometric. Alternatively an increase in phosphate ions could lower the Ca/P ratio. The evidence for this change in composition is illustrated in Fig. 3.4c, where the XRMA spectrum generated by OI type II bone was overlaid on a spectrum generated by normal foetal bone. The difference in the peak height can be observed only in the phosphorus peak with no observed difference in the calcium peak. There appears to be no change in calcium but an increase in phosphorus in OI bone which could be due to the presence of pyrophosphates in OI bone (Professor Ali - personal communication). This is supported by the work of Solomons and Styner (1969) who found elevated levels of serum and urinary pyrophosphate in twenty eight OI patients by chemical analyses. According to Ryan and McCarthy (1995) pyrophosphate can be generated by the breakdown of adenosine triphosphate to adenosine monophosphate in the presence of the enzyme nucleoside triphosphate pyrophosphohydrolase. Cassella et al (1994b) carried out a Fourier Transform Infrared Spectroscopy (FTIR) study on bone mineral from transgenic mice resembling OI and found that it was apatite in nature despite the lower Ca/P ratio. Landis (1995) studied the oim/oim mouse model that resembles moderately severe OI by using high voltage electron microscopic tomography (3D) and reported various sizes and shapes of hydroxyapatite crystals. The orientation, location and alignment of these crystals with respect to the collagen were distinctly different from the normal calcified tissues. In normal calcified tissue there was a regularity in the staggering of collagen fibres. In the oim/oim mouse, collagen assembly in parts of the tissue was disorganised and many collagen fibrils were twisted and kinked and the characteristic 67 nm D-period was out of register across some adjacent fibrils.

However, apart from these abnormalities there may be other factors affecting the normal mineralisation. The thinner collagen fibres observed in OI type II (chapter 4), the abnormal amounts of proteoglycan found in some of the type II/III bone (chapter 7) and other ultrastructural abnormalities (chapter 2) could be playing an important role in abnormal mineral formation. Undoubtedly the lower Ca/P ratio of OI type II bone mineral observed in this study may be a key factor with regards to the bone fragility in OI. Future work should clarify the causes of this abnormal mineralisation especially in OI type II.
Chapter 4

Histomorphometry of Type I collagen fibrils in the osteoid of OI bone

4.1 Introduction

Collagens are the major extracellular proteins of the body. The unmineralised region of bone is mainly composed of type I collagen and it is generally accepted that most of the OI phenotypes are caused by mutations in the genes encoding for type I collagen. The documented biochemical and genetic abnormalities have been described in detail in chapter 1.

In the fibril, collagen molecules are aligned in a parallel, but staggered manner, overlapping neighbours on both sides by a total of three quarters of their length. The collagen molecules attract and reinforce each other optimally in their configuration. In this arrangement orthogonal bands of polar amino acids take up heavy metal stains in a repeated 'bar code' pattern visible in electron microscopy (Scott 1995). In the linear arrangement there is a 35 nm separation between the adjacent ends, and each collagen molecule is staggered to its lateral neighbour by 67 nm (Hodge and Pertruska 1963). The repeating D-period of the collagen fibril is considered to have two regions, a densely packed overlap region (0.4D in length) and a gap region (0.6D in length). Hodge and Pertruska (1963) suggested that this gap region would be an ideal location for apatite crystals to nucleate in the process of mineralisation.

Fibril diameter can vary with the tissue and the developmental stage of that tissue. The young fibrils are cylindrical and often thinner than in the older tissue (Parry et al 1982). The fibril-forming process can be regulated by several mechanisms. It can be regulated by other collagens (i.e. types III and V), extracellular matrix proteins and polymers (proteoglycan, link protein), and also by structural variations in the collagen chains such as retention of amino terminal propeptides or the presence of hydroxylysine which may interfere with cross-linking.

A few authors have measured the diameter of type I collagen in bone, skin, periosteum and aorta in OI patients. Apart from the studies conducted by Stoss and Freisinger (1993) and Cassella et al (1994a), most of these reports comprised the measurements from one or two specimens and there seems to be some controversy over the diameter of osteoid collagen fibrils. Stoss and Freisinger claimed that the OI bone osteoid contained collagen of smaller diameter than normal controls while Cassella et al (1994a) reported that the OI bone osteoid contained collagen of larger diameter.
Riley et al. (1973) reported that OI bone collagen diameter was normal but corneal collagen fibril diameter was thinner than normal. Jones et al. (1984) carried out a morphometric study of bone osteoid collagen from 4 patients with OI type I and found that the bone collagen fibril diameters were between 40-60 nm, whereas the normal bone collagen fibril diameters ranged between 60-80 nm. Shapiro et al. (1983) examined a bone biopsy from a patient with OI and Paget's disease, using TEM, and reported that the bone collagen fibrils were reduced in number and decreased in diameter. Brenner et al. (1993) reported variable diameter collagen fibrils in a patient with Bruck syndrome and OI.

Small diameter collagen has also been observed in OI patients by several workers (de Matteis and Bonnucci 1968; Haebara et al. 1969; Smith et al. 1975; Byers et al. 1983; Balle et al. 1984; Flint et al. 1984).

Stoss and Freisinger (1993) measured the bone osteoid collagen diameter from 82 cases of OI and reported smaller collagen diameters in all OI types. Furthermore when classified according to clinical types, OI type II showed the smallest diameter, type I showed a slightly larger diameter and type III and IV showed the largest diameter. Although they stated that the smaller the diameter, the more severe the clinical picture, they have not given an explanation for the markedly decreased diameter observed in type I, the mildest type of OI, nor have they given the mean normal bone collagen diameter or mentioned the details of the controls (whether age- and site-matched).

Cassella et al. (1994a) carried out a morphometric analysis of the bone osteoid collagen diameter from 23 OI patients and 20 normal controls, and reported that the mean diameter for OI was 61 nm and for normal controls was 53 nm. They did not classify the patients according to the clinical types of OI.

Agerholm et al. (1994) recently performed a morphometric study on collagen diameter in skin, tendon and ligaments from eight calves with OI, and reported that the skin collagen diameter was slightly decreased but ligament and tendon collagen diameters were greatly reduced when compared with normals.

de Matteis and Bonnucci (1968) reported the periodicity of collagen as 340 Å while Haebara et al. (1969) reported it to be 200-400 Å in OI type II. Cassella (1993) could not find any difference between the normal and OI bone collagen periodicity.

**Aims of this chapter**

There seems to be some discrepancy over the range of bone collagen diameter in OI as published in the literature and there is not sufficient information regarding normal bone
osteoid collagen diameter. The ultrastructural observations described in chapter 2 on OI specimens showed thinner collagen fibrils in the osteoid of OI type II patients. The aim of this chapter was to resolve this matter by measuring osteoid collagen fibrils in 42 OI and 25 normal control subjects. The majority of these specimens have been age- and site-matched to scale out artefacts. Furthermore an attempt has been made to correlate the corresponding findings with the clinically different types of OI.

The reported abnormalities in collagen fibrillogenesis in the majority of OI patients, together with the documented evidence of misalignment of the gap region of bone collagen in the omi/omi mouse model of OI (Landis 1995; Fratzl et al 1996), intiated the necessity for measuring the periodicity, at least on patients with known collagen abnormalities. Hence measurements of the periodicity of bone collagen from 15 patients of different OI types, 2 normal foetal and 3 normal juvenile/adult control specimens was also undertaken.
4.2 Materials and Methods

Bone specimens studied were from 15 OI type I patients ages ranging from 3 to 42 years, 10 type II foetuses ages ranging from 16 weeks gestation to full term, 8 OI type II/III foetuses ages ranging from 18 weeks gestation to full term, 5 OI type III patients ages ranging from birth to 7 years, 4 OI type IV patients ages ranging from 9 to 28 years, 7 normal foetuses ranging from 19 to 24 weeks gestation and 18 normal juvenile/adults ages ranging from 2 to 30 years.

Bone tissue from OI patients and normals (mostly from mid-shaft femur) was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Agar Scientific Ltd., Essex, England) for 2 hours. During the first hour of fixation, specimens were sub-divided into 1 mm³ pieces. After two washes in buffer, the secondary fixation with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer was carried out for 1 hour at room temperature. These specimens were dehydrated, infiltrated with araldite CY212 resin and embedded as described in chapter 2. Sectioning procedures and staining protocols were also as described in chapter 2.

The stained sections were viewed on a Philips CM12 electron microscope. A suitable area of osteoid showing good cross-sectional collagen fibrils was selected and micrographs were taken in a random sweep along the osteoid at a standardised magnification of 45,000. At the beginning and end of each session, a diffraction grating replica (carbon grating 2150 lines/mm; Agar Scientific Ltd) was photographed at the same magnification in order to calibrate the microscope for standardised measurements of collagen fibre diameter.

Histomorphometry is the indirect measurement of the size and configuration of three dimensional structures obtained from a number of representative two dimensional sections, which enables subjective observations to be converted into quantitative information. In this study histomorphometric analysis of osteoid collagen diameter was carried out on an Apple Macintosh Quadra 900 computer using an OptiLab 24/2.2 image analysis program.

The electron micrographs were placed on a light box and the edges of the collagen fibres were circled using a fine tip marker pen. Images were captured using a CCD camera (Fig. 4.1) and each micrograph was calibrated in the computer. The areas of the digitised images of collagen fibres (Fig. 4.2) were measured by the OptiLab 24/2.2 image analysis program. The diameter of each fibril was determined using a formula \( \pi r^2 \) on a Microsoft Excel 4.0 program on an Apple Macintosh Performa 475 computer. Wherever possible a minimum of 300 fibrils were measured in each specimen. A reproducibility test was performed to see whether subjective errors had occurred by circling the collagen fibres manually. The collagen fibres were circled on ten prints from the same micrograph, the
diameters were measured and the coefficient of variation (CV) calculated. This procedure was repeated twice (Table 4.1).

The periodicity of the collagen fibres was measured for a few patients with known collagen abnormalities. Ultra-thin sections were stained with 5% phosphotungstic acid (Agar Scientific Ltd.) for one hour, with uranyl acetate for ten minutes, and viewed on a Philips CM12 electron microscope. Over fifty periods from about 20 fibrils were measured for each specimen. Micrographs were taken at 60,000 magnification and periods were measured manually. The microscope was calibrated using a catalase crystal grid (8.67 nm; Agar Scientific Ltd.) before each session.
4.3 Results

Ultrastructurally the morphological appearance of collagen fibres from OI patients (Figs. 4.3b, 4.3d, 4.3f) was similar to the normal controls (Figs. 4.3a and 4.3c) apart from OI type III. The collagen fibrils of three patients from this group were spiralled and did not contain a smooth outline (Fig. 4.3e). The collagen fibrils from all the patients from OI type II were visibly smaller than age-matched control collagen fibrils (Fig. 4.3d).

The results of the reproducibility test for the diameter measurements of collagen fibrils are shown in Table 4.1. The coefficient of variation for one set was 1.9% and for the other set it was 3%. These results indicate that this method is highly reproducible for estimating fibril diameter.

The results of the normal foetal and juvenile bone collagen diameter are shown in Tables 4.2.1 and 4.2.2. The diameter for normal foetal bone collagen ranged from 60-73 nm and the normal juvenile bone collagen diameter ranged from 64-88 nm.

The OI type I bone collagen diameters ranged from 50-64 nm (Table 4.3), while type II bone collagen diameters ranged from 36-53 nm (Table 4.4), which confirms the visual appearance in EM. The diameters for other OI types ranged from 51-72 nm for type II/III, 62-80 nm for type III and 60-69 nm for type IV (Tables 4.5, 4.6 and 4.7).

The bar graph showing computed mean diameter for bone collagen in OI and normal controls is shown in Fig. 4.4. All groups were tested for parametric distribution and were subjected to analysis of variance (ANOVA). Comparison of group means after ANOVA was performed by the Tukey-Kramer Honestly Significant Difference (HSD) test. The results indicate that there is a highly significant difference between OI type II and normal foetal bone collagen diameter and between OI type I and normal control bone collagen diameter (p<.01), but there is no significant difference between the OI types III and IV with normal bone collagen diameter (Fig. 4.4). The bone collagen fibres from type III patients were not circular but were angular or spiralled (Fig. 4.3e), hence the larger diameter observed in OI type III bone in this study (see chapter 2).

A study of the frequency of bone collagen fibril diameter was carried out on a selected number of patients from different OI types and normals. Normal juvenile and foetal bone collagen diameters gave a normal distribution curve with the majority of fibres belonging to the 71-80 nm group (Figs. 4.5 and 4.9). OI types III (Fig. 4.7) and type IV (Fig. 4.8) also gave normal distribution curves but the majority of fibres were of smaller diameter (51-60 nm for type III and 61-70 nm for type IV). The collagen diameters for OI type I demonstrated a very narrow range between 41-70 nm (Fig. 4.6) with the vast majority of fibres measuring 51-60 nm. The frequency distribution for type II collagen
fibres was a skew curve (Fig. 4.10) with the majority of fibres measuring 31-40 nm, which was significantly lower than the normal foetal bone collagen diameter (Fig. 4.9).

The periodicity of collagen fibres was measured on a selected number of patients with known collagen abnormalities and the results are shown in Table 4.8; Baby V.D.K, OI type II/III had not been included in the study of collagen diameter. The specimens were stained with 5% PTA and 2% uranyl acetate as reported by Chapman (1974). The estimated axial period for resin-embedded collagen fibres is about 64 nm, while in wet state collagen fibres it is around 67 nm (Hodge and Pertruska 1963). In this study the axial period obtained for the five normal controls ranged from 60 - 65 nm. Obtaining a value that is in good agreement with the estimated collagen fibril periodicity shows that the methodology used here was appropriate.

All groups were tested for parametric distribution and were subjected to analysis of variance (ANOVA). Comparison of group means with the controls was performed by the Dunnett's method. There was a significant difference between OI type II collagen fibril D-periods and those of normal controls. Foetus W (25 weeks), with modified collagens and retained pN propeptides (Fig. 4.11c), demonstrated the shortest period of 52 nm which varied markedly from normal foetal collagen period (mean D-period 63 nm). There seems to be no statistical difference between the periodicity of collagens in other OI types and normal controls but greater number of samples should be measured in order to confirm this finding.

Photographs of gel electrophoresis of the collagen chains from selected patients used in this study (Fig. 4.11) is included here to illustrate some of the different biochemical abnormalities (supplied by Dr. M. Pope).
Fig. 4.1   Apple Macintosh Quadra Image analysis system used to measure the collagen fibres in this study. The micrograph with the circled collagen fibres was placed on the light box and the image was captured by the CCD camera. Digitised images were analysed by the computer.

Fig. 4.2   The analogue images of the fibrils which have been outlined are converted into diameters.
Fig. 4.3a  Electron micrograph of transverse collagen fibres from a section of normal juvenile bone (F.D, 10 years).
Bar = 104 nm

Fig. 4.3b  Electron micrograph of transverse collagen fibres from a section of OI type I bone (A.C, 10 years) shown at the same magnification as the control (Fig. 4.3a).
Bar = 104 nm

Fig. 4.3c  Electron micrograph of the transverse collagen fibres from a section of normal foetal bone (Foetus Sy, 22 weeks).
Bar = 104 nm

Fig. 4.3d  Electron micrograph of transverse collagen fibres from a section of OI type II bone (Foetus Me, 16 weeks) shown at the same magnification as the foetal control (Fig. 4.3c). Note the markedly reduced diameter in OI type II collagen fibres when compared with the age- and site-matched control.
Bar = 104 nm

Fig. 4.3e  Electron micrograph of transverse collagen fibres from a section of OI type III bone (H.S, 3 years) shown at the same magnification as the control (Fig. 4.3a). Note the spiralled collagen fibres.
Bar = 104 nm

Fig. 4.3f  Electron micrograph of transverse collagen fibres from a section of OI type IV bone (J.D, 9 years) shown at the same magnification as the control (Fig. 4.3a).
Bar = 104 nm
Table 4.1

Reproducibility test for collagen fibril measurements.

