Detecting cooked bone in the archaeological record: A study of the thermal stability and deterioration of bone collagen

by

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Abstract

Cooked bone must account for a large proportion of the skeletal finds from archaeological sites yet it is still not possible to detect cooked bone unless it has become charred. The reason for this is that mild heating (80°C, one hour), such as is usual in cooking, does not lead to detectable changes in any biochemical parameter yet measured. The aim of thesis was to develop a technique which could detect this level of heat damage. This was attempted by analysing the collagen component of heated bone with microscopy and calorimetry.

Differential scanning calorimetry was only able to detect catastrophic damage which occurred after extensive heating (six hours at 100°C or > 145°C). However using transmission electron microscopy it was possible to visualize heat-damage at the level of the collagen fibril after very mild heat treatment. The approach was tested on modern, forensic and archaeological material. Buried bone that had not been cooked showed some alteration similar to that caused by cooking, nevertheless paired cooked and uncooked bones could always be distinguished. Analysis of modern human material with different ages-at-death showed an apparent age-related stability; the collagen fibrils from older individuals were more resistant to thermal alteration and swelling in acid. This was also observed, more dramatically, in artificially cross-linked bone collagen. Conversely bones buried in acidic soils for a short period showed a rapid fragmentation of the collagen fibrils.

This analysis has given an insight into the very early stages of collagen collapse within bone and has shown that even when protected by mineral, it is prone to damage. Degradation of bone collagen appears to be governed by the integrity of the collagen fibril and if viewed in this way can help to explain why bone collagen can remain intact far into the archaeological record, why severely diagenetically altered bone can yield undamaged collagen molecules and why bone collagen is such a reliable material for isotopic dietary analysis and ^{14}C dating.

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List of abbreviations

AAR	Amino acid racemization
AGEs	Advanced glycation end-products
Al	Aluminium
Ala	Alanine
Asp	Aspartic acid
α1(I) and α2(I)	Alpha chains 1 and 2 from type I collagen
C	Carbon
^{14}C	Radioactive isotope of carbon
°C	Degrees celsius
Ca	Calcium
$Ca_{10}(PO_4)_6.(OH)_2$	Hydroxyapatite
C:N	Carbon to nitrogen ratio
CNBr	Cyanogen bromide
cm	Centimetres
-CO	Carbonyl group
-COOH	Carboxylic acid group
d-spacing	Measurement of the combined length of one gap and overlap region in a collagen fibril
D/L	Ratio of D-amino acid to L-amino acid
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
Ea	Activation energy
EDTA	Ethylenediaminetetraacetic acid
EXO	Exothermic
F	Fluorine
g	Gram
Gla	γ-carboxyglutamic acid
Glu	Glutamic acid
Gly	Glycine
HCl	Hydrochloric acid
HLKNL	Hydroxylysino-5-ketonorleucine
HL-Pyr	Hydroxylysyl-pyridinoline
HPLC	High Performance Liquid Chromatography
hr	Hour

HT	High tension
Hyp	Hydroxyproline
ICP-OES	Inductive-Coupled-Plasma-Optical-Emission-Spectrometer
IR	Infra red spectroscopy
J g^{-1}	Joules per gram
k J mol^{-1}	Kilojoules per mole
kV	Kilovolt
km	Kilometre
LKNL	Lysino-5-ketonorleucine
L-Pyr	Lysyl-pyridinoline
Lys	Lysine
M	Molar concentration
m	Metre
mbar	Millibar
min	Minute
ml	Millilitre
mg	Milligram
mm	Millimetre
µg	Microgram
µm	Micron
N	Nitrogen
N	Normal
Na	Sodium
NaOH	Sodium hydroxide
NCPs	Non-collagenous proteins
$-NH_2$	Amine group
nm	Nanometers
O	Oxygen
OC	Osteocalcin
OH	Hydroxide
Pb	Lead
PBS	Phosphate Buffer Saline
pH	Measure of acidity or alkalinity from the power of the hydrogen ion concentration
Pro	Proline
-R	Side chain
Ra	Radium

SAXS	Small angle x-ray scattering
S.D	Standard deviation
SEM	Scanning electron microscopy
SF	Infra red crystal field splitting factor
Sr	Strontium
SRS	Synchrotron Radiation Source
T_d	Temperature of denaturation (°C)
Tel	Telopeptide
TEM	Transmission electron microscope or microscopy
T_m	Melting temperature (°C)
T_s	Shrinkage temperature (°C)
w/v	Weight for volume
X	The second amino acid position in the collagen triplet repeat unit
Y	Yellow hue
Y	The third amino acid position in the collagen triplet repeat unit
YR	Yellow-red hue

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Declaration

I hereby certify that the work described in this thesis is all my own work, except where otherwise acknowledged, and has not been submitted previously for a degree at this, or any other, university

Hannah Koon

General introduction to the thesis

The ability to identify cooked bone has far reaching implications for many disciplines, especially within the fields of paleo-anthropology, archaeology and forensic science. Evidence of heated bone from archaeological sites can be used not only to imply human influence, but also to elucidate dietary habits, cooking techniques, socio-cultural and funerary practices; yet the literature on low temperature heated bone remains limited. This is not due to a lack of significance, but rather due to the difficulty in identifying bone which has been cooked but which has not reached a temperature sufficient to cause charring to the bone surface. The aim of this research was to examine whether damage within bone collagen fibrils might be used as a means of detecting cooked archaeological bone. The difficulty with this approach is that little is known about how the collagen fibrils within bone are degraded by heat or in the burial environment. Therefore a number of objectives had first to be addressed and these are outlined below:

- to examine the early stages of deterioration in mineralized and non-mineralized collagen fibrils,
- to identify the role played by the mineral and/or mature cross-links in preventing failure of the collagen fibril,
- to explore the use of TEM and DSC analysis as techniques to identify cooked bone,
- to examine how the burial environment can impact on the ability of these approaches to detect heat induced damage.

The thesis is divided into four parts. Part 1 includes two introductory chapters, the first discusses background technical information fundamental to this thesis, including the structure of collagen and bone and the factors responsible for the thermal stability of bone collagen. The second chapter discusses why it is important to be able to detect cooked bone in the archaeological record, and an overview of the present methods of detection and their associated problems is used to explain why this has not already been accomplished. Part 2 contains four chapters which deal with the methods used, method development and preliminary experiments. This includes heating experiments which were

conducted on fresh material and analysis from experimental burials. Part 3 describes the results of case studies. Some of this work is set out in the form of articles which will be submitted for publication. The first of the two case study chapters looks at forensic applications to this research and the second is comprised of an archaeological case study in which the TEM method was tested to detect cooked bone from an Anglo-Scandinavian urban site. Finally, Part 4 provides a synopsis of the thesis which is followed by a synthesis of findings, conclusions of the research and suggestions for further work.

The CD at the back of the thesis holds a collection of digital TEM images of collagen fibrils which were taken during this research. It also includes all of the TEM images that are presented within the thesis.

Part 1 literature review

Chapter 1 Introduction to collagen and bone

In later chapters the changes that occur to bone as a result of heat (Chapters 2, 4 and 5) and burial (Chapters 2 and 6) are considered, but first it is necessary to provide an overview of the structure and composition of fresh unaltered bone. The research questions that are addressed within the thesis focus specifically on alteration to collagen, a protein which accounts for 90% of the organic content within bone. Therefore in this section there is a brief description of bone followed by a more detailed discussion of those aspects which are fundamental to this thesis, including: the hierarchical structure of collagen, its position within bone, and the different elements which provide bone collagen with its unusually high thermal stability.

1.1 What is bone?

Fresh bone has three primary constituents; water, a mineral component and an organic portion. Bone mineral, often referred to as bone- or bio- apatite is comprised of a non-stoichiometeric form of hydroxyapatite. It plays an important role in an organism, not only in providing rigidity to bone but also as a reservoir for minerals (Glimcher and Krane, 1968; Weiner et al., 1999). It has a general unit cell formula of $Ca_{10}(PO_4)_6.(OH)_2$ although it can accommodate a large number of trace elements by surface and lattice substitutions (Millard, 2001). For instance, the Ca^{2+} ions may be replaced by Sr^{2+}, Ra^{2+} or Pb^{2+} and the OH^- groups by F^-. There is also some substitution of phosphate ions for carbonate (Glimcher and Krane, 1968) and other ions, mostly metals can also be attached to the surface of the hydroxyapatite crystals by adsorption (Katz, 1996; O'Connor, 2000). The mineral contributes between 60-80% to the weight of bone (Glimcher and Krane, 1968). A further 25-30% is provided by the organic component, of which approximately 90% is made up of type I collagen (Glimcher and Krane, 1968; Millard, 2001). Collagen is a protein thus it is made up of chains of amino acids. It forms long rope-like structures which are impregnated with mineral crystallites. The remainder of the organic portion is a 'ground substance' of other organic compounds, containing various lipids and non-collagenous proteins (NCPs) including osteocalcin, osteonectin and proteoglycans (Glimcher and Krane, 1968; O'Connor, 2000). Their roles in bone are not yet fully understood although they are likely to play a part in: the initiation and control of mineralization (Scott, 1988), regulating the diameter of collagen fibrils (Parry et al., 1982; Scott,

1988) and binding collagen and mineral (Prigodich and Vesely, 1997). The exact proportion of each constituent of bone is dependent upon a number of factors such as; species, age, sex, nutritional state and bone type (Nicholson, 1996; Nielsen-Marsh et al., 2000b).

If one the major components of bone is removed this will have a drastic effect on its properties. For instance; if the mineral is dissolved away in acid the bone will become very rubbery and translucent; if a bone is boiled and the collagen is gelatinized and leached out, the bone will be extremely brittle and susceptible to breakage (Seeley et al., 1992). The intimate association of protein fibres and mineral crystals not only provides fresh bone with flexibility and tensile strength, but also provides a mutual protection which enables bone to survive for long periods after death. Whilst biological materials, such as skin, tendon and other soft tissues rarely survive into the archaeological record, bone collagen can persist for hundreds of years in a relatively unaltered state, and thus is used as a reliable substrate for biomolecular analysis. It is thought that the mineral inhibits access to destructive micro-organisms and stabilizes the collagen component against gelatinization (Nielsen-Marsh et al., 2000a; Collins et al., 2002; Kronick and Cooke, 1996). Collagen also provides protection for the mineral (Trueman and Martill, 2002). Bone apatite crystals are vulnerable to dissolution in the burial environment because they are extremely small, with a correspondingly high surface area of around 200 m^2g^{-1} (Weiner and Price, 1986). Their small size means that they are reactive and can dissolve in water that is drawn in from the surrounding environment through pores in the bone (Karkanas, 2000; Nriagu, 1983). It has been suggested that as bone crystals are held within a framework of collagen, the protein protects the surface of the apatite crystallites, and this is why fresh bone does not rapidly dissolve (Trueman and Martill, 2002).

1.2 What is collagen?

Collagen is a long chain structural protein, forming a major component of the hard and soft tissues in vertebrates. It forms cables of tightly packed molecules that strengthen tendons and ligaments, and vast meshes of interwoven strands that support skin and internal organs. Collagen is the main protein component of bones and teeth where it is combined with mineral crystals. As a result of its ubiquity within the animal kingdom the structure and stability of collagen has been subject to considerable investigation, not only within the field of biology. Collagen derivatives are utilized and have been studied within a whole range of

materials such as parchment and leather (Larsen, 2002), medical biomaterials (Lee et al., 2001; Meena et al., 1999) and foodstuffs (Snowden and Weidemann, 1978).

There are currently twenty-seven known types of collagen, with molecules from types I-III, V, XI, XXIV and XXVII capable of forming fibrils (Hulmes, 2002; Pace et al., 2003; Sato et al., 2002). The most extensively studied of these is type I collagen. This fibrillar form is the dominant collagen of skin, tendon and bone and thus will be the focus herein.

1.3 Structural hierarchy of type I collagen

Collagen has an intricate structure with a number of levels of organization which can be found at the sub-molecular scale within individual polypeptide chains of amino acids, right up to the collagen fibrils which are the building blocks for tissues such as skin, tendon and bone. Bone collagen has yet higher levels of organization; it is encased with mineral crystals and these mineralized fibrils are arranged in different conformations to form microscopic and macroscopic structures within bone. A brief description of the structural hierarchy of bone collagen is provided below and this is followed by a more detailed description of each key structural level. The relationship between these levels is depicted in Figure 1.1 where a scale bar is included to show the size of these different structural components relative to each other.