<table>
<thead>
<tr>
<th>Negative number</th>
<th>J988 diameter (nm)</th>
<th>J962 diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.24</td>
<td>73.99</td>
</tr>
<tr>
<td>2</td>
<td>65.88</td>
<td>73.79</td>
</tr>
<tr>
<td>3</td>
<td>64.84</td>
<td>72.03</td>
</tr>
<tr>
<td>4</td>
<td>67.13</td>
<td>70.98</td>
</tr>
<tr>
<td>5</td>
<td>64.66</td>
<td>70.44</td>
</tr>
<tr>
<td>6</td>
<td>68.53</td>
<td>68.19</td>
</tr>
<tr>
<td>7</td>
<td>66.50</td>
<td>71.96</td>
</tr>
<tr>
<td>8</td>
<td>66.50</td>
<td>68.69</td>
</tr>
<tr>
<td>9</td>
<td>66.38</td>
<td>74.70</td>
</tr>
<tr>
<td>10</td>
<td>64.58</td>
<td>71.14</td>
</tr>
<tr>
<td>Mean</td>
<td>66.22</td>
<td>71.59</td>
</tr>
<tr>
<td>SD</td>
<td>1.27</td>
<td>2.17</td>
</tr>
<tr>
<td>SE</td>
<td>0.15</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 4.2.1
Results of normal foetal control bone collagen fibril diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus Gy</td>
<td>19 weeks, male</td>
<td>femur</td>
<td>659</td>
<td>63.50</td>
<td>12.66</td>
<td>1.42</td>
</tr>
<tr>
<td>Foetus D</td>
<td>20 weeks, male</td>
<td>femur</td>
<td>334</td>
<td>64.59</td>
<td>9.14</td>
<td>1.03</td>
</tr>
<tr>
<td>Foetus M</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>167</td>
<td>65.49</td>
<td>12.51</td>
<td>1.36</td>
</tr>
<tr>
<td>Foetus Sy</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>194</td>
<td>67.09</td>
<td>9.62</td>
<td>1.25</td>
</tr>
<tr>
<td>Foetus T</td>
<td>23 weeks, male</td>
<td>femur</td>
<td>463</td>
<td>72.40</td>
<td>8.80</td>
<td>1.04</td>
</tr>
<tr>
<td>Foetus G</td>
<td>24 weeks, male</td>
<td>not known</td>
<td>496</td>
<td>60.47</td>
<td>7.54</td>
<td>0.97</td>
</tr>
<tr>
<td>Foetus L</td>
<td>24 weeks, male</td>
<td>femur</td>
<td>389</td>
<td>72.72</td>
<td>10.22</td>
<td>1.19</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>66.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td>4.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td></td>
<td></td>
<td>1.71</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2.2
Results of normal juvenile/adult control bone collagen fibril diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.N.D</td>
<td>2 years, male</td>
<td>iliac crest</td>
<td>348</td>
<td>69.25</td>
<td>8.90</td>
<td>1.18</td>
</tr>
<tr>
<td>M.D</td>
<td>4 years, male</td>
<td>not known</td>
<td>436</td>
<td>69.38</td>
<td>11.82</td>
<td>1.50</td>
</tr>
<tr>
<td>B.A</td>
<td>8 years, male</td>
<td>not known</td>
<td>250</td>
<td>85.09</td>
<td>12.92</td>
<td>1.31</td>
</tr>
<tr>
<td>H.C</td>
<td>8 years, female</td>
<td>femur</td>
<td>483</td>
<td>63.98</td>
<td>8.54</td>
<td>1.04</td>
</tr>
<tr>
<td>R.M</td>
<td>9 years, male</td>
<td>not known</td>
<td>480</td>
<td>65.54</td>
<td>9.66</td>
<td>1.32</td>
</tr>
<tr>
<td>F.D</td>
<td>10 years, male</td>
<td>iliac crest</td>
<td>440</td>
<td>76.31</td>
<td>13.94</td>
<td>1.85</td>
</tr>
<tr>
<td>M.F</td>
<td>10 years, male</td>
<td>femur</td>
<td>460</td>
<td>77.26</td>
<td>11.63</td>
<td>1.35</td>
</tr>
<tr>
<td>V. B</td>
<td>10 years, female</td>
<td>femur</td>
<td>360</td>
<td>72.72</td>
<td>12.09</td>
<td>1.42</td>
</tr>
<tr>
<td>M.K</td>
<td>11 years, male</td>
<td>femur</td>
<td>473</td>
<td>68.50</td>
<td>10.73</td>
<td>1.34</td>
</tr>
<tr>
<td>A.H.A</td>
<td>12 years, male</td>
<td>tibia</td>
<td>250</td>
<td>66.23</td>
<td>11.70</td>
<td>1.41</td>
</tr>
<tr>
<td>K.S</td>
<td>13 years, female</td>
<td>not known</td>
<td>606</td>
<td>85.96</td>
<td>11.89</td>
<td>1.21</td>
</tr>
<tr>
<td>D.S</td>
<td>14 years, male</td>
<td>not known</td>
<td>530</td>
<td>88.06</td>
<td>13.65</td>
<td>1.39</td>
</tr>
<tr>
<td>P.K</td>
<td>16 years, male</td>
<td>femur</td>
<td>493</td>
<td>68.48</td>
<td>13.44</td>
<td>1.88</td>
</tr>
<tr>
<td>P.C</td>
<td>21 years, male</td>
<td>iliac crest</td>
<td>440</td>
<td>79.22</td>
<td>11.96</td>
<td>1.29</td>
</tr>
<tr>
<td>J.C</td>
<td>21 years, female</td>
<td>iliac crest</td>
<td>337</td>
<td>74.23</td>
<td>9.25</td>
<td>1.10</td>
</tr>
<tr>
<td>N.G</td>
<td>25 years, female</td>
<td>iliac crest</td>
<td>524</td>
<td>75.05</td>
<td>13.17</td>
<td>1.17</td>
</tr>
<tr>
<td>D.St</td>
<td>27 years, male</td>
<td>tibia</td>
<td>195</td>
<td>70.88</td>
<td>15.23</td>
<td>1.76</td>
</tr>
<tr>
<td>D.F</td>
<td>30 years, male</td>
<td>femur</td>
<td>309</td>
<td>66.54</td>
<td>7.53</td>
<td>0.91</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>73.48</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>7.30</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SE</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>1.77</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.3

Results of OI Type I bone collagen diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. S. A</td>
<td>type IA</td>
<td>3 years, male</td>
<td>femur</td>
<td>314</td>
<td>59.39</td>
<td>12.07</td>
<td>1.46</td>
<td>not tested</td>
</tr>
<tr>
<td>S. V</td>
<td>type IA</td>
<td>8 years, male</td>
<td>tibia</td>
<td>674</td>
<td>52.48</td>
<td>9.80</td>
<td>1.39</td>
<td>not tested</td>
</tr>
<tr>
<td>R. M</td>
<td>type IB</td>
<td>9 years, male</td>
<td>femur</td>
<td>506</td>
<td>53.49</td>
<td>8.48</td>
<td>1.17</td>
<td>not tested</td>
</tr>
<tr>
<td>M. G</td>
<td>type IA</td>
<td>9 years, male</td>
<td>iliac crest</td>
<td>926</td>
<td>58.65</td>
<td>8.31</td>
<td>1.25</td>
<td>not tested</td>
</tr>
<tr>
<td>H. M</td>
<td>type IB</td>
<td>10 years, female</td>
<td>iliac crest</td>
<td>801</td>
<td>55.52</td>
<td>7.18</td>
<td>0.91</td>
<td>not tested</td>
</tr>
<tr>
<td>A. C</td>
<td>type IB</td>
<td>10 years, male</td>
<td>iliac crest</td>
<td>671</td>
<td>64.35</td>
<td>10.35</td>
<td>1.19</td>
<td>not tested</td>
</tr>
<tr>
<td>G. A</td>
<td>type IA</td>
<td>10 years, female</td>
<td>tibia</td>
<td>365</td>
<td>61.72</td>
<td>10.35</td>
<td>1.21</td>
<td>not tested</td>
</tr>
<tr>
<td>N. L</td>
<td>type IA</td>
<td>12 years, male</td>
<td>tibia</td>
<td>687</td>
<td>54.28</td>
<td>7.50</td>
<td>0.96</td>
<td>overmodified α1 &amp; α2 chains</td>
</tr>
<tr>
<td>A. A</td>
<td>type IA</td>
<td>12 years, male</td>
<td>femur</td>
<td>985</td>
<td>51.74</td>
<td>6.60</td>
<td>0.88</td>
<td>not tested</td>
</tr>
<tr>
<td>A. Mc</td>
<td>type IA</td>
<td>14 years, male</td>
<td>not known</td>
<td>368</td>
<td>60.41</td>
<td>9.60</td>
<td>1.26</td>
<td>not tested</td>
</tr>
<tr>
<td>S. D</td>
<td>type IA</td>
<td>14 years, female</td>
<td>tibia</td>
<td>401</td>
<td>59.99</td>
<td>11.18</td>
<td>1.33</td>
<td>not tested</td>
</tr>
<tr>
<td>A. M</td>
<td>type IB</td>
<td>16 years, male</td>
<td>iliac crest</td>
<td>514</td>
<td>50.87</td>
<td>7.48</td>
<td>1.00</td>
<td>not tested</td>
</tr>
<tr>
<td>J. W</td>
<td>type IA</td>
<td>30 years, female</td>
<td>ulna</td>
<td>473</td>
<td>56.17</td>
<td>9.27</td>
<td>1.26</td>
<td>not tested</td>
</tr>
<tr>
<td>C. W</td>
<td>type IA</td>
<td>42 years, male</td>
<td>iliac crest</td>
<td>412</td>
<td>60.62</td>
<td>11.11</td>
<td>0.34</td>
<td>not tested</td>
</tr>
<tr>
<td>N.N</td>
<td>type IA</td>
<td>not recorded</td>
<td>not recorded</td>
<td>487</td>
<td>49.76</td>
<td>8.98</td>
<td>1.32</td>
<td>not tested</td>
</tr>
</tbody>
</table>

Average                                | 56.63 |
SD                                        | 4.45  |
SE                                         | 1.15  |
# Table 4.4
Results of OI type II bone collagen diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus Me type II</td>
<td>16 weeks, male</td>
<td>femur</td>
<td>513</td>
<td>40.27</td>
<td>5.82</td>
<td>0.91</td>
<td>overmodified α1 &amp; α2 chains</td>
<td></td>
</tr>
<tr>
<td>Foetus Ki type II</td>
<td>16 weeks, female</td>
<td>rib</td>
<td>541</td>
<td>43.31</td>
<td>5.16</td>
<td>0.74</td>
<td>double α1 &amp; α2 chains</td>
<td></td>
</tr>
<tr>
<td>Foetus F type II</td>
<td>19 weeks, female</td>
<td>femur</td>
<td>614</td>
<td>47.60</td>
<td>6.90</td>
<td>1.01</td>
<td>overmodified α1 chain</td>
<td></td>
</tr>
<tr>
<td>Foetus S type II</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>1034</td>
<td>36.25</td>
<td>5.33</td>
<td>0.80</td>
<td>not tested</td>
<td></td>
</tr>
<tr>
<td>Foetus D type II</td>
<td>22 weeks, female</td>
<td>rib</td>
<td>452</td>
<td>50.14</td>
<td>7.75</td>
<td>1.04</td>
<td>not tested</td>
<td></td>
</tr>
<tr>
<td>Foetus Mo type II</td>
<td>22 weeks, femur or tibia</td>
<td>femur</td>
<td>274</td>
<td>50.81</td>
<td>6.69</td>
<td>1.01</td>
<td>not tested</td>
<td></td>
</tr>
<tr>
<td>Foetus T-VI type II</td>
<td>23 weeks, male</td>
<td>femur</td>
<td>1052</td>
<td>46.33</td>
<td>5.88</td>
<td>0.94</td>
<td>modified α1 &amp; α2 chains (Fig. 4.11b)</td>
<td></td>
</tr>
<tr>
<td>Foetus W type II</td>
<td>25 weeks, female</td>
<td>femur</td>
<td>547</td>
<td>44.77</td>
<td>5.38</td>
<td>0.78</td>
<td>overmodified α1 &amp; α2 chains retention of pN propeptides (Fig. 4.11c)</td>
<td></td>
</tr>
<tr>
<td>Baby Ko type II</td>
<td>perinatal, male</td>
<td>femur</td>
<td>763</td>
<td>41.91</td>
<td>4.09</td>
<td>0.67</td>
<td>overmodified α1 &amp; α2 chains</td>
<td></td>
</tr>
<tr>
<td>Baby A type II</td>
<td>perinatal, female</td>
<td>femur</td>
<td>265</td>
<td>52.54</td>
<td>7.70</td>
<td>0.95</td>
<td>double α1 chain. (Fig. 4.11a)</td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.62</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 4.5
Results of OI type II/III bone collagen diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus O</td>
<td>type II/III</td>
<td>18 weeks, male</td>
<td>rib</td>
<td>446</td>
<td>53.17</td>
<td>9.70</td>
<td>1.38</td>
<td>not tested</td>
</tr>
<tr>
<td>Foetus Ar-II</td>
<td>type II/III</td>
<td>20 weeks, male</td>
<td>femur or tibia</td>
<td>301</td>
<td>72.31</td>
<td>11.39</td>
<td>1.24</td>
<td>normal α1 &amp; α2 chains</td>
</tr>
<tr>
<td>Foetus F.G</td>
<td>type II/III</td>
<td>22 weeks, female</td>
<td>rib</td>
<td>630</td>
<td>66.56</td>
<td>12.26</td>
<td>1.63</td>
<td>overmodified α1 &amp; α2 chains</td>
</tr>
<tr>
<td>Foetus Ar-III</td>
<td>type II/III</td>
<td>22 weeks, male</td>
<td>femur</td>
<td>403</td>
<td>60.00</td>
<td>9.77</td>
<td>1.33</td>
<td>normal α1 &amp; α2 chains</td>
</tr>
<tr>
<td>Foetus Gr</td>
<td>type II/III</td>
<td>22 weeks, male</td>
<td>not known</td>
<td>432</td>
<td>64.98</td>
<td>12.12</td>
<td>1.50</td>
<td>not tested</td>
</tr>
<tr>
<td>Baby G- III</td>
<td>type II/III</td>
<td>perinatal, female</td>
<td>tibia</td>
<td>670</td>
<td>51.40</td>
<td>13.87</td>
<td>1.97</td>
<td>overmodified α1 &amp; α2 chains</td>
</tr>
<tr>
<td>Baby G-II</td>
<td>type II/III</td>
<td>not recorded</td>
<td>rib</td>
<td>328</td>
<td>62.66</td>
<td>14.46</td>
<td>1.77</td>
<td>not tested</td>
</tr>
<tr>
<td>Baby Ak-II</td>
<td>type II/III</td>
<td>not recorded</td>
<td>femur</td>
<td>694</td>
<td>53.16</td>
<td>8.58</td>
<td>1.04</td>
<td>modified α1 &amp; α2 chains</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60.53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.03</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.6
Results of OI type III bone collagen diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.S</td>
<td>type III</td>
<td>still birth, female</td>
<td>femur</td>
<td>478</td>
<td>62.51</td>
<td>7.77</td>
<td>0.95</td>
<td>not tested</td>
</tr>
<tr>
<td>B.G</td>
<td>type III</td>
<td>3 years, male</td>
<td>not known</td>
<td>285</td>
<td>66.52</td>
<td>10.51</td>
<td>1.19</td>
<td>normal α1 &amp; α2 chains</td>
</tr>
<tr>
<td>H.S</td>
<td>type III</td>
<td>3 years, male</td>
<td>femur</td>
<td>330</td>
<td>79.53</td>
<td>17.27</td>
<td>1.76</td>
<td>normal α1 &amp; α2 chains (Fig. 4.11b)</td>
</tr>
<tr>
<td>H.G</td>
<td>type III</td>
<td>6 years, female</td>
<td>not known</td>
<td>482</td>
<td>65.62</td>
<td>12.62</td>
<td>1.54</td>
<td>normal α1 &amp; α2 chains</td>
</tr>
<tr>
<td>K.W</td>
<td>type III</td>
<td>7 years, female</td>
<td>femur</td>
<td>365</td>
<td>61.73</td>
<td>12.35</td>
<td>1.35</td>
<td>not tested</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>63.09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.17</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

143
### Table 4.7
Results of OI type IV bone collagen diameter measurements and other sample details.

<table>
<thead>
<tr>
<th>Name</th>
<th>OI type</th>
<th>Age &amp; sex</th>
<th>Site of biopsy</th>
<th>Number of fibrils</th>
<th>Computed mean diameter (nm)</th>
<th>SD</th>
<th>SE</th>
<th>Collagen abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.D</td>
<td>type IV</td>
<td>9 years, male</td>
<td>femur</td>
<td>709</td>
<td>64.50</td>
<td>8.93</td>
<td>1.03</td>
<td>not tested</td>
</tr>
<tr>
<td>Z.A</td>
<td>type IV</td>
<td>14 years, female</td>
<td>iliac crest</td>
<td>660</td>
<td>62.34</td>
<td>8.78</td>
<td>1.20</td>
<td>not tested</td>
</tr>
<tr>
<td>M.K.</td>
<td>type IV</td>
<td>25 years, female</td>
<td>tibia</td>
<td>596</td>
<td>59.99</td>
<td>9.01</td>
<td>1.12</td>
<td>not tested</td>
</tr>
<tr>
<td>K.L</td>
<td>type IV</td>
<td>28 years, male</td>
<td>not known</td>
<td>205</td>
<td>68.81</td>
<td>12.81</td>
<td>1.67</td>
<td>not tested</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>63.91</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>3.75</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>1.88</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

144
Fig. 4.4  Bar graph showing the computed mean diameter of bone osteoid collagen in OI types and normal foetal and normal young controls. Each error bar represents the standard error of the mean.
Fig. 4.5 The frequency distribution of type I collagen fibres for a normal control. The majority of fibres are in 71-80 nm group.

![Frequency distribution for normal control](image)

Fig. 4.6 The frequency distribution of type I collagen fibres for an OI type I patient. The majority of fibres are in the 51-60 nm group.

![Frequency distribution for OI type I patient](image)
Fig. 4.7 The frequency distribution of type I collagen fibres for an OI type III patient. The majority of collagen fibres are in the 51-60 nm group.

Fig. 4.8 The frequency distribution of type I collagen fibres for an OI type IV patient. The majority of collagen fibres are in 61-70 nm group.
Fig. 4.9  The frequency distribution of type I collagen fibres for a normal foetal control. The majority of collagen fibres are in 71-80 nm group.

Fig. 4.10  The frequency distribution of type I collagen fibres for an OI type II patient demonstrating abnormal distribution. The majority of collagen fibres are in the 31-40 nm group.
Table 4.8  Measurements of bone collagen fibre periodicity.

<table>
<thead>
<tr>
<th>Name</th>
<th>Age &amp; sex</th>
<th>OI type /normal</th>
<th>periodicity (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus Sy</td>
<td>22 weeks, male</td>
<td>normal</td>
<td>60.08</td>
</tr>
<tr>
<td>Foetus L</td>
<td>24 weeks, male</td>
<td>normal</td>
<td>65.05</td>
</tr>
<tr>
<td>M.D</td>
<td>4 years, male</td>
<td>normal</td>
<td>60.00</td>
</tr>
<tr>
<td>F.D</td>
<td>10 years, male</td>
<td>normal</td>
<td>64.90</td>
</tr>
<tr>
<td>N.G</td>
<td>25 years, female</td>
<td>normal</td>
<td>65.17</td>
</tr>
<tr>
<td>N.L</td>
<td>12 years, male</td>
<td>type I</td>
<td>58.87</td>
</tr>
<tr>
<td>S.D</td>
<td>14 years, female</td>
<td>type I</td>
<td>56.69</td>
</tr>
<tr>
<td>C.W</td>
<td>42 years, male</td>
<td>type I</td>
<td>66.35</td>
</tr>
<tr>
<td>Foetus Mc</td>
<td>16 weeks, male</td>
<td>type II</td>
<td>54.51</td>
</tr>
<tr>
<td>Foetus Ki</td>
<td>16 weeks, female</td>
<td>type II</td>
<td>60.93</td>
</tr>
<tr>
<td>Foetus F</td>
<td>19 weeks, female</td>
<td>type II</td>
<td>58.75</td>
</tr>
<tr>
<td>Foetus S</td>
<td>22 weeks, male</td>
<td>type II</td>
<td>54.81</td>
</tr>
<tr>
<td>Foetus W</td>
<td>25 weeks, female</td>
<td>type II</td>
<td>52.28</td>
</tr>
<tr>
<td>Foetus Ar-II</td>
<td>20 weeks, male</td>
<td>type II/III</td>
<td>60.17</td>
</tr>
<tr>
<td>Foetus Ar-III</td>
<td>22 weeks, male</td>
<td>type II/III</td>
<td>60.17</td>
</tr>
<tr>
<td>Baby V.D.K</td>
<td>full term, male</td>
<td>type II/III</td>
<td>58.87</td>
</tr>
<tr>
<td>B.G</td>
<td>3 years, male</td>
<td>type III</td>
<td>55.63</td>
</tr>
<tr>
<td>K.W</td>
<td>7 years, female</td>
<td>type III</td>
<td>65.70</td>
</tr>
<tr>
<td>J.D</td>
<td>9 years, male</td>
<td>type IV</td>
<td>60.48</td>
</tr>
<tr>
<td>K.L</td>
<td>28 years, male</td>
<td>type IV</td>
<td>60.64</td>
</tr>
</tbody>
</table>
Fig. 4.11a Radio-labelled collagen α chains secreted into the culture medium and separated by polyacrylamide gel electrophoresis. Normal control patterns are shown in tracks 1 & 3 compared with OI type II (Baby A) shown in tracks 2 & 4. The latter show two forms of α1(I) and slightly smeared α2(I) chains.

a = α1(I) chains, b = α2(I) chains, c = α1(III) chains.

Fig. 4.11b Radio-labelled collagen α chains secreted into the culture medium and separated by polyacrylamide gel electrophoresis. Lanes 2, 3 & 4 are from three normal controls. Lanes 1 & 5 are from two different forms of OI. In lane 1 (OI type III, H.S) there is no change in α chain migration (arrowhead), whilst in lane 5 (OI type II, Foetus T-VI), α1(I) and α2(I) are overmodified (stepped) and there is a relative increase of α1(III) chain.

a = α1(I) chains, b = α2(I) chains, c = α1(III) chains.

Fig. 4.11c Procollagen 'ladder' from severely affected OI type II foetus (Foetus W) in lanes 2 & 3 compared with normal controls in lanes 1 & 4. The affected foetus processes collagen poorly so that there are multiple gaps in the progression from procollagen type I to α1(I) and α2(I) collagen. There is a retention of pN α1(I) and no α2(I) chains.

a = α2(I), b = pN α2, c = α1(I), d = pro(I), e = pro(III)

Fig. 4.11d Normal procollagen 'ladder' in a series of controls showing good conversion of procollagen type I (*) to α1 and α2.

a = α2(I), b = pN α2, c = α1(I), d = pro(I), e = pro(III)
4.4 Discussion

The distribution of collagen fibril diameter in this study of OI bone does not necessarily correlate with disease severity. Thus, the OI type II (the severest type) demonstrated the smallest diameter (45 nm), followed by OI type I (the mildest form) with a mean diameter of 57 nm. The diameters obtained for type III (67 nm) and type IV (64 nm) were lower than the normal control mean diameter (73 nm) but did not show a statistical difference.

To date there have been no in-depth reports on the normal human bone osteoid collagen diameter, apart from the study conducted by Cassella et al (1994a). They measured the bone osteoid collagen diameter from twenty normal controls (ages ranging from 1 to 26 years) and reported the bone collagen mean diameter as 53 nm, which is lower than the value obtained in this study. In the present study seven normal foetal and eighteen juvenile/adult controls were used to measure the bone collagen diameter; the mean value for foetal bone was 67 nm and for juvenile/adult bone was 73 nm. This value is similar to the value of 60-80 nm reported by Jones et al (1984) after studying four normal bone specimens.

The tissue processing for TEM which involves fixation, dehydration, infiltration and embedding might artefactually alter the collagen fibril diameter. Since the same procedure was followed for normal and OI specimens, these artefactual changes of tissue processing are unlikely to account for the differences observed; the comparative values presented here are not absolute but relative.

The collagen fibril diameter can be controlled by several factors. Parry et al (1982) reported that tissues with the smallest diameter have a high level of hyaluronic acid, and the tissues with largest diameter have a high concentration of dermatan sulphate; they suggested that lateral growth of fibrils beyond 60 nm is inhibited by the presence of excessive hyaluronic acid, and this inhibitory effect may be removed by an increasing concentration of chondroitin sulphate or dermatan sulphate. Fedarko et al (1992) reported high levels of hyaluronan in OI bone cells from type II patients, and the smallest collagen fibril diameter observed in OI type II bone could be due to this reason. Furthermore chondroitin sulphate PG observed in type II/III bone (chapter 7) may be a possible explanation for the larger diameter observed in this group. However the majority of mutations in OI type II have glycine substitutions in the triple helical domain of pro-α1 or pro-α2 chains which results in abnormal and unstable type I collagen (Vogel et al 1988); there is also a complementary increase in type III and V collagens (Pope et al 1980). All these factors may contribute to the reduced diameter observed in OI types II and I.
Scott and Parry (1992) proposed that collagen fibrils grow by fusion from protofibrils of about 10 nm diameter, and that the process is controlled by interfibrillar proteoglycans and the polarity of neighbouring fibrils. In young tissues, fibrils are prevented from making intimate contact by abundant proteoglycans, while the polarity mechanism becomes important when fibrils go through cycles of stress and relaxation; frequent and forceful contacts between the fibrils do occur. In addition, if the fibrils are anti-parallel, fusion of protofibrils is prevented.

The collagen fibril diameter can also be regulated by other collagens i.e. collagens III and V. It was suggested that type V collagen plays a fundamental role in the control of fibrillogenesis by forming a core within the fibrils. Another characteristic of this collagen is the partial retention of the N-propeptide extensions in the tissue form (Fichard et al 1994). The retention of amino terminal propeptide of the collagen chain can also affect the diameter and will lead to the formation of thin fibrils (Miyahara et al 1984). It was shown by Fleischmajer et al (1987) that thick type I collagen fibrils apparently lack the amino propeptide as demonstrated by immunolabelling. It was interesting to note that Foetus W (OI type II), who showed overmodified α1 and α2 chains and retained the pN propeptides (Fig. 4.11c), demonstrated a markedly reduced collagen fibril diameter of 45 nm.

Another reason for the reduced diameter of type I collagen fibrils observed in OI type I bone could be the result of a failure of type I collagen to mature to the normal diameter. This could be due simply to insufficient collagen being produced per cell as histopathology suggests in many patients with OI type I, or it could be due to the presence of type III collagen of smaller diameter. Certain mutations of the collagen genes in OI type I can cause reduced synthesis of type I collagen (Willing et al 1992, 1994), hence increasing the type III/I ratio.

As described in chapter 2, OI type III demonstrated angular or hieroglyphic collagen with no definite outline. This group showed the largest diameter which could be due to the packing defect of individual protofibrils as previously described (Scott and Parry 1992).

Stoss and Friesinger's (1993) study showed a similar pattern in diameter variation within the OI groups although the diameter they obtained was smaller than in this study.

The importance of osteoid collagen in mineralisation is well documented and described in detail in chapter 3. The thinner fibrils in OI types I and II observed in this study, in combination with mineral abnormalities, may play an important role with regards to the bone fragility in OI.
The shorter D-period in collagen fibrils observed in some of the OI type II samples is important as it implicates the alterations of the gap region caused by distortions (shortening or bending) of triple helical molecules. As suggested by many authors (Hodge and Pertruska 1963; Glimcher 1985; Traub et al 1992; Landis 1995), if the initial mineral nucleation occurs in the gap region, the altered gap in OI type II could not accommodate the mineral crystals as well as the normal gap regions. This may lead to alterations in mineral propagation within the collagen fibres.
Chapter 5

Immunogold localisation of collagen types in OI bone

5.1 Introduction

As described in the previous chapters, bone is composed of an inorganic mineralised phase and an organic matrix produced by osteoblasts. The organic matrix contains various proteins, glycoproteins, peptides, carbohydrates, lipids and non-collagenous proteins, but the bulk of the osteoid is made up of type I collagen. Recent observations suggest that there are other collagen types, apart from type I, present in human bone; Keene et al (1991) reported that type III collagen was present in discrete fibre bundles throughout the bone cortex but was more concentrated at the Haversian canal surface and in the periosteum in foetal and young bone. Collagen type VI was present in discrete fibrils separate from type III collagen in foetal bone but restricted to the margins of bone surfaces and cells in young bone.

Type I collagen, as described in chapter 1, is made up of two α1 chains and one α2 chain wound together to form a right-handed super triple helix. These combine to form large diameter 67 nm-banded fibrils. Type III collagen is a homotrimer of three α1 chains, α1(III)3, which form small diameter 67 nm-banded fibres. Type IV collagen is non-fibrillar and has independently associated α1, α2, α3, α4 and α5 (IV) components. Type V collagen forms 12 nm non-banded fibrils comprised of three components (α1, α2 and α3 V) which assemble in various combinations (Cole 1994).

There have been numerous biochemical reports of elevated levels of type III and V collagens in OI bone (Müller et al 1977; Pope et al 1980; Kirsch et al 1981; Bateman et al 1986). Müller et al (1977) were the first to observe small amounts of type III collagen in the non-fibril structures in discrete areas of compact bone from a patient with OI tarda (OI type I). Pope et al (1980) confirmed this finding and furthermore reported that there were detectable quantities of type V collagen in the long bones from patients with OI congenita (OI type II). Type III collagen was absent and type V was barely detectable in normal controls. Sykes et al (1977) and Francis et al (1981) reported an abnormally high ratio of α(III)/α(I) chains in pepsin-digested skin from OI patients. They implied that this could be due to the reduction in type I collagen. The biochemical observations do not correlate with the ultrastructural demonstration of these collagen types in OI bone. In fact, apart from the short communication on immunolocalisation at light microscopy level of OI bone by Nerlich et al (1993), to date there are no reports on ultrastructural immunolocalisation of collagen types in OI bone. There was one report recently by Waltimo et al (1994) demonstrating an increased
reactivity of type III collagen and the presence of type IV collagen in dentin from Dentinogenesis Imperfecta by immunoelectron microscopy.

Immunolocalisation is a powerful tool to localise proteins and identify the biochemical composition of a specific structural entity. This method has been successfully applied over the years to study protein secretion (Roth et al 1978; Bendayan et al 1987), especially in soft tissues. It can be used to investigate extracellular matrix assembly, and cell-matrix and matrix-mineral relationships in the mineralised tissues.

The conventional techniques of fixation, dehydration and embedding for electron microscopy make considerable alterations in the native state of biological macromolecules due to denaturation and molecular displacement (Hunziker 1993). These factors become important when it is necessary to preserve ultrastructure and antigenicity for immunolocalisation. The high cross-linking ability of the routine TEM fixative glutaraldehyde, and its reaction with amino groups of the lysyl and hydroxylsyl groups of the collagen triple helix, alters the secondary and tertiary conformation of proteins (Bowes and Cater 1968), making glutaraldehyde unsuitable for immunological studies. Paraformaldehyde reacts similarly but does not form polymeric cross-links and it is therefore the fixative of choice.

Aims of this chapter

There have been no systematic studies of co-ordinated biochemistry and immunolocalisation of various collagen types in OI bone. The inherent problems one encounters when dealing with mineralised tissues and the poor accessibility or availability of collagen epitopes make these studies difficult to perform. To overcome these problems this study has taken the following steps. First the fixation was carried out using mild paraformaldehyde fixative for a short period of time. Second pre-embedding immunogold labelling and freeze substitution embedding of unfixed bone specimens were carried out on selected specimens, in order to avoid the loss of antigenicity of collagens by the chemical fixatives. This chapter was designed to bridge the gap between biochemical findings and ultrastructural observations by examining the localisation of type I, III, IV and V collagens in the bone osteoid. It also aimed to compare the pattern and intensity of OI bone collagen staining with that of normal bone collagen. The understanding of the relative distribution of collagen types is important to gain a proper understanding of the pathophysiology of this crippling disease.
5.2 Materials and Methods

Patients' details

Bone specimens from one OI type I (S.D, 14 years), three OI type II (Foetus Mc, 16 weeks; Foetus F, 19 weeks; Foetus W, 25 weeks), two OI type II/III (Foetus Ar-II, 20 weeks; Foetus Ar-III, 22 weeks), one OI type III (H.S, 3 years), one normal foetus (Foetus Sy, 22 weeks) and two normal juveniles (M.K, 11 years; A.H.A, 12 years) were used in this part of the study.

Tissue processing for fixed tissue

The tissue for immunolabelling was embedded by the method of Roth et al. (1978). The tissue was sub-divided and one half was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for various TEM studies. For immunolocalisation the second half of the specimen was fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 hours, then quenched in 0.5 M ammonium chloride for 30 minutes; it was washed in buffer and dehydrated through a graded methanol series at progressively lower temperatures (PLT), 4°C to -20°C, and infiltrated and embedded in lowicryl HM20 resin at -20°C. Polymerisation of the specimens was carried out by UV irradiation at -20°C. The sectioning procedure was as described in chapter 2. Ultra-thin sections were collected on formvar-coated nickel grids for immunogold labelling.

Tissue processing for unfixed tissue by freeze substitution

This work was performed in a Leica AFS freeze substitution machine. Femoral bone samples from an OI type II foetus (Foetus F, 19 weeks), a type III patient (H.S, 3 years), a normal foetus (Foetus Gy, 19 weeks) and a normal juvenile (M.K, 11 years) were frozen in liquid nitrogen, placed in Reichert flow-through capsules (small) and substituted with pure methanol for 48 hours with a fresh methanol change after 24 hours. At the substitution stage the initial temperature was -85°C with a gradual increase of 10°C over 3.5 hours and at the final stages of substitution the temperature was held at -50°C for 22 hours. The specimens were then infiltrated with 1:1 lowicryl HM20 : methanol mixture for one hour at -50°C followed by 2:1 HM20: methanol mixture for two hours at -50°C. After several changes of pure resin over 22 hours at -50°C, specimens were embedded in gelatin capsules, and polymerised with UV irradiation for 48 hours at -50°C. The ultra-thin sectioning procedure was as
described in chapter 2. Ultra-thin sections were collected on formvar-coated nickel grids for immunogold labelling.