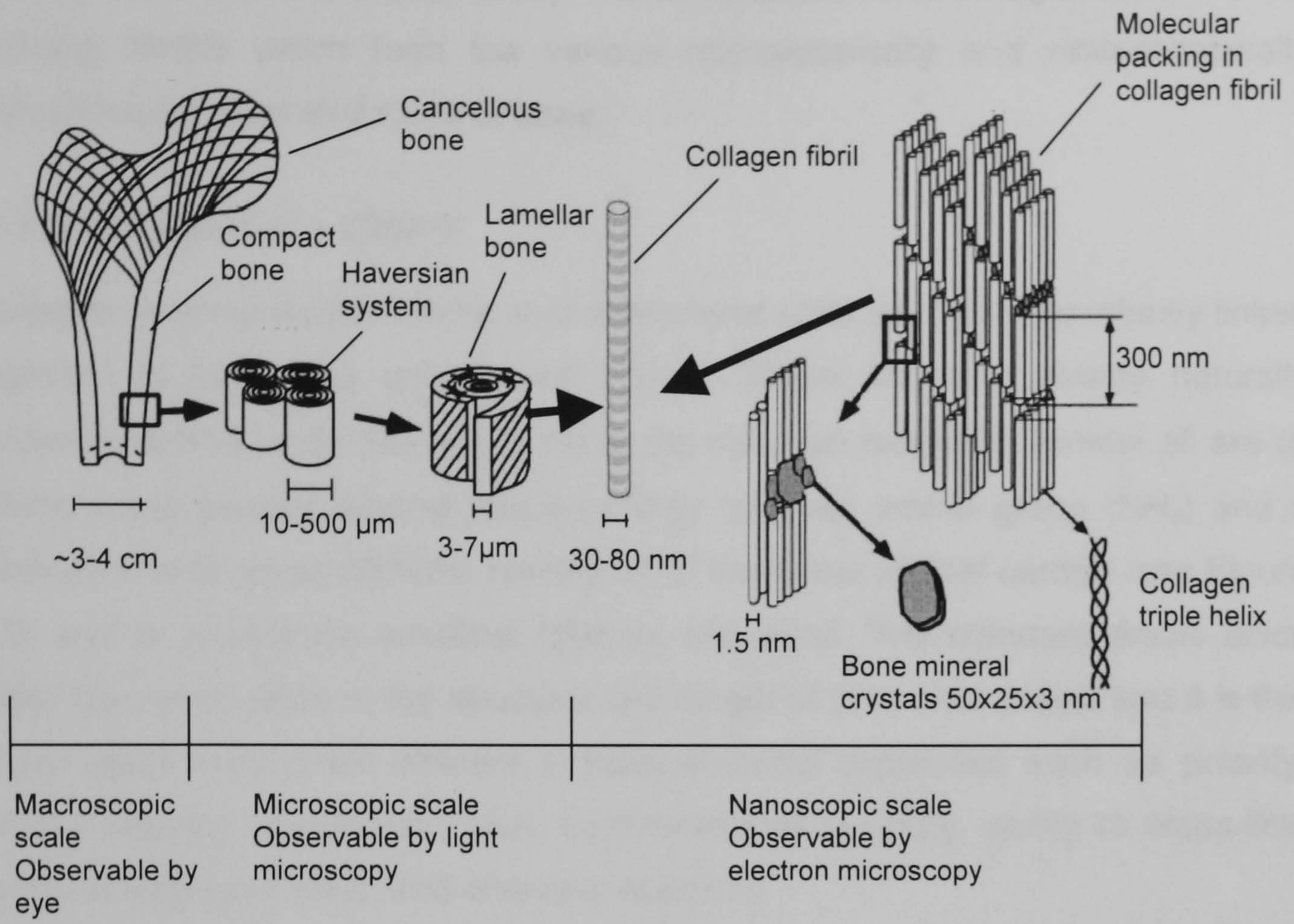


Figure 1.1 Schematic diagram showing the hierarchical structure of bone collagen, adapted from Rho et al. (1998), Figures 1 and 2, p:93, 97.

Type I collagen is composed of amino acids, which combine to form long polypeptide strands or α-chains. The basic collagen molecule is approximately 300 nm in length and 1.5 nm in diameter (Kadler et al., 1996). It consists of three α-chains each of which are twisted into a left-handed helix and also coiled around each other to form a right-handed triple helix. The molecules self-assemble via a staggered arrangement into larger structures known as fibrils which are the tensile strength bearing components of connective tissues and bone (Fratzl et al., 1998; Kadler et al., 1996). Once the molecules have been aligned into the fibril they are stabilized by a network of covalent cross-links. The fibrils are cylindrical with diameters ranging between 10 and 500 nm and extend to several microns in length (Hulmes et al., 1995). Fibrils of larger diameter are generally found in highly tensile structures such as tendon and ligaments, where they are packed into tight parallel bundles and aligned in the direction of force (Culav et al., 1999; Ottani et al., 2001). Collagen fibrils in bone are smaller and typically range from 30-80 nm in diameter (Tzaphlidou, 2005; Tzaphlidou and Berillis, 2005). In mineralized tissues the fibrils are also surrounded and impregnated by small mineral crystallites, only tens of nanometres in length and width and 2-4 nm in

thickness (Weiner and Traub, 1992). The mineralized bone collagen fibrils are the building blocks which form the various microscopically and macroscopically distinct hierarchical structures of bone.

1.3.1 Collagen α - chains

Collagen is composed of amino acid monomeric units which are covalently linked together to form long unbranched chains. There are over twenty naturally occurring amino acids that are found in the collagen molecule. Almost all are α-amino acids (except proline) because they have an amine group (NH_2) and a carboxylic acid group (COOH) coming off of the same central carbon (see Figure 1.2) and all except the smallest, glycine, are chiral. The standard amino acids differ from each other in the structure and length of their side chains and it is this which gives them their different physico-chemical properties such as polarity, acidity, basicity, aromaticity, bulk, conformational flexibility, ability to cross-link, ability to hydrogen bond, and chemical reactivity.

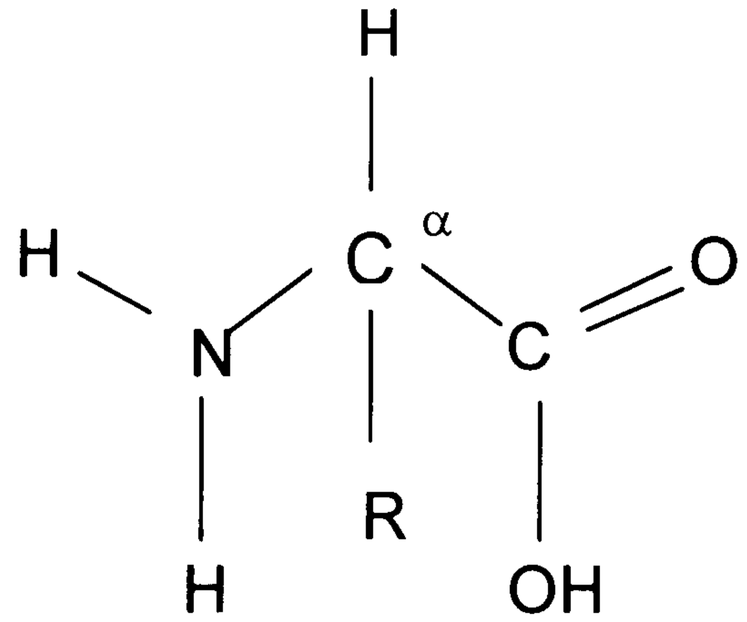


Figure 1.2 General chemical structure of amino acids. Both the amino group and the carboxyl group are attached to the α-carbon. The R group represents the side chain which differs between amino acids.

The amino acids are held together by peptide bonds which occur by the elimination of a water molecule between a carboxyl group and an amino group of two adjacent amino acids (Figure 1.3) (Freemantle, 1995). The resulting polypeptides, referred to as α-chains, are comprised of a repeat unit of three amino acids which extend for over 1000 residues in length (Collins et al., 1995; Woodhead-Galloway, 1980). The basic formula for this unit is (Gly-X-Y)n, where every third residue is glycine (Gly). Proline (Pro) and its hydroxylated form hydroxyproline (Hyp) commonly occupy the X and Y positions respectively

(Kuivaniemi et al., 1997; Woodhead-Galloway, 1980) and are essential for providing stability to the collagen molecule (Jenkins and Raines, 2002; Prockop and Kivirikko, 1995; van der Rest and Garrone, 1991).

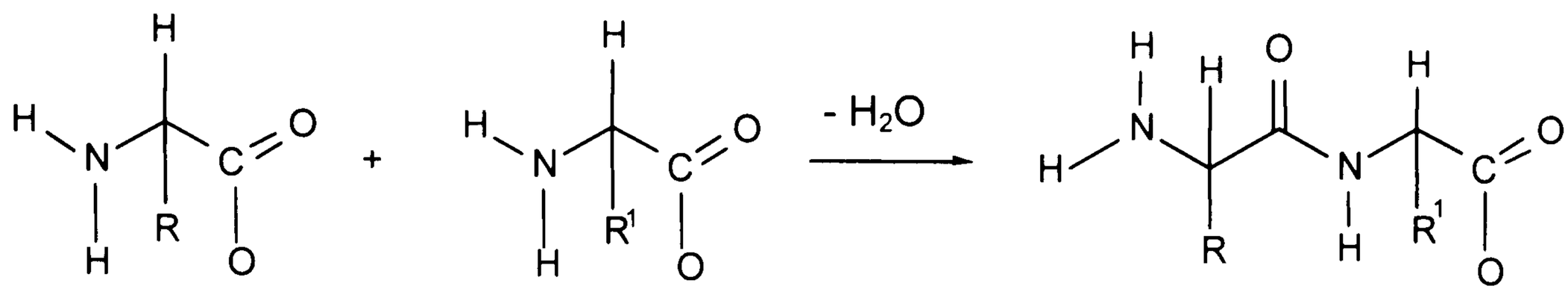
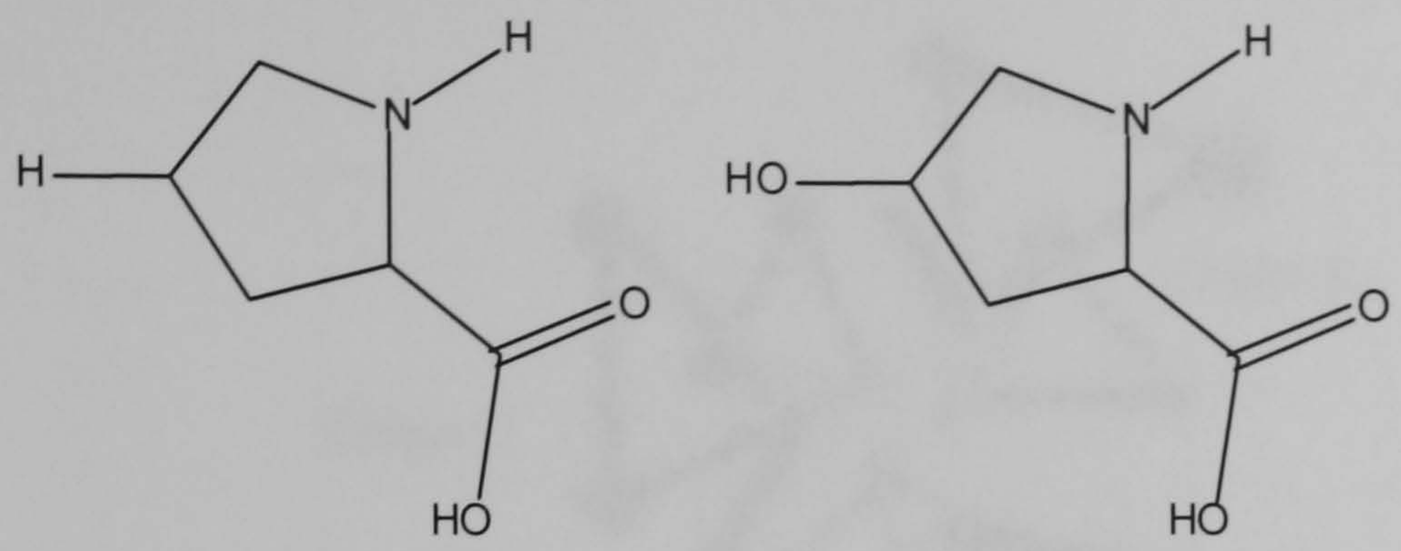


Figure 1.3 Formation of a peptide bond through the condensation of two amino acids and the removal of water.

Hyp is formed in situ by hydroxylation of Pro, rather than being incorporated into the chain in the hydroxylated state (Kivirikko and Prockop, 1967). As mentioned above Pro has a different structure to the standard amino acids found in collagen, instead of the amino group being attached to the α-carbon, it is actually part of a heterocyclic ring (Figure 1.4). The cyclic structures of Pro and Hyp cause the polypeptide backbone to bend, this produces a series of kinks which form a left-handed α-helix. These unusual rigid structures also prevent movement and rotation which stabilizes the helix (Jenkins and Raines, 2002; McClain and Wiley, 1972). In this way collagen α-chains are similar to the poly-L-proline II helix which is stabilized by the steric repulsion between the bulky pyrrolidine rings on the Pro residues (McClain and Wiley, 1972).

The central helical region of the collagen α-chain is approximately 1000 residues in length and is flanked at either end by short regions, referred to as telopeptides, which do not have the (Gly-X-Y)n repeat sequence and are not helical (Vitagliano et al., 1995; Wess et al., 1998). These regions are essential for directing fibril formation and for stabilizing the fibril structure by contributing to covalent cross-link formation (see section 1.3.6).



proline hydroxyproline

Figure 1.4 Chemical structures of proline and hydroxyproline.

1.3.2 Collagen molecule

The type I collagen molecule, *tropocollagen*, is a semi-flexible rod consisting of three α-helices each of which are coiled around each other to form a right-handed triple helix (Ramachandran, 1967; Rich and Crick, 1961) (Figure 1.5). Two of the chains are identical and denoted as (α1) and the other has a slightly different amino acid sequence and is denoted as (α2). The α2 chain is thought to increase the stability of the collagen molecule, by increasing hydrophobic interactions within the heterogeneous triple helix (Miles et al., 2002). Within the macromolecule the relatively small Gly residues, contributed alternately by the three chains, run down the centre of the molecule, whereas the more bulky amino acids are directed towards the outside (Piez, 1981) (see Figure 1.6).

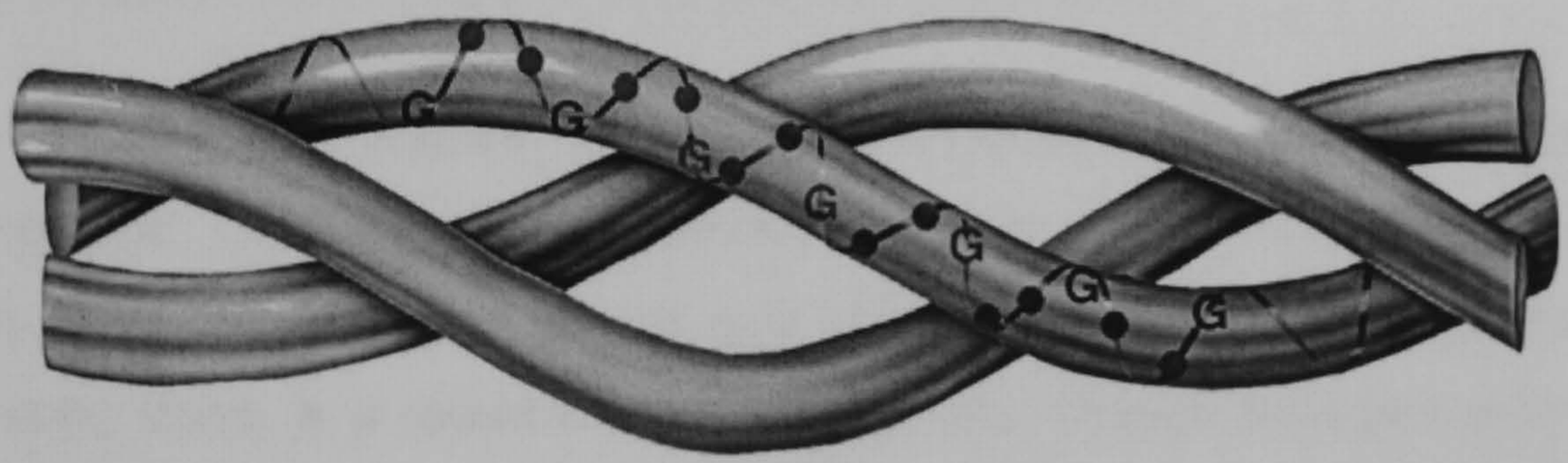


Figure 1.5 Portion of a collagen molecule showing the super helix structure, from Culav et al. (1999), Figure 2, p: 311. Individual alpha chains are coiled to form a triple helix and within each chain, the amino acids are similarly arranged in a helix, with glycine (G) facing the centre of the triple helix. The other amino acids are represented by the black dots.