**Pre-embedding immunogold localisation**

Thick sections (100µm) of femoral bone from selected patients and from a normal control were incubated with pepsin at 1mg/ml (Sigma) in phosphate buffered saline (PBS) for 30 minutes at 37°C in eppendorf centrifuge tubes, and then washed twice with PBS. The specimens were pre-incubated with 3% bovine serum albumin (BSA) plus 1% Tween 20 and 1% normal goat serum in PBS, pH 7.2, (assay buffer) for 15 minutes. The specimens were treated for 2 hours at room temperature with antibodies to collagen type I, type II, type III (Biogenesis, Poole, UK), type IV (ICN Flow, Thame, UK) and type V (Monasin, Uden, The Netherlands) at a dilution of 1:10 for anti-type I and anti-type II collagen, 1:30 for anti-type III and anti-type IV collagen and 1:5 for anti-type V collagen. Antibody dilutions were determined by dilution assay experiments and selected to give the optimum labelling intensity with minimum background labelling. The specimens were washed 3 times in assay buffer for 5 minutes each, and then incubated with goat anti-rabbit IgG gold (GAR IgG gold, 15 nm) for types I, III and IV as these were polyclonal antibodies raised in rabbit, and goat anti-mouse IgG gold (GAM IgG gold, 10 nm) for types II and V collagen antibodies as these were monoclonal antibodies raised in mouse. The gold conjugates were used at 1:100 dilution in assay buffer and incubated for one hour at room temperature. For negative controls the primary antibodies were replaced by non-immune mouse or rabbit serum as appropriate at 1:500 dilution. After washing, the labelled specimens and controls were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours at room temperature and processed as described for TEM specimens in chapter 2.

**Post-embedding immunogold localisation**

All immunolocalisation studies were carried out on ultra-thin sections, and the incubations were performed by floating the grids, with sections facing down, on droplets on parafilm. The labelling protocol was as described for pre-embedding immunogold labelling. Primary antibody was omitted for the negative control. Normal bone (age- and site-matched) was labelled as described above for collagen types I, III, IV and V. After immunolabelling, grids were washed twice in PBS then rinsed in a stream of distilled water. Sections were stained with 2% aqueous uranyl acetate and lead citrate for 5 minutes in each solution and viewed using a Philips CM12 electron microscope.
5.3 Results

As shown in Table 5.1.1, normal young and foetal bone specimens gave a strong signal for type I collagen (Figs. 5.1a and 5.1e) but weak labelling for type III collagen (Figs. 5.2a and 5.2c). Collagen types IV (Fig. 5.3a) and V (not shown) were not detectable in normal bone.

In OI type I, the labelling pattern for type I collagen (Table 5.1.2 and Fig. 5.1b) appeared to be reduced compared with the normal controls but increased for type III collagen (Fig. 5.2b). Some labelling for type IV collagen (Fig. 5.3c) was also present. Type V collagen was not detectable in OI type I bone (not shown).

OI type II showed lower labelling for collagen type I (Table 5.1.2 and Fig. 5.1f) than control foetal bone (Fig. 5.1e) but type III labelling appeared to be increased (Fig. 5.2d) compared with control foetal bone (Fig. 5.2c). There was weak labelling for type IV collagen (not shown) while types II and V collagen were not detectable in OI type II bone (not shown).

OI type II/III showed a similar pattern to OI type II for collagen types I and III (Table 5.1.2 and Figs. 5.1g and 5.2e) but increased labelling for type IV collagen (Fig. 5.3b). Clusters of gold particles were localised between collagen fibres. A similar labelling pattern for type IV collagen was observed by Karttunen et al. (1989) in reticular fibres in human lymph node tissue. Collagen types II (chapter 7- Fig. 7.3b) and V (not shown) were not detectable in OI type II/III bone.

OI type III bone collagen showed strong labelling for type I collagen (Table 5.1.2 and Fig. 5.1c) but reduced labelling for type III collagen (Fig. 5.5b). There was negligible staining for type IV and V collagens (not shown).

OI type IV bone samples were not available for immunolocalisation studies and have therefore been omitted.

There was a two fold increase in labelling intensity in the freeze-substituted specimens for collagen type I labelling in both normal and OI bone (Figs. 5.4a and 5.4c). As the specimens were not cryo-protected, there was extensive ice crystal damage especially in the OI type II and normal foetal bone specimens. The diminished osteoid in these samples made it difficult to observe the labelling patterns.

The negative controls were clear of non-specific labelling (Figs. 5.1d, 5.1h, 5.2f, 5.4b, 5.4d) for most of the experiments although some gold particles were present in the negative control for type IV collagen (Fig. 5.3d).
Pre-embedded immunogold labelling was only successful in localising type I collagen (Figs. 5.6a and 5.6b). Other collagen types could not be detected by this method.

Table 5.1.1 Immunogold staining intensities of different collagen types in normal bone.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Method of Fixation</th>
<th>Collagen type I</th>
<th>Collagen type III</th>
<th>Collagen type IV</th>
<th>Collagen type V</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal foetal</td>
<td>para fixed</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PLT to HM20</td>
<td>Fig. 5.1e</td>
<td>Fig. 5.2c</td>
<td>Fig. 5.3a</td>
<td></td>
</tr>
<tr>
<td>normal foetal</td>
<td>unfixed</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>FS to HM20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal juvenile</td>
<td>para fixed</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PLT to HM20</td>
<td>Fig. 5.1a</td>
<td>Fig. 5.2a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>normal juvenile</td>
<td>unfixed</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>FS to HM20</td>
<td>Fig. 5.4a</td>
<td>Fig. 5.5a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: +++ = very strong labelling, ++ = strong labelling, + = moderate labelling, +/- = weak labelling, - = no labelling.

FS= Freeze Substitution, HM20= Lowicryl HM20 resin, para fixed = 4% paraformaldehyde fixed, PLT = progressively lowering temperature.
Table 5.1.2  Immunogold staining intensities of different collagen types in OI bone.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Method of Fixation</th>
<th>Collagen type 1</th>
<th>Collagen type III</th>
<th>Collagen type IV</th>
<th>Collagen type V</th>
</tr>
</thead>
<tbody>
<tr>
<td>OI type I</td>
<td>para fixed</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PLT to HM20</td>
<td>Fig. 5.1b</td>
<td>Fig. 5.2b</td>
<td>Fig. 5.3c</td>
<td></td>
</tr>
<tr>
<td>OI type I</td>
<td>no FS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI type II</td>
<td>para fixed</td>
<td>++</td>
<td>++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PLT to HM20</td>
<td>Fig. 5.1f</td>
<td>Fig. 5.2d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI type II</td>
<td>unfixed</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>FS to HM20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI type II/III</td>
<td>para fixed</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PLT to HM20</td>
<td>Fig. 5.1g</td>
<td>Fig. 5.2e</td>
<td>Fig. 5.3b</td>
<td></td>
</tr>
<tr>
<td>OI type II/III</td>
<td>no FS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI type III</td>
<td>para fixed</td>
<td>++</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PLT to HM20</td>
<td>Fig. 5.1c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OI type III</td>
<td>unfixed</td>
<td>+++</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>FS to HM20</td>
<td>Fig. 5.4c</td>
<td>Fig. 5.5b</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Key:** +++ = very strong labelling, ++ = strong labelling, + = moderate labelling, +/- = weak labelling, - = no labelling.

FS = Freeze Substitution, HM20 = Lowicryl HM20 resin, para fixed = 4% paraformaldehyde fixed, PLT = progressively lowering temperature.
Fig. 5.1a  Ultra-thin lowicryl section of normal juvenile bone (M.K, 11 years) labelled with antibody to type I collagen followed by GAR IgG gold. Gold particles are seen in the cross striated collagen fibrils.  
Bar = 230 nm

Fig. 5.1b  Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled with antibody to type I collagen followed by GAR IgG gold. Gold labelling is localised in the collagen fibrils with no gold label on the mineral.  
Bar = 230 nm
Fig. 5.1c Ultra-thin lowicryl section of OI type III bone (H.S, 3 years) labelled with antibody to type I collagen followed by GAR IgG gold. Gold particles are seen in cross striated collagen fibrils. 
Bar = 230 nm

Fig. 5.1d Ultra-thin lowicryl section of normal juvenile bone (M.K, 11 years); note the absence of gold labelling when the primary antibody to type I collagen was omitted in the labelling protocol. This confirms the specificity of labelling.
Bar = 230 nm
Fig. 5.1e  Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled with antibody to type I collagen followed by GAR IgG gold. Many gold particles are seen in the cross striated collagen fibrils. 
Bar = 230 nm.

Fig. 5.1f  Ultra-thin lowicryl section of OI type II foetal bone (Foetus F, 19 weeks) labelled with antibody to type I collagen followed by GAR IgG gold. Note the reduced labelling and the thin fibrils in OI type II bone when compared with the normal foetal control shown above at the same magnification. 
Bar = 230 nm.
Fig. 5.1g  Ultra-thin lowicryl section of OI type II/III foetal bone (Foetus Ar-II, 20 weeks) labelled with antibody to type I collagen followed by GAR IgG gold.  
Bar = 230 nm

Fig. 5.1h  Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks); note the absence of labelling when the primary antibody to collagen type I was omitted in the labelling protocol.  
Bar = 230 nm
Fig. 5.2a Ultra-thin lowicryl section of normal juvenile bone (M.K, 11 years) labelled with antibody to type III collagen followed by GAR IgG gold. Very few gold particles are present in the collagen fibrils. Bar = 230 nm

Fig. 5.2b Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled with antibody to type III collagen followed by GAR IgG gold. Many gold particles are present in the collagen fibrils indicating the presence of type III collagen. Bar = 230 nm
Fig. 5.2c  Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled with antibody to type III collagen followed by GAR IgG gold. There appears to be more gold labelling than in the normal juvenile control (Fig. 5.2a).

Bar = 230 nm

Fig. 5.2d  Ultra-thin lowicryl section of OI type II foetal bone (Foetus F, 19 weeks) labelled with antibody to type III collagen followed by GAR IgG gold. Many gold particles are present on the collagen fibrils indicating significant amounts of type III collagen. Note the thin fibrils in OI type II bone when compared with the normal foetal control shown above at the same magnification.

Bar = 230 nm
Fig. 5.2e Ultra-thin lowicryl section of OI type II/III foetal bone (Foetus Ar-III, 22 weeks) labelled with antibody to type III collagen followed by GAR IgG gold.
Bar = 230 nm

Fig. 5.2f Ultra-thin lowicryl section of OI type II foetal bone (Foetus Mc, 16 weeks); note the absence of gold labelling when the primary antibody to collagen type III was omitted from the labelling protocol.
Bar = 230 nm
Fig. 5.3a Ultra-thin lowicryl section of normal foetal bone (Foetus Sy, 22 weeks) labelled with antibody to type IV collagen followed by GAR IgG gold. Very few gold particles are present in the collagen fibrils.

Bar = 230 nm

Fig. 5.3b Ultra-thin lowicryl section of OI type II/III bone (Foetus Ar-II, 20 weeks) labelled with antibody to type IV collagen followed by GAR IgG gold. Clusters of gold particles (arrowed) are present between the collagen fibrils.

Bar = 230 nm
Fig. 5.3c  Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled with antibody to type IV collagen followed by GAR IgG gold. Many gold particles are present between the collagen fibrils and at the periphery of the osteoblasts.
Bar = 230 nm

Fig. 5.3d  Ultra-thin lowicryl section of OI type I bone (S.D, 14 years) labelled in the absence of primary antibody to type IV collagen. Occasional gold particles are present between the collagen fibrils.
Bar = 230 nm
Fig. 5.4a  Unfixed freeze-substituted lowicryl-embedded normal juvenile bone (M.K, 11 years) ultra-thin section labelled with antibody to type I collagen followed by GAR IgG gold. Many gold particles are localised in the collagen fibrils. Note that no gold particles are present in the spaces between the fibrils.
Bar = 230 nm

Fig. 5.4b  Unfixed freeze-substituted lowicryl-embedded normal juvenile bone (M.K, 11 years) ultra-thin section showing the absence of labelling when the primary antibody to type I collagen was omitted in the labelling protocol.
Bar = 230 nm
Fig. 5.4c  Unfixed freeze-substituted lowicryl-embedded OI type III bone (H.S, 3 years) ultra-thin section labelled with antibody to type I collagen followed by GAR IgG gold. Although gold particles are localised in the collagen fibrils, staining appears to be reduced compared with the normal control (Fig. 5.4a).
Bar = 230 nm

Fig. 5.4d  Unfixed freeze-substituted lowicryl-embedded OI type III bone (H.S, 3 years) ultra-thin section showing the absence of labelling when the primary antibody to type I collagen was omitted in the labelling protocol.
Bar = 230 nm
Fig. 5.5a  Unfixed freeze-substituted lowicryl-embedded normal juvenile bone (M.K, 11 years) ultra-thin section labelled with antibody to type III collagen followed by GAR IgG gold. Note the absence of gold particles indicating the absence of type III collagen in normal juvenile bone.
Bar = 230 nm

Fig. 5.5b  Unfixed freeze-substituted lowicryl-embedded OI type III bone (H.S, 3 years) ultra-thin section labelled with antibody to type III collagen followed by GAR IgG gold. Clusters of gold particles are seen among the thin fibrils.
Bar = 230 nm
Fig. 5.6a  Ultra-thin section of normal juvenile bone (A.H.A, 12 years) showing pre-embedding immunogold labelling for type I collagen. Bar = 230 nm

Fig. 5.6b  Ultra-thin section of OI type II bone (Foetus Mc, 16 weeks) showing pre-embedding immunogold labelling for type I collagen. Bar = 230 nm
5.4 Discussion

This is the first report of ultrastructural immunogold localisation of collagen types in OI bone. In this study the challenging task of collagen labelling has been examined using three different techniques.

Immunolabelling was observed on most of the cross striated fibrils of type I collagen in the osteoid of normal and OI bone, but the intensity was higher in normal bone specimens. Bateman et al (1986) biochemically studied seventeen cases of lethal OI. They reported a reduction in type I collagen content by weight, and interpreted this reduction as a decreased production or an increased degradation of type I collagen. The reduced type I collagen labelling observed in this study could be due to the impairment of the quality and/or quantity of type I collagen in OI bone.

Immunogold labelling for type II collagen has been attempted on selected OI type II and II/III bone specimens, but showed no positive reaction (see chapter 7). This lack of labelling could be due to the absence of type II collagen in these bone samples or it may be that the epitope for type II collagen is sensitive to chemical fixation, hence no gold labelling would be detected.

The increased labelling of type III collagen in OI type II bone specimens is in agreement with the biochemical findings of other authors (Pope et al. 1980; Bateman et al 1986). Bateman et al suggested the increase in type III collagen in OI type II bone may be a part of the fracture repair process. Recently Sokolov et al (1992) also reported a higher level of type III collagen in OI bone. This increase in type III could be one of the reasons for the smaller diameters observed in OI type II collagen fibrils as explained in chapter 4.

The inability to demonstrate type V collagen in this study may be because, as suggested in the literature (Birk et al 1988), it exists as a core fibril of type I collagen. To expose the epitopes partial digestion of type I collagen is needed. As reported by Bateman et al (1986), most of the OI fibroblast cultures contained type V collagen in small amounts. These small amounts may not be sufficient for ultrastructural immunolocalisation. The epitope recognised by the commercially available type V collagen antibody is also sensitive to chemical fixation. Future studies of immunolocalisation should be carried out either on ultra-thin cryo-sections (Tokuyasu 1983) or using high pressure cryo-fixation and freeze substitution as described by Young et al (1995) to preserve both antigenicity and ultrastructure.

The decreased type I collagen in OI bone as demonstrated in this study may be important as type I collagen plays a significant role in the maintenance and retention of mineral in bone. In addition the increased amounts of type III collagen may affect fibril
formation, resulting in collagen fibrils of reduced diameter, which may in turn have important effects on bone fragility.
Chapter 6

Enzyme histochemical localisation of alkaline phosphatase activity in OI bone

6.1 Introduction

The enzyme alkaline phosphatase is thought to be involved in the process which leads to mineral formation in tissues like calcifying cartilage and bone. Robison (1923) initially introduced the idea that alkaline phosphatase enhanced mineralisation by showing that hexose monophosphate esters are soluble in the presence of calcium salts, but when these esters are hydrolysed by alkaline phosphatase, orthophosphates are released and precipitate as calcium phosphate. He examined the distribution of this enzyme in various tissues and proved that it was present where calcification occurred in collagenous tissues. Furthermore, Robison (1923) reported bone to be twice as rich in phosphatases as kidney and twenty times richer than liver.