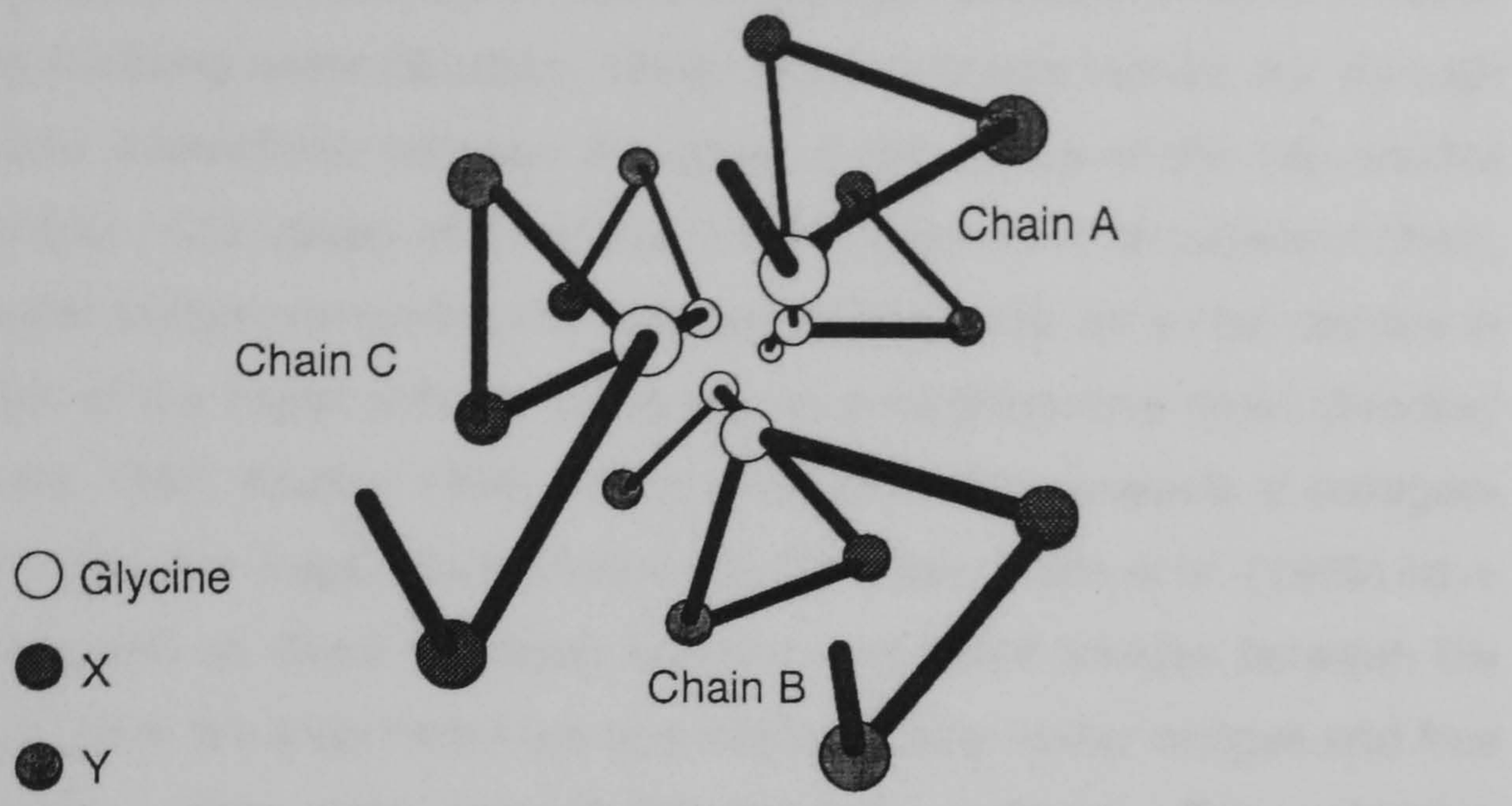


Figure 1.6 A schematic diagram showing a cross section through the collagen triple helix, adapted from van der Rest and Garrone (1991), Figure 1, p: 2815. The small glycine residues (shown in white) sit in the center of the triple helix. The X (shown in black) and Y (shown in grey) positions face away from the molecule and are occupied by other amino acids.

There are two contrasting models which describe the twist of the helical region; the 10/3 model, based upon 10 amino acid residues per three full turns of the helix (Fraser et al., 1979; Rich and Crick, 1955); and the 7/2 model, based upon 7 residues per two helical turns (Okuyama et al., 1999). However based on observations of collagen model peptides it has been suggested that the helical region of the collagen molecule may in fact contain a combination of both (Kramer et al., 1999). With the regions which are rich in Pro and Hyp having a 7/2 symmetry whereas other regions having a 10/3 symmetry.

The triple helix is held together by interactions between adjacent side chains of the amino acid residues. Weak bonds can form from interactions between oppositely charged polar side chains and stronger hydrogen bonds can also be created when there is a redistribution of electronic charge between a hydrogen atom and a highly electronegative atom such as oxygen (Freemantle, 1995; Woodhead-Galloway, 1980). The highly extended nature of the polypeptide helix prevents the formation of intra-chain hydrogen bonds, but hydrogen bonds do occur between chains (Collins et al., 1995).

In the past there has been considerable debate as to the nature and number of the hydrogen bonds between the α-chains (Ramachandran and Kartha, 1954; Ramachandran and Chandrasekharan, 1968; Rich and Crick, 1961). It is now

generally accepted, that there are in fact two hydrogen bonds per Gly-X-Y triplet, one of these involving water (Brodsky, 1999). The two bonds formed are: through direct inductive interactions between the amino (-NH) group of the Gly residue and the carbonyl (-CO) group of a residue in the X position of an adjacent chain; and via a water bridge connecting the hydroxyl (-OH) group on a Hyp residue in the Y position of the triplet with an -NH group in a neighbouring chain (Brodsky and Ramshaw, 1997; Kadler, 1994). Using X-ray diffraction analysis of collagen-like peptides, $(Gly\text{-}Pro\text{-}Hyp)_4$-Ala-Pro-Hyp-$(Gly\text{-}Pro\text{-}Hyp)_5$, Bella et al. (1995) have shown that as well as direct hydrogen bonding and water bridges between the polypeptides, there are also intrachain and intermolecular water bridges and free water molecules which pack around the collagen molecule. This extensive network of water molecules comprises a hydration cylinder around the collagen molecule which is anchored to the peptide chains by the Hyp residues.

1.3.3 Role of hydroxyproline (Hyp) in the thermal stability of the collagen molecule

When heated to a sufficient temperature the hydrogen bonds between the α-chains of the collagen triple helix are ruptured. This causes the chains to separate and unravel into random coils (Flory and Garrett, 1958). The temperature at which type I collagen molecules become denatured, often referred to as the melting temperature (T_m), is related to the Pro and Hyp content, which in turn is closely related to the body temperature of the organism (Prockop and Kivirikko, 1995). For instance cod skin collagen contains a low Pro and Hyp content and the T_m for individual molecules is just 12°C, whereas calf and chick skin molecules have T_m values of 39°C and 41°C respectively, and higher quantities of these amino acids (Burjanadze, 1979; Woodhead-Galloway, 1980).

The importance of Pro and Hyp has been confirmed using model polypeptides where the X and Y positions of the Gly-X-Y triplet have been replaced with different amino acids. A study by Chan et al., (1997) showed that the Gly-Pro-Hyp triplet produced the highest T_m. The greater thermal stability of this triplet is thought to be due to the ability of Hyp to form hydrogen bonded water bridges through its –OH group (Chan et al., 1997; Bella et al., 1995). This is supported by evidence that the thermal stability of the triple helix is dependent on the content of Hyp and not Pro, which lacks the -OH to form water bridges (Burjanadze, 1979). However there is contradictory evidence to suggest that water bridges do not significantly contribute to the stability of collagen molecules (Engel and

Prockop, 1998). Holmgren et al. (1998) found that by substituting the -OH group for -F (a more electro-negative group) in collagen-like peptides, the thermal stability of the triple helices is increased. As fluoroproline does not provide a site for hydrogen bonding of water, the authors conclude that the stability conferred by Hyp cannot be explained by additional water bridges. Instead they explain their results by the greater electron-drawing inductive effect that fluorine has compared to oxygen. This is also consistent with results from Engel et al. (1977) who found that Hyp residues enhance the thermal stability of collagen-like peptides even in a completely anhydrous environment where water-bridges could not be formed.

Miles and Bailey (2001) put forward a theoretical model in which they take into account the importance of Hyp in dictating the thermal stability of collagen molecules. They propose that a collagen molecule differs in thermal stability along its length and that the unfolding of the triple helix is not random but originates in a specific region of the molecule, 65 residues long near the C-terminus of the molecule, which is deficient in Hyp and is therefore more thermally labile.

1.3.4 Role of hydroxylysine residues

As with hydroxyproline the hydroxylation of lysine occurs after it has been incorporated into the polypeptide chain (Kivirikko and Prockop, 1967). Hydroxylysine is almost exclusively found in the Y positions of the repeating Gly-X-Y sequences in the collagen triple helix and it is also found in the short non-triple-helical telopeptide regions at the ends of the molecule (Knott and Bailey, 1998). The hydroxyl groups of hydroxylysine residues have two important functions: they stabilize the intramolecular and intermolecular collagen cross-links and they also serve as attachment sites for carbohydrate units (Kivirikko and Pihlajaniemi, 1998; Knott and Bailey, 1998). The functions of the hydroxylysine-linked carbohydrate units are not fully understood, but it has been suggested that they may regulate the packing of collagen molecules into supramolecular assemblies, as they are situated on the surface of the collagen molecules (Kivirikko and Pihlajaniemi, 1998). Studies of fibrillar collagens have indicated that collagens containing high amounts of hydroxylysine and hydroxylysine-linked carbohydrates form very thin fibrils, whereas collagens with low amounts of these modifications form thick fibrils (Notbohm et al., 1999).

1.3.5 Collagen fibril

Hodge and Petruska (1963) put forward a model for the organization of type I collagen molecules into fibrils, based on TEM micrographs of negatively stained collagen. In this widely accepted model, molecules are aligned longitudinally with a gap region of 36 nm between neighbouring triple helices in the same file; the molecules are also aligned horizontally in rows of five molecules, but staggered by approximately one quarter of their length. This produces a series of gap and overlap regions, for every five molecules in an overlap zone only four will traverse the gap zone. The periodicity of a fibril is measured by the d-spacing which comprises one gap and overlap region (Glimcher and Krane, 1968). The d-spacing of type I collagen fibrils is approximately 67 nm, although this varies slightly between tissues (Kadler, 1994) and decreases to around 64 nm if the fibril is dehydrated (Woodhead-Galloway, 1980). The repeat sequence of alternating overlap and gap zones is responsible for the characteristic banded appearance of collagen when negatively stained and viewed under an electron microscope (Woodhead-Galloway, 1980) (Figure 1.7).

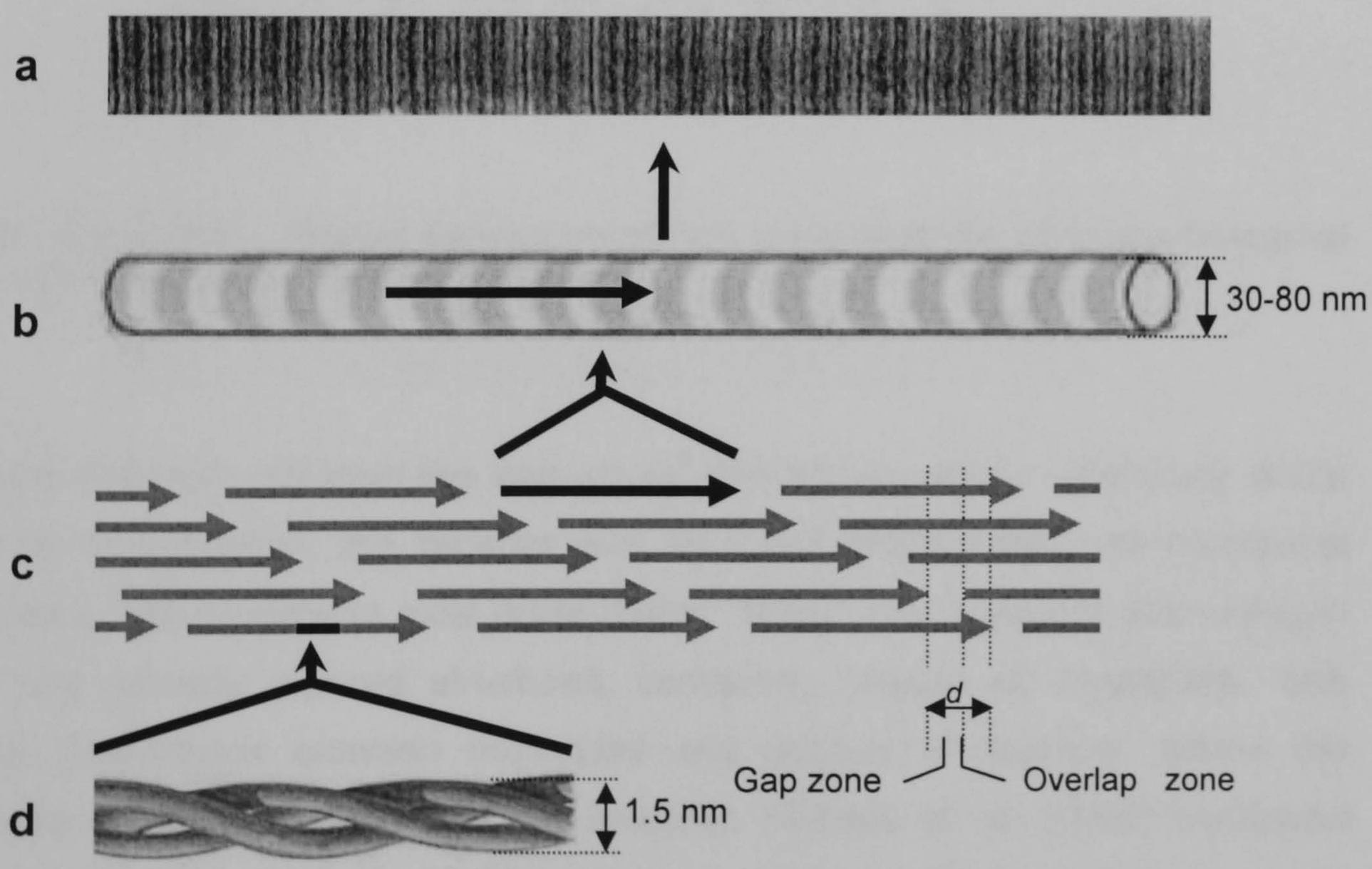


Figure 1.7 Schematic diagram showing the longitudinal arrangement of collagen molecules within fibrils. A is a TEM image showing a section of a positively stained collagen fibril. B is part of a collagen fibril showing the repeat sequence of gap (grey) and overlap (white) regions, the arrow shows the position of a collagen molecule within the fibril. C shows the quarter stagger alignment of collagen molecules within a fibril, each arrow represents a molecule. The combined length of one gap and overlap region is known as a d-spacing. D is a section of the triple helix of a collagen molecule.

Although the axial arrangement of the collagen molecules into fibrils is fairly well understood, the way in which the molecules are arranged laterally is less clear. There have been a number of models proposed to explain the lateral packing of collagen molecules into fibrils (see Wess et al. (1998)). Smith (1968) first proposed a sub-fibrillar model of a cylindrical arrangement formed from five collagen molecules, known as a Smith pentafibril or microfibril. A rather different model was then suggested by Hulmes and Miller (1979) in which in cross-section the molecules are arranged in a quasi-hexagonal array. A combination of these two models led to a new model in which microfibrils are compressed so as to fit a hexagonal array (Trus and Piez, 1980) (Figure 1.8). This arrangement was later confirmed by Orgel et al. (2001) using x-ray diffraction analysis of tendon.

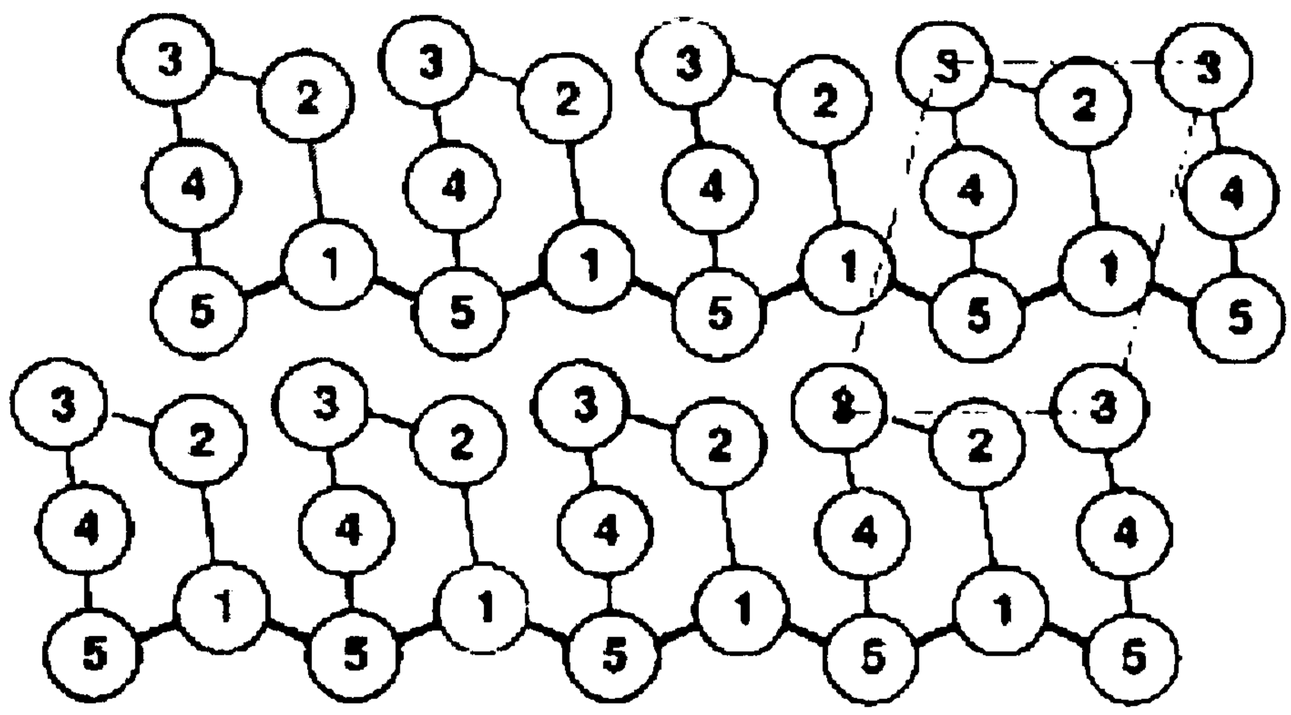


Figure 1.8 Schematic diagram showing microfibrils compressed so as to fit a hexagonal array, from Orgel (2001), Figure 4, p:1066.

Analysis of tendon collagen has also shown that the equatorial reflections, which relate to lateral order, are broader and less well defined than the meridional reflections, which relate to axial order (Miller, 1982). This suggests that collagen fibrils are partially ordered structures, containing regions of crystallinity, with regular interactions between molecules and regions of disorder, where the interactions between molecules are irregular. Hulmes et al. (1995) compared several proposed models for the lateral packing of fibrillar structures with observed x-ray diffraction data and concluded that a cylindrical structure where the compressed microfibrils are packed radially could account for both the ordered and disordered phases of collagen molecular packing within the fibril (Figure 1.9). In this model each concentric layer is separated by a distance of

approximately 4 nm which is consistent with the observed diameters of collagen fibrils in young animals (Parry and Craig, 1984) and *in vitro* studies of self-assembled collagen fibrils (Christiansen et al., 2000) both of which show discrete increments of approximately 8 nm.

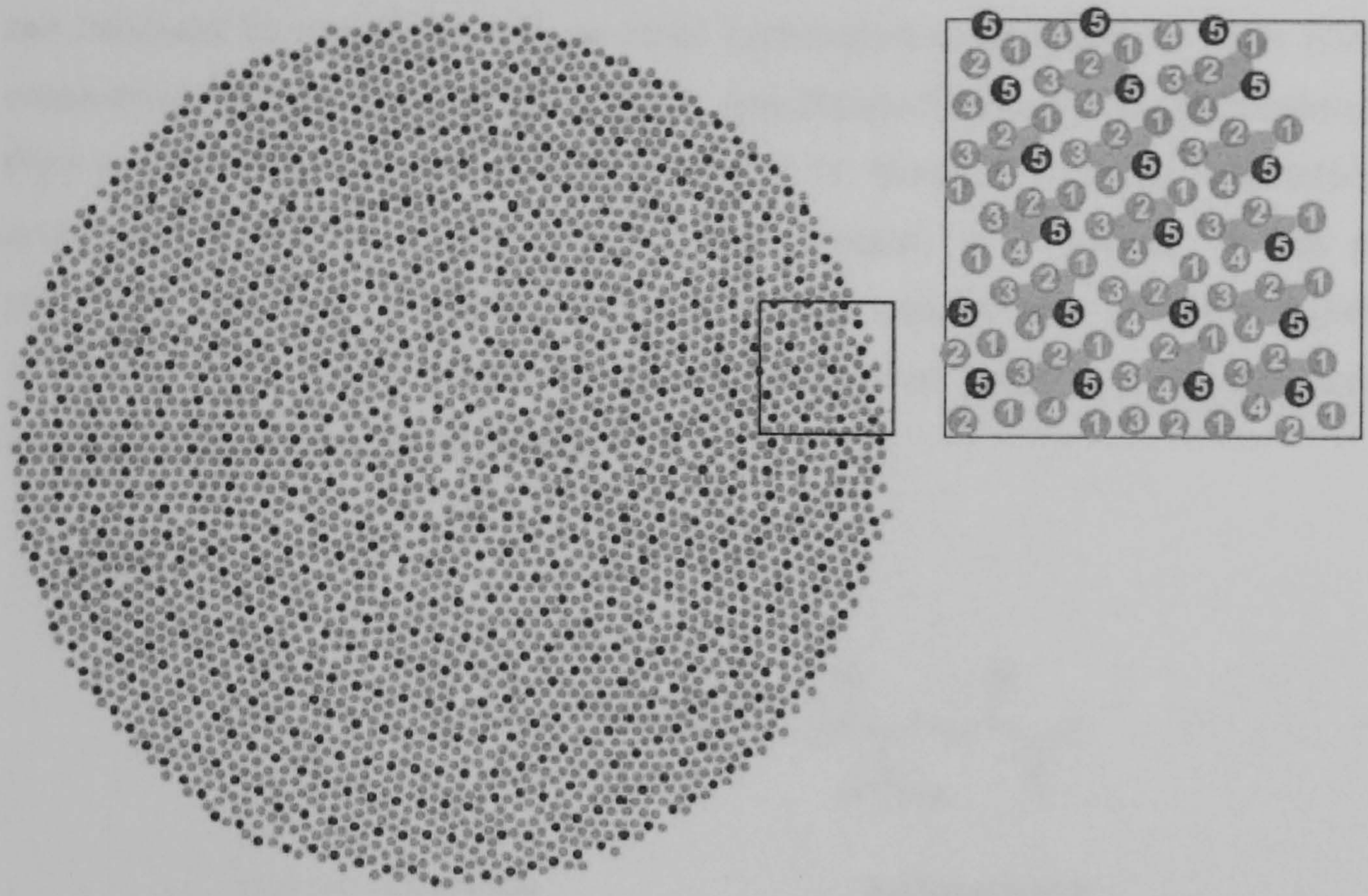


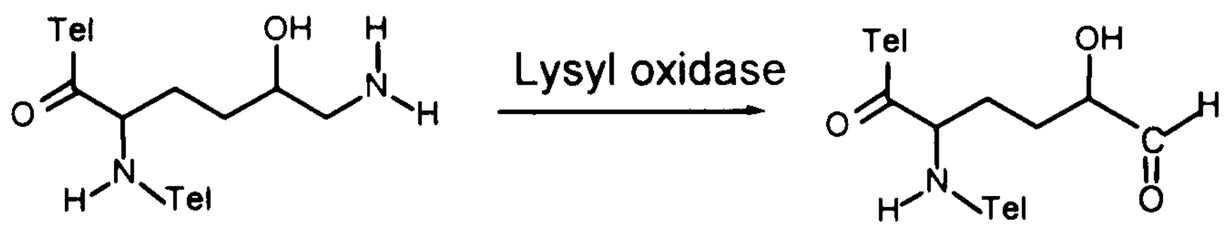
Figure 1.9 Schematic diagram of the proposed lateral organization of collagen microfibrils shown as cylindrical structure where compressed microfibrils are packed radially. From Hulmes et al. (2002), Figure 3, p:5.

1.3.6 Stabilization of collagen fibrils

The formation of covalent intramolecular and intermolecular cross-links is the final step in collagen biosynthesis and occurs once the collagen molecules have aligned into fibrils (Kadler et al., 1996). The covalent cross-links prevent the molecules sliding past each other under stress and thus provide tensile strength and stability to the collagen fibril (Bailey, 2001). Cross-linking of collagen is initiated by the enzyme lysyl oxidase which modifies certain lysine and hydroxylysine residues situated in the non-helical telopeptide regions of collagen molecules. The enzyme catalyzes the oxidative deamination of the ε-amino group on these residues to form reactive aldehydes (allysine and hydroxyallysine) (Kagan and Li, 2003) (Figure 1.10, Step 1). These aldehydes then form cross-links, by condensation with a lysine or hydroxylysine residue in the helical portion of a neighbouring molecule (Knott and Bailey, 1998). These divalent bonds are heat labile and hydrolysable in acid (Robins, 1988) but can be stabilized *in vivo*

by maturation and also *in vitro* by reduction with sodium borohydride. The major cross-link is a keto-imine called hydroxylysino-5-ketonorleucine (HLKNL) (Eyre, 1981), but in mineralized collagen a significant proportion of the cross-links are formed with non-hydroxylated Lys residues (lysino-5-ketonorleucine, LKNL) (Knott and Bailey, 1998) (Figure 1.10, Step 2). During maturation the keto-imines are believed to condense with another hydroxylysine aldehyde to form trivalent cross-links (Robins and Duncan, 1987), specifically hydroxylysyl-pyridinoline (HL-Pyr) and lysyl-pyridinoline (L-Pyr) (Figure 1.11, Step 3). Another non-reducible, enzymatic cross-link found in bone (and tendon) is a pyrrole. It has been proposed that this is formed when a HLKNL reacts with a lysine aldehyde (Kuypers et al., 1992), and is thought to occur when there is a reduced level of hydroxylation (Bailey et al., 1998).