In mature bone, alkaline phosphatase enzyme has been shown to be present in the periosteum, endosteum, superficial osteocytes (Bourne 1942; Lorch 1947) and most importantly it was shown to be firmly bound to the plasma membrane of osteoblasts and pre-osteoblasts (Göthlin and Ericsson 1971). Furthermore it was shown to be located in matrix vesicles (Ali et al 1970; Anderson 1976). Various authors have reviewed the possible role of alkaline phosphatase activity in calcification (Bourne 1972; Wuthier and Register 1985). According to the latter, alkaline phosphatase produces phosphate ions and acts as a transporter of these ions which couple with calcium ions to produce bone salt. It also helps the local destruction of mineral growth inhibitors, such as pyrophosphates, through its phosphohydrolase activity, thus keeping the surface of bone crystals free of inhibitors and permitting the continued growth of crystals (Ali et al 1970; Ali 1983, 1992).

To date there is only one report on ultrastructural alkaline phosphatase localisation in OI bone. Doty and Mathews (1971) studied four patients with OI tarda (type I) using histochemical localisation of alkaline and acid phosphatase, and reported reduced activity in alkaline phosphatase (osteoblast activity) although the acid phosphatase activity (osteoclast activity) was comparable to that of the normal tissue. They concluded, on the basis of the above observations and other ultrastructural features, that the skeletal genetic defect in OI tarda was expressed by the osteoblast population. Since this report 26 years ago no one has attempted histochemical localisation of alkaline phosphatase in OI and normal bone. One reason for this could be because of the inherent problems associated with the technique.
The ultrastructural histochemical localisation of alkaline phosphatase depends on the formation of an electron opaque reaction product which can be visualised on EM (Lewinson et al 1982). In this method the incubation medium contains lead nitrate as the capture reagent, which causes precipitation of lead phosphate at the site of enzyme activity; this can be used as a qualitative measure of osteoblast reactivity. At EM level alkaline phosphatase has been studied in calcifying cartilage but, apart from Doty and Mathew's work in 1971, not in bone. The distribution of alkaline phosphatase in growth plate has been described by Matsuzawa and Anderson (1971) and Ralphs and Ali (1986), in fracture callus by Göthlin and Ericsson (1971) and Volpin et al (1986), in condylar cartilage by Meikle (1976) and Lewinson et al (1982) and in human articular cartilage by Rees and Ali (1988).

**Aims of this chapter**

The aim of this chapter was to investigate the presence of alkaline phosphatase in normal and OI bone and to assess whether there is an osteoblast dysfunction in OI. Alkaline phosphatase was measured using an adaptation of the above mentioned method (Lewinson et al 1982) and the reaction products compared on bone specimens from four OI patients and two normal controls. In addition two OI foetal growth plates and one normal foetal growth plate (age-matched) were examined using the same technique.
6.2 Materials and Methods

Patients' details

Specimens from three OI type II foetal femoral bones (Foetus Mc, 16 weeks; Foetus F, 19 weeks; Foetus W, 25 weeks), two OI type II growth plates (Foetus Mc, 16 weeks; Foetus F, 19 weeks), one normal foetal femoral bone and growth plate (Foetus Gy, 19 weeks), one OI type III femoral bone specimen (H.S, 3 years) and one normal bone (A.H.A, 12 years) were examined.

Localisation of alkaline phosphatase

A lead nitrate method (Lewinson et al 1982) modified in this laboratory (Ralphs and Ali 1986; Rees and Ali 1988) was used to identify alkaline phosphatase reactivity. Bone and growth plate sections (50 μm-100 μm thick) were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C for 1 hour, followed by washing in 0.1 M sodium cacodylate buffer. Sections were incubated in 10 ml of 40 mM Tris/HCl buffer pH 9.0 (Merck, Poole, UK) containing 9 mM sodium β-glycerophosphate (Merck), 5 mM magnesium chloride (Merck) and 3.6 mM lead nitrate (Merck) at 37 °C for 30 minutes. In control samples, 2 mM levamisole hydrochloride (Aldrich, Gillingham, UK), a potent inhibitor of alkaline phosphatase, was added to the incubating medium. After incubation, specimens were washed briefly in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed for 30 minutes in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, and processed as for the standard TEM processing technique described in chapter 2.

Sections for TEM were cut on a Reichert-Jung Ultracut E ultramicrotome, picked up onto copper grids and viewed unstained on a Philips CM12 electron microscope so that the electron dense reaction product of lead phosphate could be seen easily.

178
6.3 Results

The black electron dense reaction product of lead phosphate which indicates the presence of alkaline phosphatase was easily demonstrable on the cell membrane of the osteoblasts, as focal concentrations in the collagen osteoid and on the mineralisation front of normal bone (Figs. 6.1a & 6.4a). In normal bone the intensity of the reaction seemed to be stronger than in OI bone (Figs. 6.2a, 6.3a, 6.5a) and appeared as a continuous black line along the osteoblast cell membranes. In OI bone (Figs. 6.2a, 6.3a, 6.5a) the reaction product only appeared as a few electron dense beads along the osteoblast cell membrane. The osteoblasts of one sample of OI type II bone seemed to be necrotic although the specimen was processed immediately after termination (Fig. 6.3a). In both normal and OI bone, the phosphatase reaction product seemed to be on the periphery of the osteoblasts with no visible reaction in the internal organelles (Figs. 6.1a & 6.2a). The osteoid of OI bone did not contain any focal concentrations of the reaction product. The specificity of the phosphatase reaction was confirmed by the absence of the reaction product when sections were incubated in the presence of levamisole inhibitor (Figs. 6.1b, 6.2b, 6.3b, 6.4b & 6.5b). The black reaction product seen in the mineral front in these sections (levamisole controls) represents a non-specific reaction due to the binding of lead to the rapidly growing crystal nodules. This was seen only in foetal tissues and was negligible in juvenile tissue samples (Figs. 6.4b & 6.5b).

In the growth plate, electron opaque deposits of lead phosphate indicative of alkaline phosphate activity, were clearly visible on the cell membranes of chondrocytes, matrix vesicles within the extracellular matrix and on the longitudinal septae in normals (Figs. 6.6a & 6.6b). The reaction product was not apparent in levamisole treated growth plate sections (Fig. 6.9a).

In the OI foetal growth plate, the reaction products around the cell membranes of chondrocytes appeared to be less intense (Figs. 6.7a, 6.7b, 6.8a & 6.8b) compared with normals (Figs. 6.6a & 6.6b). The reaction product was absent in cell organelles in both normal and OI chondrocytes.
Fig. 6.1a  Normal foetal bone (Foetus Gy, 19 weeks) demonstrating alkaline phosphatase activity as shown by an intense lead phosphate reaction product (electron dense deposits) around the osteoblasts and the mineral front. Bar = 1.6 μm

Fig. 6.1b  Normal foetal bone (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor showing no reaction product on the osteoblasts. This confirms the specificity of the alkaline phosphatase reaction around the osteoblasts in Fig. 6.1a. The relatively reduced black reaction product seen in the mineral front is a non-specific reaction due to the binding of lead to the rapidly growing crystal nodules. This is seen only in foetal tissues and is negligible in juvenile tissue samples (Figs. 6.4b & 6.5b). Bar = 1.6 μm
Fig. 6.2a OI type II foetal bone (Foetus F, 19 weeks) showing diffuse reaction product indicating low alkaline phosphatase activity around the osteoblasts. The strong staining in the mineral front is due to the dissolution of mineral in this area.
Bar = 2.5 µm

Fig. 6.2b OI type II foetal bone (Foetus F, 19 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor showing little or no reaction on the osteoblasts but non-specific staining in the mineral front is still present.
Bar = 1.6 µm
Fig. 6.3a  OI type II foetal bone (Foetus W, 25 weeks) stained for alkaline phosphatase activity showing a diffuse reaction product around the osteoblasts with strong staining in the mineral front.
Bar = 2.5 μm

Fig. 6.3b  OI type II foetal bone (Foetus W, 25 weeks) stained for alkaline phosphatase activity in the presence of levamisole inhibitor showing no reaction product on the osteoblasts.
Bar = 1 μm
Fig. 6.4a   Normal juvenile bone (A.H.A, 12 years) stained for alkaline phosphatase activity showing an intense reaction product (electron dense deposits) around the osteoblasts layer and the mineral front.  
Bar = 1.3 μm

Fig. 6.4 b   Normal juvenile bone (A.H.A, 12 years) stained for alkaline phosphatase activity in the presence of levamisole inhibitor showing no reaction on the osteoblasts.  
Bar = 620 nm
Fig. 6.5a  OI type III bone (H.S, 3 years) stained for alkaline phosphatase activity showing diffuse reaction product around the osteoblasts with strong staining in the mineral front.
Bar = 1 µm

Fig 6.5b  OI type III bone (H.S, 3 years) stained for alkaline phosphatase activity in the presence of levamisole inhibitor showing no reaction of the osteoblasts. Some residual non-specific reaction product is present in the mineral front.
Bar = 1.6 µm
Fig. 6.6a  Normal foetal growth plate (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity showing an intense reaction product (electron dense deposits) around the chondrocytes.
Bar = 3.5 µm

Fig. 6.6b  Normal foetal growth plate (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity and shown at a higher magnification than in Fig. 6.6a. Note the intense reaction product (electron dense deposits) around the chondrocytes indicating a high alkaline phosphatase activity. The internal organelles are clear of any deposits.
Bar = 940 nm
Fig. 6.7a  OI type II foetal growth plate (Foetus F, 19 weeks) stained for alkaline phosphatase activity showing diffuse reaction product (electron dense deposits) around the chondrocytes.  
Bar = 3.5 \mu m

Fig. 6.7b  OI type II foetal growth plate (Foetus F, 19 weeks) stained for alkaline phosphatase activity and shown at a higher magnification than in Fig. 6.7a. Note the reduced reaction product around the chondrocytes when compared with normal chondrocytes (Fig. 6.6b).  
Bar = 1 \mu m
Fig. 6.8a  OI type II foetal growth plate (Foetus Me, 16 weeks) stained for alkaline phosphatase activity showing sparse reaction product (electron dense deposits) around the chondrocytes.
Bar = 3.5 μm

Fig. 6.8b  OI type II foetal growth plate (Foetus Me, 16 weeks) stained for alkaline phosphatase activity and shown at a higher magnification than in Fig. 6.8a. Note the reduced reaction product around the chondrocytes when compared with a normal chondrocyte (Fig. 6.6b).
Bar = 1 μm
Fig. 6.9a Normal foetal growth plate (Foetus Gy, 19 weeks) stained for alkaline phosphatase activity in the presence of the levamisole inhibitor showing no reaction product around the chondrocytes. This confirms the specificity of the reaction for alkaline phosphatase activity shown in Figs. 6.6a and 6.6b.
Bar = 1.75 µm

Fig. 6.9b OI type II foetal growth plate (Foetus F, 19 weeks) stained for alkaline phosphatase activity in the presence of the levamisole inhibitor showing no reaction product around the chondrocytes. This confirms the specificity of the reaction for alkaline phosphatase activity shown in Figs. 6.7a, 6.7b, 6.8a and 6.8b.
Bar = 1.75 µm
6.4 Discussion

As demonstrated above in the results section, the bone tissues assayed for alkaline phosphatase activity using the modified method of Lewinson et al (1982) showed a different labelling pattern for OI and normal bone. However this method does not distinguish enzyme activity from the total amount of enzyme protein present. There appears to be reduced and diffuse reaction product on OI osteoblasts, thus implying either a reduced level and/or altered activity of alkaline phosphatase and hence a dysfunction of osteoblasts. This confirms the finding of Doty and Mathews (1971) of the impaired activity of alkaline phosphatase in OI osteoblasts. Furthermore McKusick (1972) also suggested that the defect in OI was the functional impairment of the osteoblasts resulting in the decreased formation of apparently normal bone matrix. The specimens examined in the present study clearly show a reduced activity of alkaline phosphatase in OI bone. Even in the OI growth plate, hypertrophic chondrocytes showed less intense reaction product than the chondrocytes in the normal growth plate.

Ralphs and Ali (1986) carried out a study on alkaline phosphatase activity in rabbit ulna growth plate. They reported a positive reaction in the hypertrophic zone and especially in longitudinal septae. The reaction product was absent in the resting zone and in the major part of the proliferation zone. Their results indicate that initial calcification starts in the hypertrophic zone where the level of alkaline phosphatase activity is high. The normal human growth plates used in this study showed a similar pattern, but in the OI growth plate even the hypertrophic zone, where the alkaline phosphatase activity is reported to be high, showed less intense reaction product, indicative of reduced or altered activity. Perhaps delayed calcification observed in some of the OI type II growth plates as described in chapter 2 could be a result of this reduced alkaline phosphatase activity.

Serum alkaline phosphatase levels in seven OI type I patients have been shown to be normal (Minisola et al 1994). Morike et al (1993) studied the expression of osteoblast markers on cultured cells isolated from fifteen patients in all OI types and compared these with seven foetal and postnatal normal controls. They reported that the alkaline phosphatase levels in OI were comparable to normal, but osteocalcin levels were reduced in OI type II bone cells. Although these biochemical reports indicate that alkaline phosphatase levels are normal in cultured OI cell lines, our ultrastructural histochemical observations show reduced enzyme localisation and this may suggest reduced amounts of protein or reduced activity at the tissue level.

Clearly the data obtained in the present study is not quantitative and assessing the degree of staining is subjective. In addition since this enzyme localisation was carried out on a small number of OI samples, a greater number of samples should be stained for
alkaline phosphatase activity in order to confirm the findings presented here and to arrive at a better understanding of the osteoblast function in OI bone. However from the preliminary evidence presented in this study, there seems to a reduced or altered alkaline phosphatase activity in OI osteoblasts, which may in turn result in reduced bone formation.
Chapter 7

Abnormal presence of proteoglycans found in OI bone

7.1 Introduction

Proteoglycans (PGs) form the amorphous ground substance that fills the space between the fibres of connective tissue to form the matrix. They are complex carbohydrates that contain one or more glycosaminoglycan (GAG) chains covalently bound to a core protein. These glycans can vary and may include heparan, keratan, chondroitin and dermatan sulphate chains. They consist of repeating disaccharide units, one residue of which is always hexosamine, usually with sulphate ester groups attached at the 4 or 6 positions.

The interactions of PGs and collagen have remained speculative for some time, partly because of the unresolved problem of PG translocation and the collapse of PG domains during processing of the tissue (Scott 1985). The development of cupromeronic and cuprolinic blues, two dyes designed for electron histochemical use in a 'critical electrolyte concentration' system, led Scott and co-workers (1981, 1989) to demonstrate the specific interactions of PGs and collagen type I fibrils. He proposed the hypothesis that the collagen fibril intraperiodic a and c bands were assigned to keratan sulphate PG and chondroitin-dermatan sulphate PGs were located in the d and e bands. Furthermore all four bands were in or at the step of the gap zone in the collagen fibril.

It is generally believed that PGs may be involved in matrix formation and in the regulation of mineralisation (Boskey 1984; Fisher 1985; Fisher and Termine 1985). It has been suggested that PGs also play a role in controlling collagen fibril diameter (Scott and Parry 1992), and recent evidence supports an important role in fibrillogenesis (Parry et al 1982; Kadler et al 1996).

Blumenthal et al (1979) emphasised the role of PGs in bone mineralisation; they reported that a decrease in PG content was accompanied by the deposition of mineral in cartilage. Initial calcification probably follows the removal of inhibitory compounds such as PGs (Ali 1992). Posner et al (1978) compared the effects of PG aggregates and subunits prepared from calcifying and non-calcifying cartilage in two different hydroxyapatite formation systems. Aggregates were more effective inhibitors of apatite formation in both systems whilst the subunits delayed apatite formation. Such observations indicate that PGs may inhibit bone calcification.
PG abnormalities have also been suspected in OI for some time; for example both Bleckmann et al (1971) and Engfeldt and Hjerpe (1976) reported increased amounts of GAGs in OI type II bone biopsies (old classification-OI congenita: new-type II ), when compared with normal bone. Brown et al (1975) also observed an abnormal distribution of GAGs in OI dentine and Spencer (1962) showed histochemical evidence of abnormal GAGs in OI bone. Goldberg (1978) found an increased excretion of PG in young patients with OI.

**Aims of this chapter**

The OI type II/III bone described in this study in chapter 2 appeared to contain excessive amounts of PG-like material in the enlarged osteocytic lacunae when examined by electron microscopy. The aim of this chapter was to undertake an investigation and identification of these components by light microscopy, together with electron probe X-ray microanalysis, immunogold labelling and cuprolinic blue staining at ultrastructural level. In addition the specific relationship of PG with the osteoid collagen fibrils was investigated using OI bone specimens from selected patients and these were compared with normal control bone specimens using a cuprolinic blue 'critical electrolyte concentration' method (Scott and Orford 1981).
7.2 Materials and Methods

Patients' details

Specimens of bone were obtained from ten foetuses which were diagnosed as OI type II and type II/III. Details of the five specimens which were positive for PG as assessed by a number of techniques and two control samples (age- and site-matched) are given in Table 7.1. In all foetuses, OI was initially diagnosed on routine ultrasound scans of the mother and the affected pregnancies were terminated. The clinical classification of OI type was based on clinical signs, babygram X-rays and collagen biochemistry from cultured fibroblasts.