Step 1

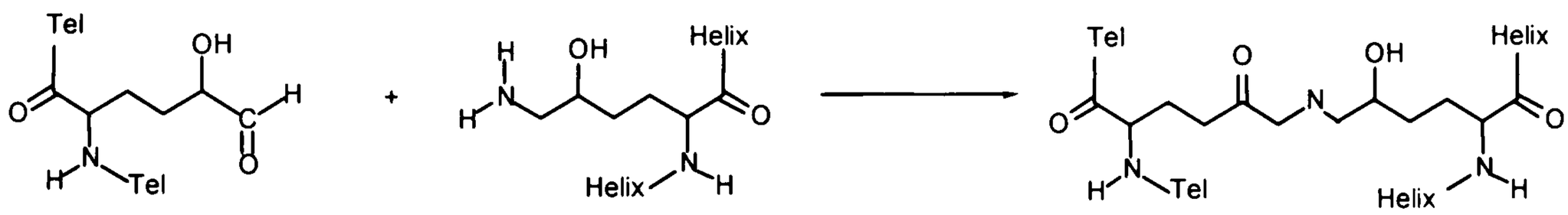


hydroxylysyl residue

hydroxyallysine
(reactive aldehyde)

Step 2

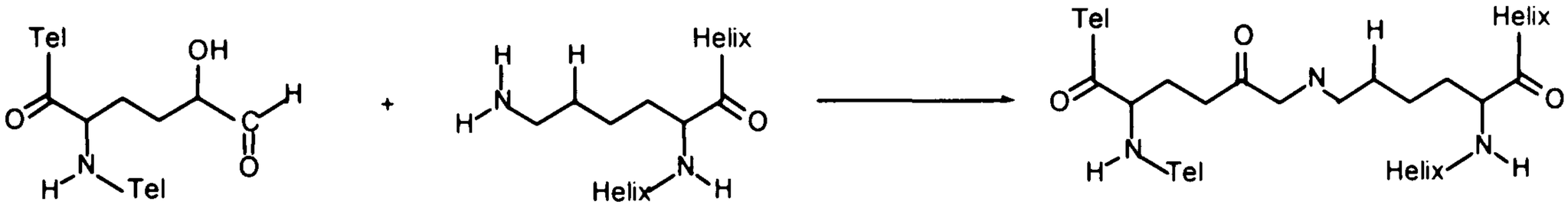
A) Immature cross-link formation with hydroxylysine



hydroxylysyl residue

HLKNL
(hydroxylysino-5-ketonorleucine)

B) Immature cross-link formation with lysine



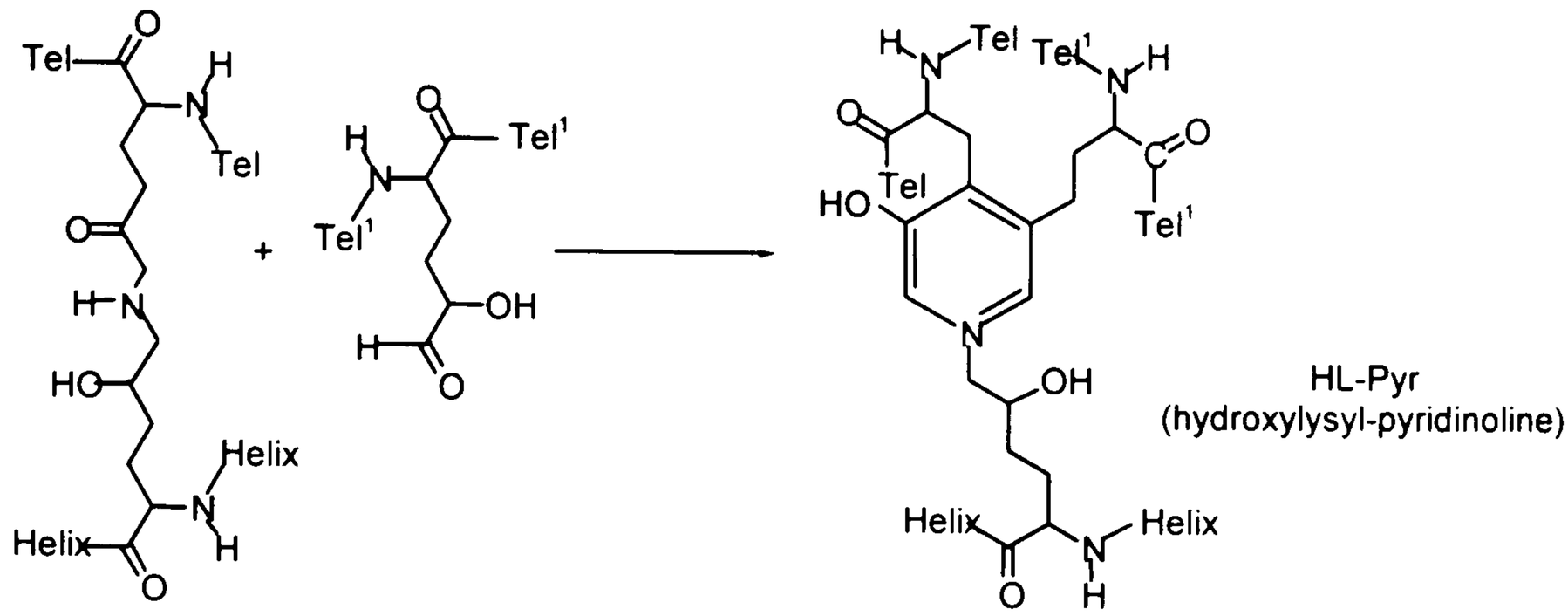
lysyl residue

LKNL
(lysino-5-ketonorleucine)

Figure 1.10 Formation of immature covalent cross-links in bone collagen. Step 1 enzyme catalysed oxidative deamination of a hydroxylysyl residue in the telopeptide region to form the reactive aldehyde, hydroxyallysine. Step 2 formation of the immature ketoimine cross-links A) HLKNL and B) LKNL through condensation of hydroxyallysine with a lysyl or hydroxylysyl residue in an adjacent α-chain. Based on Knott and Bailey (1998), Figure 1, p:182.

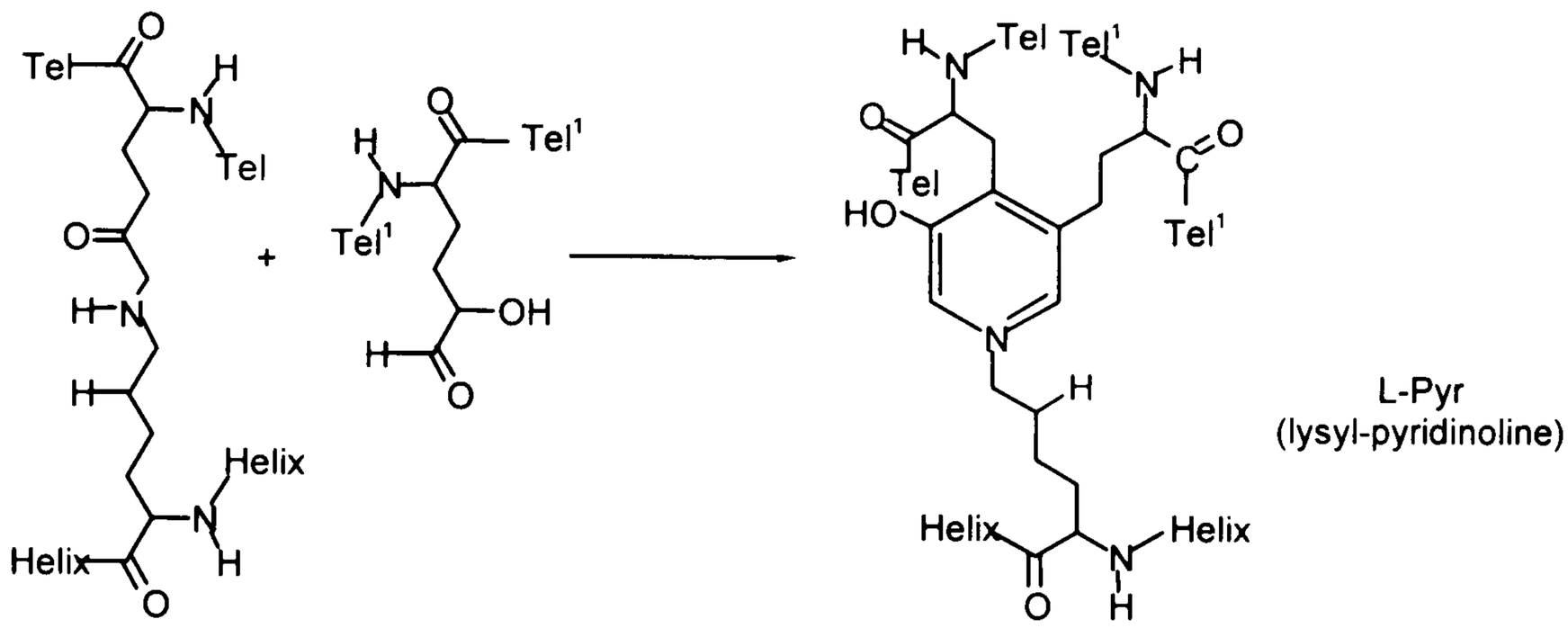
Step 3

A) Mature cross-link formation with HLKNL



HL-Pyr
(hydroxylysyl-pyridinoline)

B) Mature cross-link formation with LKNL



L-Pyr
(lysyl-pyridinoline)

Figure 1.11 Formation of mature cross-links in bone collagen. Step 3 formation of pyridinoline cross-links through further condensation of the ketoimine cross-links with a hydroxyallysine residue to form A) HL-Pyr and B) L-Pyr. Based on Kuypers et al. (1992), Scheme 2, p:135.

1.3.7 Polymer-in-a-box theory for the thermal stabilization of collagen molecules within fibrils

The denaturation temperature of collagenous tissues is known to increase if the tissue is dehydrated (Finch and Ledward, 1972). In accordance with this observation, Miles and Ghelashvili (1999) showed a negative relationship between the denaturation temperature and the volume fraction of water within collagen fibrils. The authors explained this phenomenon using a polymer-in-a-box model whereby they envisaged that the collagen molecule is confined within a box, the walls of which are formed by adjacent triple helices within the fibril. They suggest that as the fibril is dehydrated the intermolecular spacing is reduced which restricts the available space around the collagen molecule. This results in a loss of configurational entropy which causes a corresponding

increase in the Gibbs free energy of activation and consequently an increase in thermal stability. This theory also explains why, as previously mentioned, the denaturation temperature of individual collagen molecules is close to body temperature, yet in mammalian skin, tendon and demineralized bone where the molecules are closely packed within fibrils the denaturation temperature is increased by over 25°C (Miles and Bailey, 1999). Recently, Miles et al. (2005) have also suggested that the increase in denaturation temperature which is observed when collagen is synthetically cross-linked can also be explained by the polymer-in-a-box theory. They suggest that the increased stability is due to a dehydration process because the cross-links hold the collagen molecules closer together thereby exclude water.

1.3.8 Mineralized collagen fibrils

The initial nucleation of the mineral crystals, their position with regard to collagen fibrils and the dimensions of mineral crystals are still controversial issues; although they have been investigated in both mineralized tendon and bone using techniques such as electron microscopy and x-ray scattering. It is generally agreed that nucleation of the mineral occurs within the gap region that is formed by the quarter staggered arrangement of collagen molecules within fibrils (Fratzl et al., 1991; Glimcher and Krane, 1968; Woodhead-Galloway, 1980). The mineral deposits show an axial periodicity (approximately 67 nm repeat distance) along the collagen fibrils consistent with the location of the crystals corresponding to collagen gap and overlap zones (Landis et al., 1996). With continued deposition the mineral crystals grow or coalesce into larger crystals which expand into the overlap region within the fibril structure (Fratzl et al., 1991; Landis et al., 1996) and also cover the surface of the fibril. When viewed by TEM it can be seen that the mineralized fibril is encapsulated within the mineral which obscures the fibrillar banding pattern (Kadler, 1994) (see Figure 1.12).

There is some ambiguity as to the shape of the mineral crystals. They have been variously described as book-like (Millard, 2001), rod-like (Glimcher and Krane, 1968), plate-like and needle-like in appearance (Currey, 2002; Fratzl et al., 1992; Rho et al., 1998). It is unclear whether the mineral crystals found in mineralized turkey tendon are of a different shape to those found in bone. Fratzl (1992) determined by x-ray diffraction that the crystals in adult turkey leg tendon were plate-shaped with a thickness of approximately 2 nm, whereas those in bone were needle shaped with a thickness of 3-4 nm. However needle-shaped

crystallites have been observed in tendon that is just starting to mineralize (Traub et al., 1992), and studies using electron microscopy have identified plate-like crystals in bone samples from a range of different species (Kim et al., 1995; Landis et al., 1996; Weiner and Price, 1986). Weiner and Traub (1986) have also shown that plate-like crystals have a needle-like appearance when viewed on their edge.

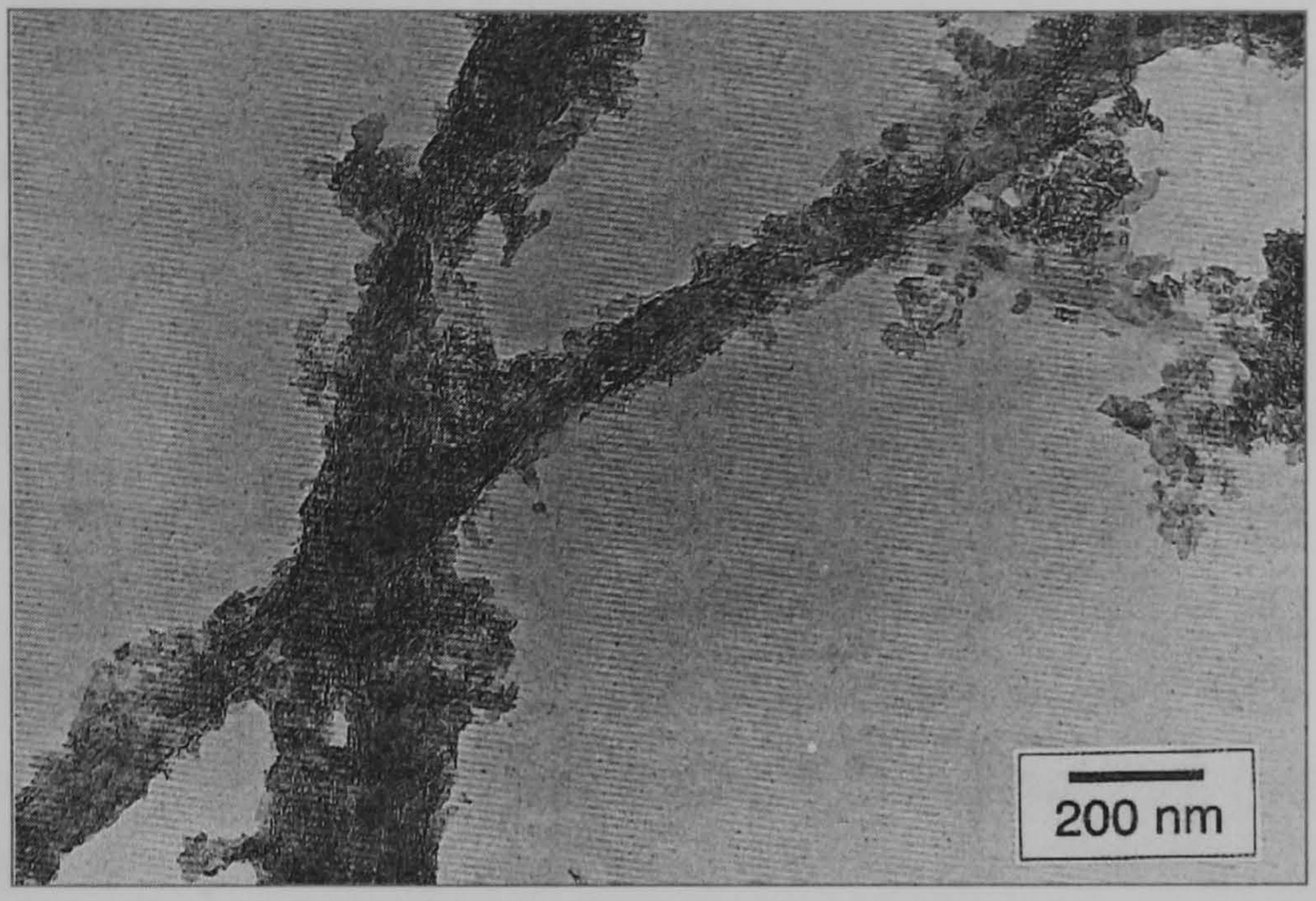


Figure 1.12 TEM image of mineralized bone collagen fibrils, from Kadler (1994), Figure 13, p:555.

The question of how much of the mineral resides within the fibril and how much is between the fibrils is also contentious. It is thought that the majority of the inorganic crystals reside within the collagen fibrils, within the gap regions (Glimcher and Krane, 1968; Katz, 1996; Weiner and Traub, 1986). The crystals are arranged in parallel layers that traverse the diameter of the fibrils. Whilst the thickness and length of the crystals are the correct dimensions to fit within the gap regions, they are too wide to exist within a single gap region (Weiner and Traub, 1986). It has been suggested that the thin plates grow along grooves within the collagen fibril which are formed by adjacent gap regions (Landis et al., 1996; Ottani et al., 2001; Weiner and Traub, 1986). Whilst in mineralized turkey tendon it is thought that all of the mineral is associated with the collagen fibrils (Weiner and Traub, 1986), Bonar et al. (1985) determined by chemical analysis that there is more mineral in bone than can be accommodated within the collagen fibrils, suggesting that mineral must also be present between the collagen fibrils. Cressy and Cressey (2003) using TEM have observed thin sheets of mineral within the inter-fibrillar space of sheep and human bone which are not associated

with the fibrils. However the same technique has been applied to fish bone and in this case mineral was found only within the collagen fibrils not within the inter-fibrillar space (Lee and Glimcher, 1991).

1.3.9 Thermal stabilization of mineralized collagen fibrils

The thermal stability of mineralized collagen has received considerably less attention than factors affecting the thermal stability of collagen molecules or non-mineralized fibrils, although it would appear that the presence of mineral has a dramatic effect on the thermal stability of bone collagen. DSC studies have suggested that the characteristic 60-65°C endothermic transition which occurs in untreated non-mineralized collagen is shifted up to 150°C in mineralized tissue (Kronick and Cooke, 1996). It has been speculated that the physical constraints imposed by the mineral suppress denaturation (Covington, 2001; Nielsen-Marsh et al., 2000a). This idea is based on the same polymer-in-a-box theory that was proposed by Miles and Ghelashvili (1999) to explain the stabilizing effect of dehydrating collagen. In this case mineral crystals surround and impregnate collagen fibrils thereby reducing the free space available for the collagen to collapse into. A thermodynamic consequence of this enforced organization is that more energy is required for denaturation to occur and thus there is an increased thermal stability.

There is evidence to suggest that in mineralized tissues the water molecules occupying free space around the collagen molecules are replaced with crystallites. As mentioned above in mineralized tissue the gap regions are occupied with mineral crystals. Fratzl et al. (1993) showed how the mineral crystals could also be deposited inside the overlap regions of collagen fibrils if they replace the water molecules (Figure 1.13). In this model the formation of large crystals pushed the collagen molecules closer together which also explains why the equatorial spacing, which corresponds to the separation of the individual molecules, is reduced as the amount of mineral increases (Bigi et al., 1988; Lees, 1987).

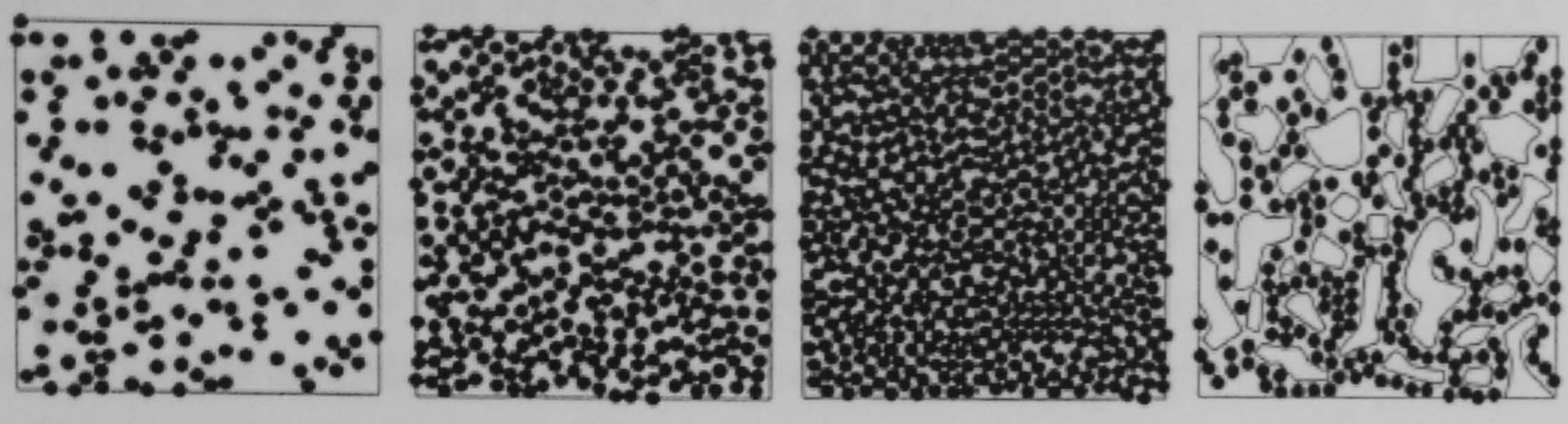


Figure 1.13 Computer models based on x-ray spectra showing the possible lateral packing of collagen molecules in (i) fully hydrated (ii) partially dehydrated (iii) fully dehydrated and (iv) mineralized turkey leg tendon, adapted from Fratzl et al. (1993), Figure 3, p: 263. The black circles represent collagen molecules and the grey bordered white structures in diagram iv represent mineral crystals. Note the amount of free space around molecules in the fully hydrated state compared to the dehydrated or mineralized states.

1.3.10 Bone structures

Mineralized fibrils form larger fibers and fiber bundles, which combine to produce three structures; Woven bone, Lamellar bone and Parallel-fibred bone. Woven bone is rapidly laid down and the mineralized collagen fibrils have no preferred orientation. This type is commonly found in newly formed bone; it is prevalent in the bones of infants and also found in adult bone at fracture sites (Currey, 2002). Lamellar bone is less densely mineralized than woven bone and the fibrils are arranged into vast sheets, approximately 3-7 μm in thickness, known as lamellae (Rho et al., 1998). In each lamellae sheet the collagen fibrils are aligned along their long axis and the orientation is different in each concentric layer of lamellae giving them a plywood-like organization (Giraud-Guille et al., 2003; Weiner et al., 1999). Parallel-fibred bone (a less common type), has a structure that is part-way between the other two (Katz, 1996; Millard, 2001).

A normal mature skeleton is made up of two types of bone which are microscopically and macroscopically distinct yet have the same chemical composition as both are formed from lamellar bone (White and Folkens, 2000). Cancellous or spongy bone is a highly porous material found predominantly in the flat (skull and ribs) and irregular (wrist, ankle and spine) bones and at the ends of long bones (leg and arm). It consists of fine interconnecting struts of bone, known as trabeculae (Millard, 2001; White and Folkens, 2000). Compact bone is the principal component of long bone shafts. For this research only compact bone

was used and all of the samples (with the exception of the rib cortical bone samples from the Palace Leas burial experiment and Hamman-Todd case study, discussed in Chapters 6 and 7 respectively) were from the mid-shaft of long bones. This type of bone is characteristically dense and unlike cancellous bone it contains microscopic blood vessels. The lamellae sheets wrap in concentric layers around a central canal to form a structure called an *osteon* or *Haversian* system which runs approximately parallel to the longitudinal axis of the bone (White and Folkens, 2000). In life the Haversian and also Volkmann's canals (which are at right angles to the Haversian canals) contain blood vessels (Seeley et al., 1992). In the burial environment the vascular network has a deleterious effect as it makes the bone porous and as a result susceptible to diagenetic alteration (see Chapter 6). Not all of the lamellae form Haversian systems; interstitial lamellae are found in the spaces between osteons and the periosteal (outer) and endosteal (inner) surface of bone is covered by a thin layer of circumferential lamellae. Microscopic analysis of mid-shaft long bone from young cow, sheep and pig has shown that it is composed solely of vascular sheets sandwiched between circumferential lamellae, with none of the lamellae organized into Haversian systems (Locke, 2004).

1.4 Bone turnover and implications for bone collagen thermal stability

The cells of the skeleton act to maintain the physical structure of bone, a process referred to as remodelling. Bone remodelling is a continuous process occurring throughout life. The turnover of collagen in mature collagenous tissues occurs within the time period of months to years compared with days for the non-structural proteins (Bailey, 2001) and bone collagen turns over up to three times more slowly than collagen found in soft tissues (excluding dermis) (Gineyts et al., 2000). A given sample of bone is therefore likely to contain a mixture of newly formed and mature bone and will possess mineralized collagen fibrils which will have different thermal stabilities.

Bone is remodelled by cells known as osteoclasts, which destroy bone and osteoblasts which lay down new bone to form a Haversian system (Vaughan, 1975). The osteoclasts form a *cutting cone* shape and advance through the bone leaving behind a cylindrical cavity approximately 200 nm in diameter (Currey, 2002). The internal cavity is then filled in with concentric lamellae, except for the very centre of the cavity which contains blood vessels and nerves (Figure, 1.14).

Li et al. (2003) argued that as bone is constantly remodelling within any single element there will be collagen in different stages of calcification, which would affect the *in situ* thermal stability of the collagen. They found an increase in the quantity of denatured collagen in bone with increased temperature and suggested that as non-mineralized collagen denatures at 60°C whereas mineralized collagen denatures at approximately 150°C, collagen denatured and lost at the lower end of this temperature range was non- or partially-calcified whereas loss at higher temperatures came from fully mineralized collagen.

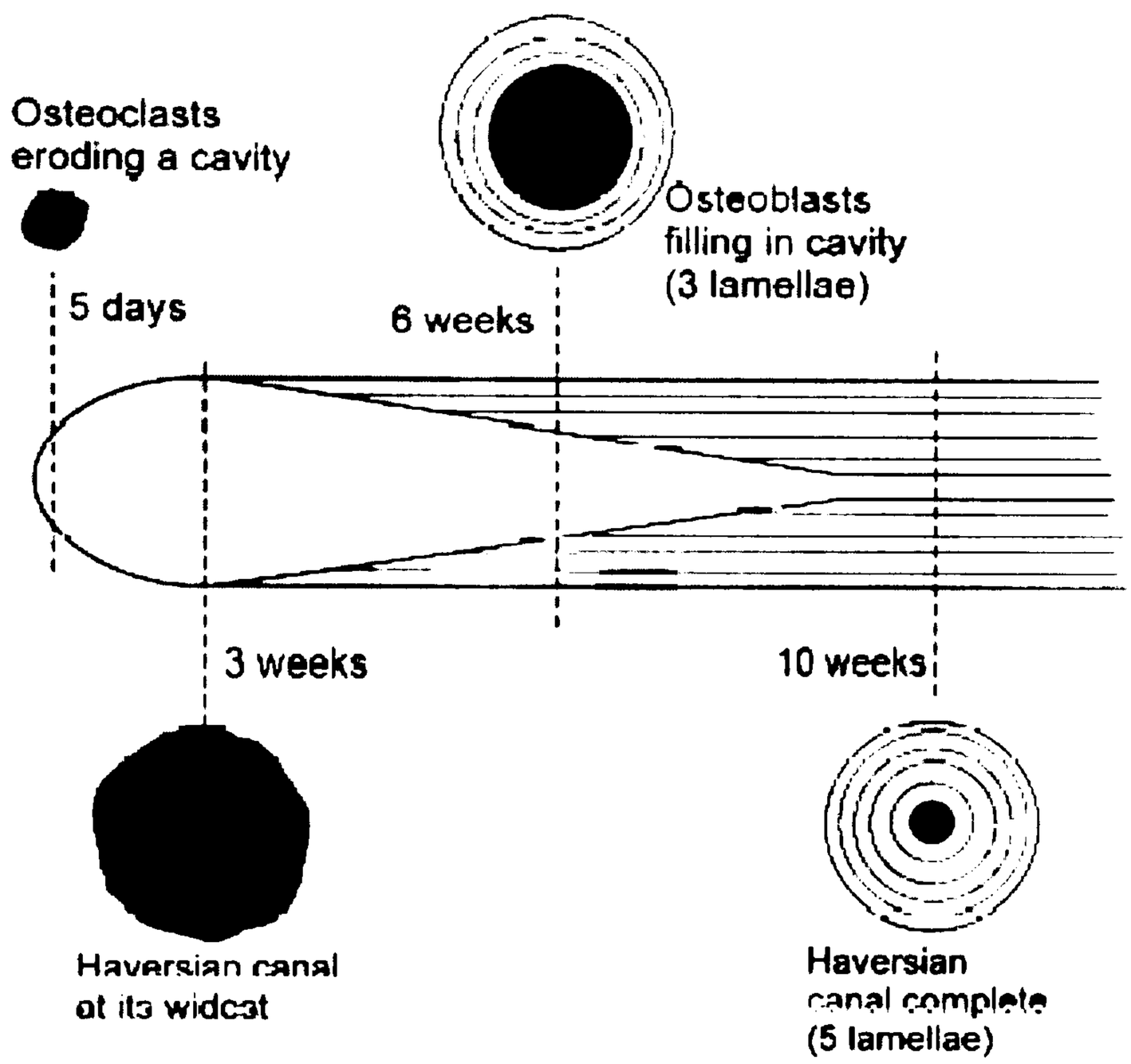


Figure 1.14 Schematic diagram showing bone being remodeled to form a new Haversian system, seen for longitudinal and cross-sectional views. The times give roughly the time course of the process in humans. In the cross sections the central cavity is shown black. At 5 days the osteoclasts are still widening the cavity in the bone. At 3 weeks the cavity is at its widest. By 6 weeks the cavity is half filled in by osteoblasts, and by 10 weeks the process is completed, although it will take longer for the bone to become completely mineralized. From Currey (2002), Figure 1.2, p: 15.