In addition to the above study bone specimens from two OI type II foetuses (Foetus F, 19 weeks; Foetus W, 25 weeks), four OI type II/III individuals (Foetus Ar-II, 20 weeks; Foetus F.G, 22 weeks; Baby G-III, full term; Baby V.D.K, full term), one OI type III patient (H.S, 3 years), three normal foetuses (Foetus Gy, 19 weeks; Foetus Sy, 22 weeks; Foetus L, 24 weeks) and two normal juveniles (M.K, 11 years; N.A, 13 years) were processed for cuprolinic blue staining to demonstrate the PG distribution patterns between the osteoid collagen fibres.

Electron microscopy processing and cuprolinic blue staining

The femoral mid-shaft was dissected at post-mortem examination of the OI foetuses. One half of the cylindrical specimen was fixed in 2.5% glutaraldehyde (Agar Scientific Ltd., Stansted, UK) in 0.1 M sodium cacodylate (Agar Scientific Ltd) buffer, pH 7.4, for routine transmission electron microscopy (TEM) and electron probe X-ray microanalysis (XRMA). The second half was fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for immunological studies.

The specimens processed for cuprolinic blue staining were fixed in 2.5% glutaraldehyde in 25 mM sodium acetate buffer (pH 5.6), containing 0.1 M magnesium chloride and 0.5% cuprolinic blue dye for 18 hours as described by Scott and Orford (1981). The unbound dye was removed by rinsing for 15 minutes in several changes of buffer solution, followed by further en bloc staining for 15 minutes in 0.5% aqueous sodium tungstate. The specimens were dehydrated in a graded ethanol series (first alcohol contained 0.5% sodium tungstate), and embedded in araldite CY212 resin (Agar Scientific Ltd.).

For routine TEM, each specimen was sub-divided (1 mm³) and post-fixed in 1% osmium tetroxide (Agar Scientific Ltd.) in 0.1 M sodium cacodylate buffer for one hour;
samples were then washed in buffer, dehydrated and embedded in araldite CY212 resin. For XRMA the osmication step was omitted because of the osmium interference in the region of the phosphorus Kα peak.

Specimens were cut using a Reichert-Jung Ultracut E ultramicrotome. Semi-thin (1μm) light microscopy survey sections were cut and stained with either (a) 1% toluidine blue in 1% borax or (b) methylene blue/azur II and basic fuchsin. Ultra-thin sections were stained with 2% aqueous uranyl acetate and lead citrate for 10 minutes in each solution, before viewing on a Philips CM12 electron microscope.

**Light microscopy processing**

As confirmatory evidence for the presence of PG in osteocytic lacunae, rib-bone specimens from the same OI and control foetuses (as indicated above) were fixed in 10% formal saline for 24 hours, and decalcified in neutral EDTA, dehydrated and embedded in paraffin wax. Thick sections (5μm) were cut and rehydrated, stained with 1% alcian blue in 1% acetic acid for 20 minutes, and counterstained with Van Gieson stain for 1 minute.

**Electron probe X-ray microanalysis (XRMA)**

XRMA was performed on non-osmicated, unstained araldite sections (100 nm) in a Philips CM 12 electron microscope with the EDAX 9800 system as described in chapter 3.

**Tissue processing for immunogold labelling**

The tissue for immunolabelling was embedded by the method of Roth *et al* (1978). The tissue was sub-divided and fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 hours, then quenched in 0.5 M ammonium chloride for 30 minutes. Samples were washed in buffer, dehydrated through a graded methanol series at progressively lower temperatures, and embedded in lowicryl K4M or HM20 resins. The sectioning procedure was as described above for araldite blocks.
Immunolabelling at light microscopy level

Immunostaining with monoclonal antibodies to chondroitin sulphates relies on the prior digestion of specimens with either chondroitinase ABC or chondroitinase AC II, as these antibodies recognise the oligosaccharide stub that remains attached to the core protein after enzyme digestion of the PGs (Couchman et al 1984).

All incubations were carried out on 1 μm lowicryl sections of OI or normal foetal bone on ultra-clean glass slides. The sections were incubated with chondroitinase ABC (0.5 units/ml; Sigma, Bournemouth, UK) for 30 minutes at 37°C. After digestion, slides were washed twice in assay buffer [phosphate buffered saline containing 3% bovine serum albumin (BSA, Sigma) and 1% Tween 20] for 15 minutes each, then incubated with antibodies to chondroitin 4-sulphate (2B6), chondroitin 6-sulphate (3B3), keratan sulphate (5D4; all from ICN Biomedicals Ltd., Thame UK) at 1:500 dilution and to the small PGs decorin (LF30) and biglycan (LF15; both donated by Dr. M. Bayliss, Kennedy Institute, London, obtained originally from Dr. Larry Fisher, USA) at 1:200 dilution for 2 hours at room temperature. The sections were then washed three times in assay buffer for 5 minutes; this was followed by the addition of gold-labelled goat anti-mouse IgG (GAM IgG, 10 nm; Bio-Cell, Cardiff, UK) for 2B6-, 3B3- and 5D4-incubated slides and goat anti-rabbit IgG gold (GAR IgG gold, 15 nm; Bio-cell, Cardiff, UK) for LF15- and LF30-incubated slides. Both antibodies were used at 1:100 dilution and staining was for 1 hour. Sections were then washed several times with distilled water and incubated with silver enhancement solution (Bio-Cell Skell 15, Cardiff, UK) in the dark for a further 15 minutes. The slides were then fixed with Unifix (Kodak Ltd., UK) at 1:10 dilution for 5 minutes, washed thoroughly in running tap water and counterstained with 1% light green solution for 5 minutes. For negative controls the primary antibody was replaced by non-immune mouse or rabbit serum as appropriate at 1:500 dilution.

All LM sections were viewed on an Olympus BH2 photomicroscope and relevant areas were photographed using Fuji Ektachrome 64T colour reversal film or Ilford Pan F 50 black and white negative film.

Immunolabelling at electron microscopy level

All immunolocalisation studies were carried out on ultra-thin sections; the incubations were performed by floating the grids, with sections facing down, upon liquid drops on parafilm.
The labelling protocol was as described for light microscopy labelling, except that a primary antibody cocktail was used at 1:200 dilution and the silver enhancement step was omitted. In addition 5D4, 2B6, 3B3 (all at 1:200 dilution), and antibodies to decorin and biglycan (at 1:50 dilution) were used separately. Immunogold localisation for type I and type II collagens was as described in chapter 5.

Immunogold labelled grids were stained with 2% aqueous uranyl acetate and lead citrate for 5 minutes in each solution and viewed using a Philips CM 12 electron microscope.
7.3 Results

Light microscopy

The type II/III OI bone viewed by LM was highly porotic with numerous vascular channels and many osteocytes. The semi-thin araldite sections (1μm), stained with methylene blue/azure II and basic fuchsin, showed osteocytes and lacunae surrounded by a blue band of osteoid-like material within a light pink mineralised matrix (Fig. 7.1a). This was completely different when compared with the age- and site-matched controls (Fig. 7.1b) which had few vascular channels, osteocytes with small lacunae and no staining of the osteoid surrounding the lacunae. When the OI sections were stained with toluidine blue, many osteocytic lacunae showed purple metachromasia indicative of PGs (Fig. 7.1c), whereas, in the normal controls, the lacunae were blue (Fig. 7.1d).

Paraffin sections of decalcified OI rib, when stained with alcian blue and counterstained with Van Gieson, showed abnormally blue-coloured osteocytic lacunae, indicating that they contained PGs, within a red mineral matrix (Fig. 7.1e). Normal age- and site-matched controls did not show the blue colour (Fig. 7.1f).

Electron microscopy and X-ray microanalysis

Ultra-thin araldite sections of femur, stained with 2% uranyl acetate and Reynold's lead citrate, showed fibrillar and stellate particles resembling PGs in the osteocytic lacunae (Figs. 7.2a1, a2 and b1, b2). Some of the large osteocyte lacunae did not show a cell profile in the plane of the section (Fig. 7.2a1), this space being occupied by the stellate particles and filamentous material.

Generally speaking OI osteocytic lacunae were larger than those of normal bone. In addition to the large cellular and pericellular space of the lacuna, there was a thick seam of unmineralised collagenous osteoid which surrounded the lacuna (Figs. 7.2a1 & b1).

Moreover, the collagen fibres and bundles were thick resembling type I collagen and were not thin fibrils as seen in epiphyseal cartilage. This was confirmed when lowicryl-embedded ultra-thin sections were labelled for type I and II collagens, a positive labelling occurred only for type I collagen (Fig. 7.3a). Therefore these are not remnants of growth cartilage which do not contain type I collagen, but are bone osteocytic lacunae.

When analysed by electron probe X-ray microanalysis, the PG-like material in the osteocytic lacuna of an unstained and non-osmicated araldite section of OI bone gave a
positive signal (Kα) for sulphur and calcium (Fig. 7.4a) whereas normal bone osteocytic lacunae gave no signal for sulphur or calcium (Fig. 7.4b). The high signal for sulphur may be due to sulphated PGs while the high signal for calcium may be an indication that these ions are binding to the PGs. The unmineralised perilacunar collagen band may be a result of this binding which could make calcium ions unavailable for mineral crystal formation.

Immunogold labelling (light microscopy)

The 1μm thick lowicryl sections of femoral bone from the OI specimens showed a positive reaction for PGs in the osteocytic lacunae when stained with a cocktail of antibodies to keratan sulphate and chondroitin sulphates (Fig.7.5a); age- and site-matched controls were negative (Fig. 7.5b). There was no staining of the OI bone serial sections when the primary antibodies were replaced with a non-immune normal mouse serum (Fig. 7.5c).

Immunogold labelling (electron microscopy)

At ultrastructural level, immunogold labelling for PG was localised with the fine fibrillar and particulate material in the pericellular region of the osteocytic lacunae (Fig. 7.6a). Very few gold particles were seen on the mineralised matrix or in the collagen band at the periphery of the lacunae. Gold label appeared to run along the filamentous material (Fig. 7.6b). This intense labelling of PG was not present in normal bone (Fig. 7.7). When the primary antibodies were replaced by non-immune mouse or rabbit sera, there was no staining of OI bone (Fig. 7.8).

When the antibodies to keratan sulphate (5D4), chondroitin 4-sulphate (2B6) and chondroitin 6-sulphate (3B3) were used separately, very strong labelling intensities were observed for keratan sulphate (Figs. 7.9 a & b), and chondroitin 4-sulphate (Figs. 7.10a & b) whilst there was only moderate labelling for chondroitin 6-sulphate (Figs. 7.11a & b). There was weak labelling for the small PGs decorin (Fig. 7.12) and biglycan (Fig. 7.13). When the primary antibodies were replaced by non-immune mouse or rabbit sera, there was no staining of OI bone (Figs. 7.14 a & b).

Cuprolinic blue staining to characterise excessive PG in the osteocytic lacunae of OI bone showed excessive amount of electron dense PG rods in the lacunae and fewer rods between the collagen fibres (Figs. 7. 15a & b).
Cuprolinic blue staining to localise PGs in the osteoid

The PG-collagen interactions demonstrated by cuprolinic blue staining varied between OI and control bone. There was an orderly pattern of distribution of PGs between the collagen fibres in normal (foetal and juvenile) bone osteoid (Figs. 7.16, 7.18, 7.20, 7.22 and 7.24). In OI bone there was a higher content of PG between the osteoid collagen fibrils (Figs. 7.17, 7.19, 7.21, 7.23 and 7.25) and furthermore there was a disturbed pattern of PG distribution in relation to collagen fibres. In OI type II/III and OI type III, which demonstrated thicker fibrils, there were long PG rods between the collagen fibres, often extending over two D-periods (Figs. 7.23 and 7.25) and arranged in parallel with the collagen fibres (Fig. 7.23).
Table 7.1 Patients' details and the techniques used to confirm the presence of proteoglycans in the osteocytic lacunae.

<table>
<thead>
<tr>
<th>Name, Age and Sex</th>
<th>Site of Biopsy</th>
<th>Other Details</th>
<th>Tol blue</th>
<th>AB/VG</th>
<th>XRMA</th>
<th>Immuno</th>
<th>CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foetus D, 20 weeks, male</td>
<td>Femur</td>
<td>Normal</td>
<td>+ve</td>
<td>NT</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Foetus M, 22 weeks, male</td>
<td>Femur and rib</td>
<td>Normal</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Foetus Ar-II, 20 weeks, male</td>
<td>Femur or tibia</td>
<td>OI type II/III, second affected foetus from normal parents</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Foetus Ar-III, 22 weeks, male</td>
<td>Femur and rib</td>
<td>OI type II/III, third affected foetus from normal parents</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Foetus Gr, 22 weeks, male</td>
<td>Femur</td>
<td>OI type II/III, family history not known</td>
<td>+ve</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Foetus F.G, 22 weeks, female</td>
<td>Rib</td>
<td>OI type II/III, fourth affected foetus from consanguinous parents</td>
<td>+ve</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+ve</td>
</tr>
<tr>
<td>Baby G -III full term, female</td>
<td>Tibia</td>
<td>OI type II/III, third affected baby from consanguinous parents</td>
<td>+ve</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Fig. 7.1a  Araldite section (1μm) of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks) stained with methylene blue/azur II and basic fuchsin, showing the porotic nature of the bone. Note the abundant vascular channels, many osteocytes with large lacunae and the dark blue band of osteoid-like material surrounding the lacunae. x 125

Fig. 7.1b  Araldite section of a normal foetal femoral bone (Foetus M, 22 weeks) stained with methylene blue/azur II and basic fuchsin as in Fig. 7.1a. Note the difference in the bone with fewer vascular channels, osteocytes with small lacunae and the absence of blue-staining osteoid surrounding the lacunae. x 125

Fig. 7.1c  Araldite section (1μm) of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks) stained with toluidine blue. Note the purple-coloured lacunae (metachromasia) indicating the presence of proteoglycan and the blue band of osteoid around the lacunae. x 500

Fig. 7.1d  Araldite section of a normal foetal femoral bone (Foetus M, 22 weeks) stained with toluidine blue, showing the absence of any purple-coloured lacunae or blue-stained band of osteoid compared with Fig. 7.1c. x 500

Fig. 7.1e  Paraffin wax section of an OI type II/III foetal rib bone (Foetus Ar-III, 22 weeks) stained with alcian blue and counter stained with Van Gieson stain. Note the blue-coloured osteocytic lacunae, indicating the presence of sulphated polysaccharides. x 500

Fig. 7.1f  Paraffin wax section of a normal foetal rib bone (Foetus M, 22 weeks) stained in a similar manner to that of Fig. 7.1e showing the absence of blue-coloured osteocytic lacunae. x 500
Fig. 7.2a1 Ultra-thin araldite section of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks). Note the thick seam of unmineralised osteoid collagen in the periphery of the lacunae.
CO- collagen, MM- mineralised matrix, PG- proteoglycan.
Bar = 1.7 μm

Fig. 7.2a2 Higher magnification of an osteocytic lacuna shown in Fig.7.2a1. Note the fine fibrillar and stellate particles resembling proteoglycans.
Bar = 150 nm
Fig. 7.2b1  Ultra-thin araldite section of an OI type II/III foetal femoral bone (Foetus Ar-III, 22 weeks). Note the perilacunar proteoglycan-like material and thick seam of osteoid collagen surrounding the lacuna.
CO-collagen, LA-osteocyte lacuna, MM-mineralised matrix, OC-osteocyte, PG-proteoglycan.
Bar = 1 µm

Fig. 7.2b2  Higher magnification of the osteocytic lacuna shown in Fig. 7.2b1. Note the fine fibrillar and stellate particles resembling proteoglycans and the thick seam of collagen band surrounding the lacuna.
Bar = 404 nm
Fig. 7.3a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) immunogold labelled for type I collagen showing a positive labelling along the collagen fibres.
Bar = 230 nm

Fig. 7.3b  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) immunogold labelled for type II collagen; note the absence of labelling.
Bar = 230 nm

Fig. 7.3c  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks); note the absence of labelling when the primary antibody was omitted in the labelling protocol. This confirms the specificity of the type I collagen labelling.
Bar = 230 nm
Fig. 7.4a  The XRMA spectrum from an osteocyte lacuna area in an unstained and non-osmicated araldite section of an OI type II/III femoral foetal bone (Foetus Ar-III, 22 weeks). Note the high signal for sulphur, which may be due to sulphated proteoglycans. The high signal for calcium may be an indication that these ions are binding to proteoglycans.