As discussed previously, the presence of mineral is a major contributor to the thermal stability of bone collagen but covalent cross-links are another key factor affecting collagen stability. A cross-section of compact bone will contain both immature divalent cross-links from newly formed bone as well as mature multivalent cross-links from older collagen. As the former are heat-labile whilst the latter can survive exposure to 200°C (Wang et al., 2002b), one might expect

that the older highly cross-linked collagen fibrils would be more thermally stable. However studies of both rat and human bone collagen by Danielsen have suggested that the situation is more complicated and newly formed bone collagen actually has a higher denaturation temperature. This is based on comparison studies of samples of different ages at death (Danielsen, 1990; Danielsen et al., 1994) and also comparing cancellous bone versus compact bone (Danielsen, 1998) (as the former is believed to remodel more rapidly and is therefore rich in immature cross-links (Eyre et al., 1988). The contributions of mineral and cross-links, and the effect of age on the structural integrity of collagen fibrils when exposed to heat are discussed in Chapter 5.

1.5 Summary

This chapter has given an overview of the current understanding of collagen and bone structure. As has been noted, the thermal stability of the collagen molecule has been explored in detail, whilst the collagen fibril has received rather less attention. This thesis is focused upon the collagen fibril, and addresses how this, often overlooked, level of structural hierarchy impacts upon the study of archaeological cooked bone. Before this examination of the thermal degradation of the collagen fibril is discussed, the following chapter explains the significance of cooked archaeological bone and how damage of the collagen fibril could be used to detect this thermal alteration.

Chapter 2 Introduction to cooked bone in the archaeological record

Definitions

Taphonomy – all processes which act upon a bone after death, these can include human modification such as butchery and cooking and also natural processes which include weathering and diagenesis

Sub-aerial weathering – above ground alteration due to natural processes such as UV radiation, breakage, trampling and carnivore activity

Diagenesis – below ground alteration such as leaching, hydrolysis by acidic groundwater and attack by micro-organisms and fungi

Cooking - mild heat treatment, including boiling or roasting in which the bone has not reached a temperature that would induce charring

2.1 Why look at cooked bone?

Archaeologists study how people lived in the past. One key aspect of their lives was food procurement and preparation. Between the death of an animal and its transformation into food, the bones will go through a number of stages of taphonomic alteration. The initial processes of hunting, butchery and disarticulation have been studied in great detail, both ethnographically and from the study of faunal remains. The processes that will affect bones after they have left the dinner plate have also featured in the archaeological literature i.e. in taphonomic and diagenetic studies of domestic refuse. Humans are the only species which actually cook their food and cooking and meat eating have been linked to key changes in the dentition, and enlargement of the brain (the expensive tissue hypothesis; Aiello and Wheeler, 1995), therefore evidence of cooking and the processing of meat is fundamental.

The problem with cooking is that, unlike burning, temperatures remain sufficiently low to transform the food whilst retaining some moisture. A technique which could identify such low temperature thermal alteration has the potential to answer key archaeological questions such as when early fire started to be used for cooking. From the nutritional standpoint, cooking would have meant an improvement in the edibility of meat both in terms of the quality and extended

use-life of meat. In addition, boiling the residual meat and extracting lipids from bones would have provided a new and important source of nutrients (Lupo and Schmitt, 1997).

Another question that has stimulated (often macabre) interest is the cooking of human bone. Finding evidence for cannibalism remains an area of active debate, especially with regard to sites in the American Southwest (Novak and Kollman, 2000; Turner and Turner, 1999) and Fiji (DeGusta, 2000). Cut-marks on bone are an indicator of human influence and have been used as a way of detecting cannibalism. However it has been argued that this evidence could represent war related mutilation or secondary burial (Lambert et al., 2000); the ability to recognize cooked human bone could help to distinguish these ritual behaviours from cannibalism. It has also been suggested that if cooked human bone could be identified the reported incidence of cannibalism would arguably increase (Hurlbut, 2000).

2.1.1 Social status

Ideas regarding improved nutrition and cannibalism are merely two high profile examples; the identification of cooked bone is of wide-ranging archaeological interest. Evidence of cooking can be used to elucidate dietary habits and cooking techniques. Food and eating habits are also strong cultural markers filled with meaning. Anthropological studies have shown that eating habits may be used as a means of non-verbal communication, with which to reinforce relationships between individuals and groups within a society (Douglas, 1972; Lévi-Strauss, 1978). Gender roles, for instance, can be manifested within food processing and consumption (Hastorf, 1991). The mode by which a meal was cooked, and the type of food stuffs that are eaten, can reflect social and economic status within a society (Goody, 1982; Grant, 2002). The division of a carcass within a group can be used as a means to reinforce social order at meal times, with higher quality cuts restricted to those of higher social standing (McCormick, 2002; Stokes, 2000).

The ritualistic meanings behind cooking are not just restricted to the living; heated bone, be it animal or human, also has a role to play in understanding funerary practices. Animal remains that are associated with human activity are usually recovered from the floor levels of habitation areas, refuse pits or middens. These remains are generally considered to represent everyday domestic waste.

However animal bones were sometimes deliberately buried and these remains are often thought to have special ritual meaning (Lauwerier, 2002). For instance throughout history animal bones are found associated with human inhumations (Dürrwächter et al., 2006). Deliberate interment of raw or cooked animal remains in a burial are interpreted as 'grave gifts', perhaps given for religious reasons, to feed the soul and to enable the deceased to make the journey to the other world (Lauwerier, 2002). The grave gifts may also be used to express status, either that of the deceased or of the deceased's relatives (Grant, 2002). Evidence for the preparation of these 'gifts' would aid interpretation of their significance.

Beliefs can also be expressed in the ways that relatives offer their deceased to the gods. Whether the deceased is offered raw, cooked or burnt may reflect what the relatives believe to be their god's wishes (Oestigaard, 2000). Whilst burnt bone is often easy to recognize, bones which have been heated to lower temperatures are often categorized and catalogued as unburnt and thus interpreted as inhumations (Oestigaard, 2000).

2.1.2 Biomolecular studies

Cooking is designed to transform foodstuffs to make them more edible. In this process the biological macromolecules are degraded by a variety of chemical processes (such as hydrolysis and oxidation). Consequently cooking diminishes biomolecular survival. Molecules such as DNA, osteocalcin and collagen are able to survive within bone for thousands of years and have been used to provide information as diverse as migration patterns, diet, genetics, sex, age and aging. Bone collagen, for example, has been utilized for radiocarbon dating (van Klinken and Hedges, 1998), stable isotope paleodietary analysis (Ambrose, 1993; Bocherens et al., 1995; Schoeninger et al., 1983) and amino acid racemization dating (AAR) (Csapó et al., 1994). Many authors have investigated the diagenesis of remains, notably bone, in order to improve the recovery of vital (and chronological) signals from ancient samples. Indeed there are a series of international conferences specifically focused upon bone diagenesis.

Remarkably, the most common and arguably one of the most detrimental processes that an archaeological bone will have suffered, cooking, remains largely unexplored. Yet it is the thermal history of a bone which is required to interpret techniques such as amino acid racemization (Csapó et al., 1994;

Marshall, 1990) and can help predict the survival of biomolecules in the archaeological record (Smith et al., 2003).

2.2 Why has cooked bone not been studied more widely?

Given the social and taphonomic importance of cooking, one would expect to find many attempts to discriminate cooked from uncooked bone, but this is not the case. It has been suggested that the apparent lack of interest in cooking practises may represent a gender bias; with emphasis on the traditionally 'male' roles of butchery and hunting but considerably less on food preparation which is seen as a 'female' role (Montón Subías, 2002). A more prosaic explanation is that cooking leaves very little evidence in the archaeological record.

One approach has been to identify taphonomic evidence for animal processing another has been to detect thermal alteration (the key component of cooking); problems with these alternative approaches are discussed below.

2.3 Taphonomic evidence of cooking from faunal remains

Some studies have attempted to identify evidence for cooking practices by looking for features of food-processing on animal bones. These include evidence of burning (Albarella and Serjeantson, 2002; Binford, 1981; Gifford-Gonzalez, 1989), the position of butchery marks (Gifford-Gonzalez, 1989), fracture patterns, and degree of fragmentation (Gifford-Gonzalez, 1989; Oliver, 1993; Outram, 2001).

2.3.1 Burning

Evidence of burning will show that a bone has been heated but can also provide information about the condition of the bone at the time it was cooked. Charring which extends over the entire surface of a bone indicates that all the flesh has been removed, but if it affects only the articular ends this indicates that the bone was cooked with some or all of the flesh attached, for instance when a joint is roasted (Albarella and Serjeantson, 2002; Gifford-Gonzalez, 1989).

2.3.2 Butchery marks

Butchery marks can show how the carcass was dismembered and this may provide an indication of how the meat would have been cooked. The distribution of cut-marks and fractures on the upper limb bones can (for instance) provide an indication of whether whole joints of meat were roasted on the bone or whether

pieces of meat were first removed from the carcass and then cooked (O'Connor, 1989). However cut-marks can sometimes be difficult to recognize due to sub-aerial weathering and diagenetic processes.

2.3.3 Fracture patterns

Fractures can be produced during butchery and dismemberment and again these can also provide an indication of cooking practice. For instance long bones processed for boiling in cooking pots often show irregular transverse fractures resulting from their breakage prior to boiling, because they have to fit into cooking pots (Gifford-Gonzalez, 1989; Turner and Turner, 1999).

There is archaeological evidence to suggest that bones were exploited for their marrow and grease content which may have involved an element of heating. Marrow has a high calorific value and can therefore be an important source of nutrition (Outram, 2002). It is extracted from the long bones by striking or chopping at the shaft to expose the marrow within (Albarella and Serjeantson, 2002; Marshall, 1989). However there is some debate as to whether butchery and marrow extraction occurs prior to, or after, the bone has been heated (Gifford-Gonzalez, 1989; Outram, 2002). It has been suggested that the types of fracture produced during this process will differ depending on whether the bone has been heated beforehand to 'loosen' the marrow (Gifford-Gonzalez, 1989; Oliver, 1993). However, Alhaique (1997) found that the fracture pattern produced during marrow extraction was not solely affected by whether the bone was cooked or not; species, age, skeletal element and the tool used all had an effect.

Grease can be extracted from fragmented bones by boiling them and then skimming the fat from the surface of the water once it has been cooled. There is ethnographic evidence of bones being boiled for grease rendering (Binford, 1978; Yellen, 1977, 1991) and the presence of sets of numerous crushed fragments of bone found within archaeological contexts have been used to indicate grease rendering (Church and Lyman, 2003; Outram, 1999, 2002, 2004). However, the presence of very small fragments of bone is not in itself a good indicator. Church and Lyman (2003) extracted grease from bones of different sizes and found that the efficiency of extraction varies little if the bone fragments are 5 cm or less largest dimension. They also cite ethnographic and archaeological studies where the bone fragment size was reported to be as large as 7-8 cm. An additional problem with using both fracture patterns and comminution to infer cooking from

bones is that natural processes of trampling and weathering can also cause fragmentation (Behrensmeyer, 1978).

One way to discriminate deliberate from natural fragmentation is to discriminate between fresh (processing) and dry (natural) breaks (Outram, 2002). In Outram's study of a Mesolithic site in the Italian Dolomites, almost all the cancellous bone on the site had been reduced to small fragments with most of the larger fragments being from the shaft. The author argued that most fragmentation was due to fresh fracture rather than post-depositional attrition and was therefore due to marrow extraction, followed by bone-grease rendering.

Whilst the results of the case study are compelling there is no direct evidence that the bones were in fact boiled or heated in any way. The evidence is circumstantial as is the case for studies which infer cooking based on the position of butchery marks, the type of bone (i.e. high meat bearing bones such as the humerus, tibia or ribs versus low meat utility bones such as the skull and feet bones) and the spatial distribution of bones at a site (i.e. a butchery site versus a domestic waste deposition). All such studies can be used to indicate human influence but only in the case of burnt bone can heat be directly implicated. If bone that was cooked, but not burnt, could be detected, then assumptions about cooking practices and diet could be tested.

2.4 Effects of heat on the appearance and composition of bone and methods for detecting cooked bone

Several different approaches have been proposed to demonstrate that a bone has been heated, and to what temperature. As a bone is heated it goes through a number of stages of alteration which become more extreme with increasing temperature until ultimately it is transformed into calcined dust.

Bone is a composite material consisting of flexible organic part, a brittle mineral part and water. During the heating process the different components of bone become affected and it is these changes which allow heated bone to be detected. The resulting modifications to both a bone's gross appearance and to its structural composition have been studied.

Whilst a number of variables come into play during the heating process, such as the presence and amount of flesh surrounding the bone, the temperature and duration of heating, and the specific method of heating applied; the key changes

to bone result from loss of water, loss of the organic content (through gelatinization or combustion) and alteration of the mineral component.