Fig. 7.4b  The XRMA spectrum from an osteocyte lacuna area in an unstained and non-osmicated araldite section of a normal foetal femoral bone (Foetus M, 22 weeks). Note that it does not show a high signal for sulphur.
Chapter 7

7.4 a

7.4 b

02-MAR-95 15:01:46 EDAX READY
RATE= 295CPS TIME= 200LSEC
FS= 548CNT FS= 200LSEC
A = OI bone osteocyte lacuna

02-MAR-95 15:05:20 EDAX READY
RATE= 21CPS TIME= 200LSEC
FS= 1613CNT FS= 200LSEC
A = Normal foetal bone osteocyte
Fig. 7.5a  Immunogold labelled and silver enhanced lowicryl section (1μm) of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks). The majority of the osteocytic lacunae are labelled by antibodies to chondroitin 4-sulphate, chondroitin 6-sulphate and keratan sulphate followed by GAM IgG gold and silver enhancement. x125

Fig. 7.5b  Immunogold labelled, silver enhanced lowicryl section (1μm) of a normal foetal femoral bone (Foetus M, 22 weeks) showing no staining for proteoglycans when labelled with the same antibodies used in Fig. 7.5a. x125

Fig. 7.5c  Serial section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing no staining for proteoglycans when the primary antibodies were replaced by non-immune mouse serum in the staining protocol. x125
Fig. 7.6a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) osteocytic lacuna immunogold labelled for proteoglycans. Labelling is localised to the fine fibrillar and particulate material in the pericellular region of the osteocytic lacuna. Very few gold particles are present in the cell or in the collagen band at the periphery of the lacuna.

Bar = 1.6 μm

Fig. 7.6b  Higher magnification of the osteocytic lacuna shown in Fig. 7.6a. Note that the gold label appears to run along the filamentous material.

Bar = 227 nm
Fig. 7.7 Ultra-thin lowicryl section of a normal foetal femoral bone (Foetus M, 22 weeks) labelled for proteoglycans. Note the osteocytic lacuna is not intensely labelled. Bar = 530 nm

Fig. 7.8 Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) showing an osteocytic lacuna. Note the absence of gold labelling when the primary antibodies were replaced by non-immune mouse serum in the staining protocol. Bar = 330 nm
Fig. 7.9a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-keratan sulphate antibody (5D4) followed by GAM IgG gold showing strong labelling of an osteocytic lacuna. Immunogold labelling is localised to the fine fibrillar and particulate material in the pericellular region. Bar = 650 nm

Fig. 7.9b  Higher magnification of the osteocytic lacuna shown in Fig. 7.9a. Bar = 196 nm
Fig. 7.10a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone
(Foetus Ar-II, 20 weeks) labelled with anti-chondroitin 4-sulphate antibody (2B6)
followed by GAM IgG gold, showing intense labelling of an osteocytic lacuna.
Bar = 1.7 μm

Fig. 7.10b Higher magnification of the osteocytic lacuna shown in Fig.7.10a.
Bar = 196 nm
Fig. 7.11a  Ultra-thin lowicryl section of an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-chondroitin 6-sulphate antibody (3B3) followed by GAM IgG gold, showing moderate labelling of an osteocytic lacuna. Bar = 833 nm

Fig. 7.11b  Higher magnification of the osteocytic lacuna shown in Fig. 7.11a. Bar = 196 nm
Fig. 7.12 Ultra-thin lowicryl section of an osteocytic lacuna from an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-decorin antibody (LF30) followed by GAR IgG gold. Few gold particles are present between collagen fibres. Bar = 196 nm

Fig. 7.13 Ultra-thin lowicryl section of an osteocytic lacuna from an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks) labelled with anti-biglycan antibody (LF15) followed by GAR IgG gold. Bar = 196 nm
Fig. 7.14a Ultra-thin lowicryl section of an osteocytic lacuna from an OI type II/III foetal femoral bone (Foetus Ar-II, 20 weeks). Note the absence of gold labelling when primary antibody was replaced by non-immune mouse serum in the staining protocol.
Bar = 1 µm

Fig. 7.14b Higher magnification of the osteocytic lacuna shown in Fig. 7.14a.
Bar = 196 nm
Fig. 7.15a  Ultra-thin araldite section of an OI type II/III bone (Foetus Ar-II, 20 weeks) *en-bloc* stained with cuproline blue (CB) showing excessive amounts of CB positive rods of proteoglycan in the osteocytic lacuna. 
Bar = 1.3 μm

Fig. 7.15b  Higher magnification of the osteocytic lacuna shown in Fig. 7.15a. Note the electron dense proteoglycan rods in the lacuna and between collagen fibres. 
Bar = 309 nm
Fig. 7.16  Ultra-thin araldite section of osteoid collagen from a normal foetal bone (Foetus Gy, 19 weeks) en-bloc stained with cuprolinic blue (CB). The section shows few CB positive rods of proteoglycan between collagen fibres.
PG = proteoglycan
Bar = 100 nm

Fig. 7.17  Ultra-thin araldite section of osteoid collagen from an OI type II foetal bone (Foetus W, 25 weeks) en-bloc stained with cuprolinic blue (CB). The section shows abundant CB positive rods of proteoglycan between collagen fibres.
PG = proteoglycan
Bar = 100 nm
Fig. 7.18 Ultra-thin araldite section of osteoid collagen from a normal foetal bone (Foetus Sy, 22 weeks) en-bloc stained with cuprolinic blue (CB). The section shows few CB positive rods of proteoglycan arranged in an orderly pattern between collagen fibres.
PG = proteoglycan
Bar = 100 nm

Fig. 7.19 Ultra-thin araldite section of osteoid collagen from an OI type II foetal bone (Foetus F, 19 weeks) en-bloc stained with cuprolinic blue (CB). The section shows abundant CB positive proteoglycan rods between collagen fibres.
PG = proteoglycan
Bar = 100 nm
Fig. 7.20  Ultra-thin araldite section of osteoid collagen from a normal foetal bone (Foetus L, 24 weeks) *en-bloc* stained with cuprolinic blue (CB). The section shows few CB positive rods of proteoglycan between collagen fibres.

PG = proteoglycan
Bar = 100 nm

Fig. 7.21  Ultra-thin araldite section of osteoid collagen from an OI type II/III bone (Baby V.D.K, full term) *en-bloc* stained with cuprolinic blue (CB). The section shows many CB positive rods of proteoglycan between collagen fibres.

PG = proteoglycan
Bar = 100 nm
Fig. 7.22 Ultra-thin araldite section of osteoid collagen from a normal juvenile bone (M.K, 11 years) en-bloc stained with cuprolinic blue (CB). The section shows very few CB positive rods of proteoglycan between collagen fibres.

PG = proteoglycan
Bar = 100 nm

Fig. 7.23 Ultra-thin araldite section of osteoid collagen from an OI type II/III bone (Baby G-III, full term) en-bloc stained with cuprolinic blue (CB). The section shows long CB positive rods of proteoglycan between collagen fibres.

PG = proteoglycan
Bar = 100 nm
Fig. 7.24 Ultra-thin araldite section of osteoid collagen from a normal juvenile bone (N.A, 13 years) en-bloc stained with cuprolinic blue (CB). The section shows very few CB positive rods of proteoglycan between collagen fibres.

PG = proteoglycan
Bar = 100 nm

Fig. 7.25 Ultra-thin araldite section of osteoid collagen from an OI type III bone (H.S, 3 years) en-bloc stained with cuprolinic blue (CB). The section shows long CB positive rods of proteoglycan between collagen fibres.

PG = proteoglycan
Bar = 100 nm
7.4 Discussion

Various workers have reported an increase in the number of osteocytes per unit area in OI bone (Doty and Mathews 1971; Falvo and Bullough 1973) This has been interpreted by some as a relatively reduced matrix synthesis rather than an increased cellular proliferation. Each osteocyte consequently lies closer to its fellows because of the reduced bone matrix (Smith 1994), giving an impression of increased numbers.

In the present study, the hyperosteoctyosis was evident in OI type II/III foetal bone and the enlarged osteocyte lacunae had an adjoining perilacunar osteoid-like band of collagen. The latter implies either restricted mineral formation or mineral dissolution. The abundant vascular channels and large osteocytes significantly increase bone matrix porosity and probably predispose this type of woven bone to fracture easily (Currey 1984).

In addition, using four different techniques such as histochemistry, immuno-electron microscopy, electron probe X-ray microanalysis and cuproline blue staining, this study demonstrated excessive amounts of PGs in the enlarged osteocytic lacunae in OI type II/III bone. Normal age- and site-matched control bone did not show such changes in PGs.

Usui et al (1989) reported apparent changes in osteocytes, such as vacuolisation of cytoplasm with floccules and cellular debris in the lacunae, when ischaemic changes were induced in normal bone. The fine fibrillar and particulate material observed in the osteocytic lacunae of OI bone in this study was PG positive, but was absent from normal bone. Since all samples were processed in a similar manner, such particulate PG material is not likely to be an artefact produced by cell autolysis during specimen processing.

The observations of this study are consistent with the histochemical findings of Spencer (1962) and the biochemical findings of Engfeldt and Hjerpe (1976). Solheim (1969), who isolated subnormal amounts of GAGs from cortical bone tissue in OI, postulated that either a qualitative or quantitative defect of GAGs could hamper normal mineralisation. Urinary PG levels in OI patients were tested, and the levels were found to be especially high in younger OI patients (Goldberg 1978). These findings suggest that there may be alterations of PGs, particularly in foetal bone, which require further investigation and explanation.

Although previous studies have demonstrated small PGs (PG 100, decorin, biglycan) rather than large PGs in human foetal bone (Bianco et al 1993; Bosse et al 1993), the type II/III OI bone biopsies studied here contained large PGs which have been identified by immunogold labelling with specific antibodies. The immunogold labelling pattern for
decorin and biglycan was rather weak when compared with the labelling intensity of the large PGs keratan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate. Takagi et al (1991) have detected PGs containing chondroitin 4-sulphate and dermatan sulphate in the walls of osteocyte lacunae, perilacunar matrix and canaliculi in rat bone using biochemical and immunocytochemical methods. Such material lay adjacent to the mineral line, but was markedly less dense than that seen in our specimens. Furthermore the osteocytic lacunae lacked the peripheral collagen osteoid which we have noted in OI type II/III specimens.

Fisher (1985) hypothesised that there are high levels of chondroitin sulphate PGs which act as a space filler in foetal and young bone and are replaced by type I collagen during later life. Sauren et al (1989, 1992) have demonstrated PG-like material in the perilacunar matrix of osteocytes in rat and human bone using the cationic dye cuprolinic blue. Treatment with chondroitinase ABC did not digest the fine filamentous and granular material in the perilacunar matrix and around canaliculi. They concluded that this material probably consisted mainly of proteins and not just PGs.

Vetter et al (1991b) reported that the decorin levels were not significantly altered in OI bone when compared with normal bone although it was slightly higher in OI type III. Fedarko et al (1992) reported a decrease in three PGs in cultured OI cells. They studied sixteen OI patients, of whom only two were type II. The substantial phenotypic and pathogenic heterogeneity of OI in general, particularly the various type II sub-types, is very well documented (Sillence et al 1984). Therefore only a proportion of OI cell lines are likely to show reduced PG synthesis, and PG profiles will probably vary significantly according to OI sub-classification and phenotype. In this study we have detected an increase in large PGs in five OI type II/III patients.

The results suggest that these individuals may belong to a new subset of OI type II. The provisional sub-classification of OI type II has been previously described by Sillence et al (1984) and Rowe and Shapiro (1990). It is possible that the excessive PG deposition is a marker for one subset of OI type II (or II/III as proposed in this study) and this requires further investigation. However Fedarko et al (1995) have recently reported an elevated level of hyaluronan in OI bone cells. Furthermore when they grouped the OI cell strains by clinical types and plotted the average of steady state levels of matrix components, chondroitin sulphate PG showed a higher level in OI type II. These observations are consistent with our findings and also clearly demonstrate the phenotypic variability within the main types of OI.

The relationship between PGs and biological mineralisation has been well documented, and many different in-vitro studies agree that PGs inhibits hydroxyapatite formation (Bowness and Lee 1966; Cuervo et al 1973; Chen and Boskey 1985). The present
Chapter 7

ultrastructural study clearly demonstrates excessive PGs in the osteocyte lacunae of OI type II/III foetal bone with simultaneous poor mineralisation; the latter would undoubtedly predispose to excessive bone fragility and thinning. In particular the osteoid-like collagen band is devoid of mineral. Similar bands of osteoid-like collagen have not been observed in either human osteomalacia (Dopping-Hepanstal et al 1981) or in Fibrogenesis Imperfecta Ossium (Ralphs et al 1985).

The excessive PG in the osteocytic lacunae of OI type II/III bone may be due to a primary subtle genetic collagen abnormality which is accompanied by a secondary up-regulation of PG production, or it could be due to the lack of degradation and removal of PGs. In connective tissue development, the presence of PGs and chondrocytes is an early event preceding bone formation (Ali 1983). Thus, in OI foetal bone the presence of PG may be a sign of persistent immaturity of the newly formed bone.

The cuprolinic blue staining result showing a higher content of PG in the bone osteoid of OI bone compared with normal bone is another new finding of this study. As implicated in the literature, a major role is played by PG in the regulation of mineralisation, matrix formation and controlling collagen fibril diameter. Thus finding abnormal amounts of PG in OI bone may be relevant to the increased bone fragility in this crippling disease.

Scott (1988,1995) and Scott et al (1981,1989) performed studies on collagen and PG interactions in various tissues, and showed that in the collagen fibril the a and c bands were assigned to keratan sulphate PGs, while chondroitin and dermatan sulphate PGs were located in the d and e bands. Furthermore all four bands were located in or at the step of the gap zone in the collagen fibril. According to Scott (1995) bone has little or no proteodermachondran sulphate (PDS); since PDS occurs at or near the gap zone of the collagen fibril in non-mineralised tissue, it was speculated that in the presence of PDS mineralisation would not occur. Scott and Glanville (1993) showed that the primary and secondary structures of small PG proteins from different species strongly resemble each other and their binding sites are similar. The small PGs bind to the a, c, d and e bands of collagen types I, II and III, in a region which contains a conserved 11 amino-acid sequence. Scott and Glanville (1993) investigated this sequence and the PG binding patterns in four animal species (man, cow, mouse, chick) and reported that the sequence is highly conserved between species. Moreover this sequence remains unaltered in cases of OI with mutated glycine residues.

In the present study the PGs seen between the collagen fibrils in the osteoid of OI bone have not been identified either by immunolocalisation or digestion with chondroitinase ABC or AC II followed by cuprolinic blue staining. (Chondroitinase ABC will digest both chondroitin and dermatan sulphates but ACII will digest only chondroitin

222
sulphates). Future work should clarify this by identifying the different PGs. The abundant PGs in OI bone observed in this study may affect mineralisation, matrix formation and the control of collagen fibril diameter, all of which are likely to affect the characteristics of bone and make it more susceptible to fracture as seen in severe types of OI.
Chapter 8

General discussion and future work

This study differs from previous studies on OI bone for many reasons. For the first time, over forty human bone specimens from OI patients have been analysed using several electron microscopical techniques, and all qualitative and quantitative changes are correlated with the equivalent clinical types of OI according to the Sillence classification. Furthermore, as far as possible, the same biopsy site was compared with normal age- and site-matched controls. Many specimens were obtained fresh from the theatres and were fixed immediately. All specimens were treated under the stringent requirements necessary to maintain the ultrastructure of the cellular and extracellular components as close to the \textit{in-vivo} situation as possible.

The morphological changes of thirty six bone specimens were studied at LM and EM level (chapter 2) and the abnormalities were listed according to the various clinical types of OI. The changes observed in this study showed a clear correlation with the clinical phenotype. OI type I, the mildest form of OI, showed the fewest abnormalities in bone ultrastructure. Most of the type I bone specimens showed lamellar organisation, thick, organised osteoid with a normal mineralisation pattern and fairly normal bone cells. Type IV closely resembled type I with only minor abnormalities in the bone cells and osteoid. Type III showed abnormalities in the structural organisation and distribution of collagen, which plays an important role in bone structure. OI type II and II/III, which are either lethal or severely crippling, revealed many bone abnormalities. For example, the osteoid collagen from OI type II was sparsely distributed and thinner than normal in diameter. In addition bone formation in the growth plate was defective. The patchy mineralisation observed in some of the OI type II samples may have been due to inhibitors of calcification in the abnormal collagen osteoid which prevented the normal propagation of mineral. Excessive amounts of proteoglycan (PG) present in some specimens (chapter 7) and other non-collagenous proteins may act as inhibitors of mineralisation. The unmineralised collagen bundles observed in OI type II bone in this study would normally be mineralised and incorporated into the mineral matrix, but somehow this process is hindered. Culbert \textit{et al} (1995) studied two OI type II infants with overmodified collagens. They isolated collagen fibres from OI and normal control bone using a liquid nitrogen bomb and examined the fibres in TEM. They reported that in OI bone only 5\% of the collagen fibrils were encrusted with plate-like hydroxyapatite (HA) crystals, whereas in the normal controls about 70\% of fibrils were encrusted with these crystals. Their observation also supports the speculation that there might be some inhibitory effect on HA incorporation into the collagen fibres in the matrix of OI bone.
Chapter 8

The abnormal collagen fibres consistently observed in OI type III bone are also important. Although no biochemical abnormalities in collagen type I were detectable in these patients by the techniques used, their bone collagen was ultrastructurally abnormal often with irregular edges and no periodicity. They were also twisted, spiralled and sparsely distributed throughout a very thick osteoid. Similar types of collagen fibrils occur in the skin of patients with Ehlers Danlos Syndrome (EDS) sub-type VII (Vogel et al 1979), which overlaps clinically and biochemically with OI (Hata et al 1988), in Fibrogenesis Imperfecta Ossium (Ralphs et al 1985) and also in the skin of cattle and sheep with dermatoparaxis (Fjolsted and Helle 1974). According to Pope and Burrows (1997), the angular collagen fibrils in EDS VII are caused by either a structural defect in the COL 1A1 or 1A2 genes, or by a defect in the enzyme responsible for cleaving the collagen propeptides. In the OI type III patients with apparently normal collagen biochemistry there must still be either a defective aggregation of collagen filaments to form compact fibrils, or a dissociation, desegregation or fraying of normal fibril packing. This implies either an abnormality in collagen processing, or, as in EDS I/II, a disruption of type I collagen fibrillogenesis by the presence of other collagens or even extracellular matrix components. Whatever the cause, these structurally abnormal collagens do not appear to provide the nucleating and scaffolding sites essential for normal mineralisation, and hence give rise to the thick unmineralised osteoid and patchy mineralisation observed in these specimens.

Abnormalities observed in OI type IV bone occurred in the osteoblasts, osteoid and periosteum. Osteoblasts showed degenerative changes similar to those reported previously (Stoss 1990). The elastin seen in the osteoid and periosteum of some specimens has been previously observed by Cassella et al (1996) but was referred to as 'fibrous bodies'. The strong resemblance to the ultrastructural appearance of elastin and the positive colour reaction to Miller's elastin stain as demonstrated in this study confirms that elastin was present in these specimens. An increase in elastin has been reported in other inherited diseases of connective tissue such as EDS IV, and has been implicated as an induced secondary response to collagen depletion (Pope et al 1996). The depletion of collagen and the presence of elastin observed in type IV bone specimens in the present study once again appear to provide poor nucleation and scaffolding sites for mineral deposition and propagation.

 Electron probe X-ray microanalysis (chapter 3) showed that the Ca/P ratio of OI bone mineral was lower than in normal controls. When correlated with the clinical types of OI, the Ca/P ratio mirrored the severity of disease with OI type II bone sections giving the lowest value (1.55). The results from the microanalysis of cryo-sections were even more emphatic. For OI type II, the Ca/P ratio was 1.49 whereas the normal foetal and normal adult cryo-sections gave a Ca/P ratio of 1.69, the difference being highly statistically significant (p<0.01). This confirmed the finding of Cassella et al (1995) that OI cortical
bone has a lower Ca/P ratio than normal controls. These results reflect a summation of 15 samples representing all types of OI. The present study has been extended by analysing thirty five OI bone specimens and twenty five age- and site-matched normal controls. The results were differentiated according to clinical types of OI based on the Sillence classification (Sillence et al 1979) and for the first time expressed separately for each type of OI. Another unique feature of this study was the use of a more reliable cryo-sectioning technique that can avoid artefactual changes in the mineral composition that may occur in aqueous media. For this part of the study, freshly dissected bone was rapidly frozen and sectioned dry in order to avoid contact with any solvent prior to examination by electron microscopy. Thus, possible artefactual demineralisation that may occur during conventional tissue processing and ultramicrotomy was avoided and the values obtained were more reliable and statistically more valid than previously. The lower Ca/P ratio in OI type II may be due to the possible substitution of calcium and phosphorus in the apatite crystals by sodium, potassium or carbonate ions, making the crystals non-stoichiometric as suggested by Brown (1966), or due to an increase in phosphate ions as demonstrated in this study. This increase could be due to the presence of pyrophosphates in OI bone (Solomans and Styner 1969). However Cassella et al (1994b) performed an FTIR study on bone mineral in transgenic mice, in a model which resembles OI, and showed that the bone mineral crystals are apatite in nature despite their lower Ca/P ratio. They proposed that the possible substitution of calcium and phosphate ions in the crystal could account for the lower Ca/P ratio.

Another striking observation was the thinness of the bone mineral crystals in OI type II cryo-sections when compared with normals (chapter 3), which is consistent with the findings of other authors (Vetter et al 1991a; Traub et al 1994). These smaller crystals are more soluble and thus more prone to substitution by other moieties such as carbonate ions, which makes the OI crystals non-stoichiometric. These far from ideal crystals could possibly account for the fragility observed in OI bone.

The histomorphometric measurement of type I collagen diameter (chapter 4) was carried out for 42 OI patients and 25 normal age- and site-matched control subjects and the corresponding findings correlated with the clinically different types of OI. The collagen diameter of OI bone in this study did not correlate with disease severity but nevertheless varied with OI type. Thus, OI type II (the most severe type) demonstrated the smallest mean collagen diameter (45 nm) whereas OI type I (the mildest form) showed a mean diameter of 57 nm. The mean diameters obtained for type III (67 nm) and type IV (64 nm) were lower than the normal mean diameter (73 nm) but the difference was not statistically significant. A similar pattern was observed by Stoss and Freisinger (1993) but their values were much smaller than those obtained in this study, and they did not report the normal bone collagen diameter. The mean diameter of normal bone collagen published by Cassella et al (1994a) was 53 nm, whereas Jones et
al (1984) reported a range from 60 nm to 80 nm; neither study included measurements on normal foetal bone. The normal foetal bone mean collagen diameter measured in this study was 67 nm and the juvenile/adult bone collagen diameter was 73 nm. Although foetal collagen was smaller than juvenile/adult collagen, there was no statistically significant difference between the two groups. However, normal foetal bone as used in this study is a more appropriate control for direct comparison of collagen diameter for OI foetal bone (types II and II/III). Many authors (Smith et al. 1975; Byers et al. 1983; Balle et al. 1984; Jones et al. 1984; Stoss and Freisinger 1993) are in agreement that type I collagen fibres from OI patients are smaller in diameter than those from normals.

The reduced diameter observed in OI type I and type II could be due to several possible reasons. Firstly, the increased amount of type III collagen as shown by immunolabelling (chapter 5) might alter the collagen type III/I ratio and hence the overall fibril diameter. The documented biochemical evidence for increased amounts of type V collagen in OI could also play a role in controlling fibril diameter. There is evidence that in other connective tissue disorders, such as EDS I/II, collagen V mutations cause distorted cauliflower-like fibrils (Burrows et al. 1996). Secondly as suggested in the literature, retention of pN propeptides could lead to reduced collagen diameter. One of the foetal bones used in this study had retained pN propeptides and showed a reduced mean collagen diameter of 45 nm. Using immunolabelling techniques, Fleischmajer et al. (1987) demonstrated that thick type I collagen fibrils lacked the amino propeptide. Thirdly, an increase in hydroxylysine levels in OI collagen as documented by several authors (Eastoe et al. 1973; Trelstad et al. 1977; Heathcote 1978) could also alter the fibril diameter. The mutant collagens undergo hydroxylation and glycosylation thus forming cross-links between fibrils and altering the overall diameter. These and other fibrils of abnormal diameter may not be able to accommodate mineral as well as those of normal diameter. Hence they may be unable to provide the nucleating sites for mineral formation and the scaffolding sites for mineral propagation.

The results for the measurement of the periodicity of collagen fibres (chapter 4) showed that OI type II bone collagen had a much reduced D-period while other OI types normally had slightly shorter periods (with individual variations) when compared with normal bone collagen. In the present study the control mean period was 63 nm; the documented value for normal collagen periodicity is 64 nm for resin-embedded specimens (Hodge and Pertruska 1963). This value correlates well with the value obtained in this study, proving that the methodology used here was correct.

The shorter collagen period observed in OI type II bone implies that the gap regions in the collagen fibrils are altered at sites where initial mineralisation occurs (Hodge and Pertruska 1963; Glimcher 1985; Traub et al. 1992; Landis 1995). Perhaps these altered gaps in OI type II bone cannot accommodate the mineral crystals correctly, which leads
to alterations in mineral propagation within the collagen fibres. Furthermore the misalignment of D-period between adjacent collagen fibres observed in some of the OI type II specimens is in agreement with the findings of Landis (1995), who studied normal and pathological calcifications including OI (omi/omi mice) by high-voltage electron microscope tomography (3D). The omi/omi mice, which are known to produce only α1(1) collagen homotrimers and resemble moderately severe OI, showed abnormalities in collagen organisation. Many fibrils were twisted and kinked, and the characteristic 64-70 nm collagen period was out of register across some adjacent fibrils. Based on these studies Landis (1995) proposed a model for collagen-mineral interactions in which normal collagen fibrils are arranged in a strictly ordered manner to generate extensive channels and grooves. This regular staggering creates highly ordered hole and overlap zones for the packaging of molecules to form macromolecules and fibrils. Nucleation of mineral occurs initially in the hole zones. Crystals develop along the long axis of collagen and into the spaces generated by channels, resulting ultimately in broad parallel sheets of mineral growing in length and width.

In abnormal conditions like OI, assembly of collagen molecules follows a random stagger, resulting in irregular hole and overlap regions which fail to maintain a strict alignment thus isolating the channel spaces into small pockets. Since these spaces are limited in this assemblage, nucleation of mineral begins in the hole zone and develops along the long axis of collagen but growth in width is restricted because of the limited channel spaces. The resultant crystals are longer than wider and do not provide uniform calcification within the collagen array. Landis (1995) suggested that this type of collagen-mineral composite which varied in shape, size, location and orientation would be weak when compared with normal bone. Although there is no direct evidence for this in the present study, the patchy mineralisation observed in OI type II bone specimens could have been due to limited channel spaces within the collagenous matrix, restricting the normal propagation of mineral.

The immunoelectron microscopic demonstration of different collagen types in OI bone (chapter 5) and the comparison of labelling intensities with normal age- and site-matched controls is another achievement of this study. It has bridged the gap between biochemical findings and ultrastructural observations. Many authors have reported the biochemical finding of type III collagen in OI bone (Müller et al 1977; Pope et al 1980; Bateman et al 1986; Culbert et al 1995); this was demonstrated ultrastructurally for the first time in the present study. Furthermore, the labelling intensity for type I collagen was reduced in OI bone when compared with the normal age- and site-matched controls. Bateman et al (1986), who studied seventeen cases of lethal OI biochemically, showed there was a consistent reduction of type I collagen content and interpreted this reduction as either a decreased production or an increased degradation of type I collagen. The reduced type I collagen labelling in OI bone observed in this study could be due to the impairment of the
quality and/or quantity of type I collagen which may be important as this protein plays an essential role in the bone mineralisation process.

Alkaline phosphatase as an osteoblast marker has been examined at EM level in this study (chapter 6), and enzyme activity appears to be reduced in OI bone cells when compared with normal controls. This confirms the findings of Doty and Mathews (1971) that OI osteoblasts have a functional impairment, thus producing diminished collagenous osteoid which is associated with defective mineralisation.

OI is clinically and biochemically an extremely heterogeneous disorder. As explained by Byers (1996), there are various steps involved in the process of collagen fibril formation from gene transcription to final mineralisation, all of which are controlled by different sets of genes. According to Rowe and Shapiro (1990), there are at least fifteen steps involved in this cascade of events and abnormalities can occur at any stage. The first step in the process is the activation of COL 1A1 and COL 1A2 genes to produce the individual collagen chains. This is followed by chain assembly and molecular folding of collagen molecules, which are then transported to the endoplasmic reticulum of the cell. The C- and N-terminal propeptides of procollagens are cleaved prior to the formation of collagen fibrils, which finally become mineralised to form the hard mineralised matrix. In OI the fundamental defect is generally abnormal type I collagen, which is caused by defective COL 1A1 and COL 1A2 genes. Since not all OI patients have mutations of collagen type I, there must be other unknown causes for their bone fragility and crippling deformities. Despite the molecular and biochemical advances in OI research, there are still many unanswered questions. In some mild forms of OI there is a marked decline in the fracture rate in adolescence and the reason for this has not been satisfactorily explained. The fracture rate rises again following the menopause in women while in men it remains low (Paterson et al 1984). It is possible that these hormonal changes could play a role in this process. In addition to collagen abnormalities, abnormal mineral composition, other non-collagenous proteins and hormones could be important factors affecting the brittleness of bone.

The unexpected and abnormal presence of PGs associated with the osteocyte lacunae of five OI type II/III bone samples was demonstrated using four different methods (chapter 7). This new finding may be important as many in-vitro studies agree that PGs inhibit hydroxyapatite formation (Bowness and Lee 1966; Cuervo et al 1973; Blumenthal et al 1979; Chen and Boskey 1985). Initial calcification of bone probably follows the removal of inhibitory compounds such as PGs (Ali 1983, 1992). The excessive PG production observed in this study could be due to a number of causes. Firstly there may be a primary abnormality of OI osteocytes. Secondly a subtle collagen abnormality may be inducing secondary up-regulation of PG production, or thirdly there may be a deficiency
in the degradation and removal of PGs. Perhaps the altered levels of PGs in OI bone affect mineralisation and predispose the bone to excessive fragility.

In the present study bone collagen and proteoglycan interactions in seven OI bone samples appeared abnormal when compared with normal controls as demonstrated by cuprolinic blue staining (chapter 7). There appears to be more PGs associated with collagen fibrils in the osteoid of OI bone, and this abnormal quantity and distribution of PGs could play an important role in controlling fibril diameter and mineral formation.

The present study aimed to explore the cellular and matrix changes seen in OI bone and to evaluate whether previously reported biochemical findings can be substantiated ultrastructurally. The use of several techniques revealed many cellular and matrix changes which have not been previously reported. In particular, the marked abnormalities observed in OI type II were quite striking. These samples showed abnormal mineral composition with a markedly lowered Ca/P ratio. The collagen fibrils were reduced in diameter, had a shorter D-period, and demonstrated many ultrastructural abnormalities and alterations of collagen types. All these abnormalities in the bone cells, the collagens, the non-collagenous proteins such as PGs, and the poorly substituted, non-stoichiometric, thin hydroxyapatite crystals observed in OI are likely to play an important and major role in bone fragility and the crippling deformities that occur in this disease.

**Future Work**

Future work should explore certain aspects of OI which could not be examined in this study. For example there are several reports of changes in non-collagenous proteins in OI bone. Dickson *et al* (1975) observed higher levels of these proteins and α2HS-glycoproteins in OI bone. Termine *et al* (1984) reported decreased levels of osteonectin and bone proteoglycan, but bone sialoprotein levels were not affected in bovine Osteogenesis Imperfecta. Vetter *et al* (1991b) studied the levels of non-collagenous proteins of bone in OI and normal controls, and found reduced levels of osteonectin, with high levels of bone sialoproteins, osteocalcin and α2HS-glycoproteins in all OI patients. There was no alteration in decorin levels in these patients when compared with the normal controls. These findings have not been matched with ultrastructural data and future work should clarify these observations by immunolocalisation of non-collagenous proteins at electron microscopy level using commercially available antibodies to osteopontin, osteonectin and bone sialoproteins. Appropriately fixed and Lowicryl-embedded OI and age-matched control bone samples are available for this part of the study.
Immunogold localisation of type V collagen was not successful in this study for two possible reasons. According to Birk et al (1988), type V collagen exists as a core fibril of type I collagen and, to expose the epitopes, digestion of type I collagen with collagenase is needed. Furthermore this should be carried out on unfixed cryo-sections as the epitopes recognised by commercially available antibodies to type V collagen are sensitive to chemical fixation.

The abnormal mineral composition as determined by electron probe X-ray microanalysis revealed a lowered Ca/P ratio especially in OI type II bone mineral. Future work should clarify the causes of this abnormal mineralisation, and whether the lower Ca/P ratio is a result of increased phosphorus and pyrophosphates in OI bone or due to the substitution of calcium by other moities. To achieve this, more detailed chemical analyses of bone mineral need to be undertaken to evaluate mineral metabolism in OI.

The histochemical localisation of alkaline phosphatase as a marker for osteoblast activity was carried out only on five OI patients, while the localisation of acid phosphatase as a marker for osteoclast activity was not attempted in the present study. As these reactions provide information about the cells and focal areas of bone formation and resorption, a systematic study should be carried out on bone samples from all OI patients and the findings correlated with severity of disease.

In the present study collagen-PG interactions were studied using cuprolinic blue staining, but PG rods in the collagenous osteoid have not been identified. It is important to know which class of PG is present in different types of OI as these molecules may play an important role in controlling collagen fibril diameter and mineralisation. Future studies should identify these PG rods by immunogold labelling using commercially available antibodies which may recognise the different PGs.
References


References


References


References


References


References


244
References


References


Vogel, B. E., Doelz, R., Kadler, K. E., Hejima, B., Engle, J. and Prockop, D. J. (1988). A substitution of cysteine for glycine 748 of the $\alpha_1$ chain produces a kink at this site in the procollagen 1 molecule and an altered N-proteinase cleavage site over 225 nm away. Journal of Biological Chemistry 263: 19249-19255.