2.4.1 Gelatinization

As bone is heated in water the collagen is transformed by a process known as gelatinization. Once gelatinized the collagen will be leached out of the bone and the longer the bone is boiled the more gelatin is created. A decrease in the amount of collagen within the bone can be used as a means of detecting cooking (see below). However whilst gelatinization can occur rapidly in non-mineralized tissues, in bone, where the collagen is protected within a mineral 'cage', this process is much slower. Roberts et al. (2002) showed that bones had to be boiled for 81 hours before there was a significant loss of the organic content. Bada et al. (1989) found changes in amino acid profile of boiled bone after more gentle heating regimes and was able to detect boiled bone based upon the extent of aspartic acid racemization (the chemical conversion of this chiral amino acid from its L - to D - enantiomer).

2.4.2 Loss of water

Prolonged heating can result in a loss of water within the bone, causing a reduction in weight, and a distortion of the macroscopic structure through warping, shrinkage and cracking (Buikstra and Swegle, 1989; Shipman et al., 1984). The pattern of cracking on the bone surface has been found to correlate to some degree with temperature and thus it has been used as a means to estimate cooking temperature (Nicholson, 1993; Shipman et al., 1984).

2.4.3 Alteration and loss of the organic component

If a bone is roasted or baked, following the boiling off of water from within the bone, temperatures are able to climb and the next major transition is the combustion of the organic matter. This results in carbonized material being deposited on the bone surface (charring), indicating breakdown and removal of the collagen fibres, non-collagenous proteins and fats in the bone tissues.

Modification and loss to the organic fraction has been investigated by analysing the elemental composition using carbon, hydrogen, and nitrogen (CHN) concentrations (Nicholson, 1998; Stiner et al., 1995) and carbon / nitrogen (C/N) ratios (Brain and Sillen, 1988). Taylor et al. (1995) found that in heated bone the amino acid profile loses its 'collagen-like' pattern. This was detected by a relative

increase in NH_3 levels and a decrease in the ratio of glycine / glutamic acid (presumably due to deamidation).

At a macroscopic level, thermally-induced colour change, associated with the combustion of the organic matter, has been used as a method to identify cooked bone and different colours have been found to correlate to some degree with increasing temperature, (Bennett, 1999; Nicholson, 1993; Shipman et al., 1984).

2.4.4 Alteration to the mineral component

At higher temperatures modification and decomposition of the mineral also occurs. This is caused by destruction of the bonds holding the apatite crystal structure together. It results in the formation of larger, more uniform, crystals and causes a reduction in space between these components (McKinley, 2000; Shipman et al., 1984). Impurities in the crystal structure are also removed (Wright and Schwarcz, 1996), and pyrophosphate is introduced, ultimately leading to the formation of β-tricalciumphosphate, a homogeneous mineral (McKinley, 2000).

The increase in mineral size and uniformity has been investigated using x-ray diffraction (Shipman et al., 1984) and infrared spectroscopy (Stiner et al., 1995)

2.4.5 Microscopic changes

At a microscopic level incremental temperature-related changes in the surface morphology of bone have also been observed. Using scanning electron microscopy (SEM) specific ultrastructural features associated with different temperatures have been identified that are not visible at lower magnifications (Holden et al., 1995b; Quatrehomme et al., 1998; Shipman et al., 1984).

2.5 Complicating factors

2.5.1 Cooking temperature

The temperatures at which the different components of fresh bone can be affected by heat highlights a major complication to identifying cooked bone. Before any significant alteration to the mineral can occur the bone must reach very high temperatures. An increase in crystallinity of the mineral component is not thought to be detectable until the oven has reached temperatures in excess of 500°C (Shipman et al., 1984). As mentioned above, scanning electron microscopy has been used to identify thermal alteration within the bone microstructure; this alteration has been detected at 285°C (Shipman et al., 1984).

However in recent studies on forensic material minimal alteration to the microstructure of bone was observed when specimens were heat-treated at temperatures below 600°C (Holden et al., 1995b; Quatrehomme et al., 1998).

This would suggest that the main areas of observable alteration at low temperatures would be degradation and combustion of the organic component (Turner and Turner, 1999). Combustion is thought to occur between 360-525°C (Holden et al., 1995b; Shipman et al., 1984) and heat-induced colour change has been observed between 200-650°C (Nicholson, 1993).

Roberts et al. (2002) found that loss of the collagen content, increasing crystallinity and increasing porosity have be observed to occur at lower temperatures during boiling, however none of these changes appear to be useful as means of uniquely identifying cooking except in the most extreme cases (e.g. soup kitchens, gelatin extraction). This was because the bone had to be heated (at 95°C) for more than nine hours before even the slightest alteration was detectable.

A consensus appears to be emerging that there is a significant problem with detecting cooking (as opposed to burning). As has already been noted by others (Shipman et al., 1984; Taylor et al., 1995), roasting does not heat the bone (which is surrounded by insulating flesh) efficiently. Indeed the roasting process is controlled to retain moisture and enhance palatability of the flesh and in all but accidental cases the presence of this moisture will prevent temperatures ever climbing above the boiling point of water. We (Koon et al., 2003) have roasted fleshed bone in oven temperatures of 180°C and 220°C and found the bone temperature rarely reached 100°C by the time that the meat was cooked, thus confirming these predictions.

Consequently, although burning can be detected in the archaeological record, cooking introduces only relatively subtle thermal changes which cannot be detected using the conventional techniques which have been used to identify thermal alteration in bone.

2.5.2 Sub-aerial weathering and diagenetic processes

Cooking alters both the mineral and organic phases (see above). However long-term burial will also lead to changes in both of these phases and it is therefore necessary to be aware of impact of the burial environment on bone. The main

pathways to bone diagenesis are discussed in Chapter 6. Below there is a brief discussion of the impact of cooking on the survivorship of bone in the burial environment, followed by a discussion of the similarities between cooked and weathered bones.

2.5.2.1 Differential survival of cooked and raw bone

Heating will weaken the bone composite by degrading the protein and at higher temperatures altering the mineral. It may be expected that heating will lead to accelerated loss of bone compared with unheated equivalents in the same burial site. Burning, by completely destroying the organic phase and generating a more stable mineral phase will conversely be expected to enhance the probability of survival (Buikstra and Swegle, 1989). Differential survival will potentially bias zoological interpretations based on the analysis of species and/or body part representation in an archaeological assemblage.

Little is known about the effect of cooking on bones. There are claims of unchanged (Chaplin, 1971) and decreased survival (Speth, 2000). Arguably, bones which have been subjected to mild heat during cooking may actually survive better because the removal of organic matter (proteins and lipids) both reduces their palatability to scavenging organisms and also diminishes microbial decay and putrefaction (Turner and Turner, 1999). Jans et al. (2004) argue that intact inhumations lead to putrefaction and microbial decay carried through the vascular system and into the bone. Conversely butchered bones display significantly less microbial attack.

Increased exposure to heat, will cause degradation of the collagen and alteration of the bone structure which will reduce the physical qualities of the bone (Turner-Walker and Parry, 1995). As the strength declines due to loss of collagen, bones will be more susceptible to fragmentation by trampling and soil pressure.

Some burial experiments have included cooked bone in their burial assemblages: however, the cooking regimes are very variable and none of these have established unequivocally whether, or how, cooked bone behaves differently when buried (Bell et al., 1996b; Nicholson, 1996,1998).

2.5.2.2 Similarities in the characteristics of cooked and buried bone

Although not commonly recognized, there are parallels between the effects of weathering and cooking, and further several forms of physical and chemical alternations which are used to indicate cooking are also used as indicators of weathering.

Bones heated to high temperatures in the presence of oxygen show characteristic colour changes with increasing temperature. The first signs of colour change can be seen at 200-300°C, at which point the bone turns from ivory to light tan and then dark brown. This colour change is associated with combustion of the organic content within the bone. At higher temperature and after prolonged exposure the bone becomes black or dark grey. Between 600-800°C all the remaining moisture and organic content is lost and the calcined bone and changes to blue-grey or white (Buikstra and Swegle, 1989; Shipman et al., 1984). When viewed at very high magnifications, surface modifications such as warping and cracking are also evident within this temperature range (Shipman et al., 1984). However some similar surface modifications have also been observed on unheated but weathered bone (Nicholson, 1993). Further colour changes are not unique to cooked bone; changes may also occur in the burial environment due to staining from trace elements and organics in the soil (Brain and Sillen, 1988; Shipman et al., 1984). In theory however careful chemical analyses should be able to discriminate between staining and charring (e.g. trace element analysis; Reiche et al., 1999).

During both weathering and cooking, the degree of crystallinity is seen to increase by a process of Ostwald ripening (a growth of larger crystals at the expense of smaller ones, (Tuross et al., 1989; Weiner and Bar-Yosef, 1990) The increase in crystallinity can occur over millennia as the bones become fossilized or through more rapid transformations of weathering over a few months or years (Stiner et al., 1995). Changes in crystallinity have been monitored by a crystallinity index based on X-ray diffraction, infra-red spectra and microscopic analysis of the crystallites (e.g. Sillen, 1989; Trueman et al., 2004; Weiner and Bar-Yosef, 1990). Stiner et al. (1995) have convincingly established the difficulty of even identifying burned bone using infra-red and X-ray diffraction, since recrystallization due to burning may be impossible to distinguish from recrystallization due to weathering above and below ground. They found no

significant difference in crystallinity values between burnt and unheated archaeological samples (Stiner et al., 1995). As a consequence of such findings researchers have cautioned against the use of crystallinity as a single criterion for determining heat-induced changes in bone (Nicholson, 1993; Shipman et al., 1984).

Direct comparisons of the impact of weathering and heating on the *organic* phase are less common. Some studies have argued that in both cases collagen is preferentially lost relative to non-collagenous proteins (Taylor et al., 1995; Weiner and Bar-Yosef, 1990). Non-collagenous proteins are generally more soluble but are also more acidic. It is reasoned that such proteins will display a higher affinity for hydroxyapatite than collagen and will persist in bone once the collagen has been degraded (Masters, 1987).

Roberts et al. (2002) looked at the effects of boiling on the different components of bone and failed to discriminate between boiled and buried bone. They looked at changes to the organic (C:H:N analysis) and mineral compositions (IR) as well as structural changes (mercury intrusion porosimetry and histology). They found that as the bone was boiled, the protein was lost and crystallinity and porosity increased. They observed a correlation between protein loss (nitrogen loss) and crystallinity (increase in splitting factor). Similar relationships have also been observed with archaeological bone; with bones that are low in nitrogen generally exhibiting higher crystallinity (Nielsen-Marsh and Hedges, 2000). This led the authors to suggest that cooked bone could not be distinguished from weathered bone, and in fact cooking could be used as an analogue for the effects of burial.

2.6 Strategies to detect cooked bone

2.6.1 TEM as a possible technique

TEM may offer a solution to the problem of differentiating low temperature heated from unheated bone. Snowden and Weidemann (1978) used TEM to analyse collagen fibrils from the tendinous sheath of the *longisisumus dorsi* muscle of mature sheep, after samples had first been heated incrementally to 80°C. At 64°C they observed 'melted regions' both along the collagen fibrils and at the ends. Based on these findings a similar approach was subsequently adopted to identify thermal alteration in fish bone vertebrae (Richter, 1986). In this instance, after the bones were heated, the collagen fibrils had to be demineralised before they could be viewed by TEM. The results of this study showed that even within

mineralized tissue thermal alteration could be observed within the collagen fibrils. Richter (1986) found that at 60°C after 30 minutes most of the fibrils showed 'melted' areas and that at 100°C after the same duration all the collagen had melted. For my MSc thesis I conducted a pilot study to determine if the same technique could be applicable to mammalian compact bone collagen which is more highly mineralized than fish bone collagen (Moyle and Cech, 1982; Nicholson, 1996), more densely packed, with less interfibrillar space (Lee and Glimcher, 1991) and is more thermally stable due to a higher concentration of hydroxyproline (Eastoe, 1957; Woodhead-Galloway, 1980; see section 1.3.2 and 1.3.3). The results of the pilot study were promising, and suggested that sheep compact bone heated at 80°C could be distinguished from uncooked bone. However, an unheated bone which had been buried for seven years in an acidic moorland soil showed similar types of alteration as the cooked samples. The results were written up and published at the beginning of my PhD studies (Koon et al., 2003).

2.6.2 DSC as a possible technique

Differential Scanning Calorimetry (DSC) is another technique which has the potential to be used to explore the early stages of collagen deterioration in cooked bone. It has many advantages including; fast analysis time (typically thirty minutes), easy sample preparation, can be used over a wide range of temperatures and it produces highly reproducible analyses. Also DSC analysis can be applied to both collagen molecules in solution and fibrillar collagen. It is a particularly useful tool for studying collagen deterioration because when heated to a sufficient temperature collagen goes through a large endothermic transition, easily observed as a peak in a DSC trace. The size, shape and location of this peak can provide information about the state of the collagen molecules within the sample (Miles et al., 1995). If the collagen is degraded, the peak on the DSC trace will probably be broader, as there will be less uniformity in the stability of the collagen molecules within the sample; the peak may also be smaller and shifted to a lower temperature, because the degraded collagen will be less thermally stable. DSC has been used to study the thermal behaviour of native collagen (Flandin et al., 1984; Miles et al., 1995). The technique has been used to study the deterioration of historical parchment and leather (Chahine, 2000) and has also once been applied to study collagen deterioration in modern cooked bone and archaeological bone (Nielsen-Marsh et al., 2000a). In this study, the DSC results showed a significant downward shift in the peak of the bone collagen

from archaeological material and the modern cooked bone compared to the unheated bone. The latter observation was, however, only based on one cooked and one unheated bone which had previously been part of a short term burial experiment, and therefore the application of DSC to identify cooked bone warrants further study.

2.7 Summary

The issue of cooking has been largely neglected in archaeological studies. This is not due to a lack of importance but most probably because of the difficulty in identifying cooked bone. There is at present no technique which is able to detect alteration within the low temperature range that is associated with cooked bone (as opposed to burnt bone). A further complication is that similar types of alteration have been observed from bones which have been exposed to weathering and diagenesis, and those which have been subjected to thermal alteration.

There is a need therefore to develop a technique that can not only determine heat induced changes from the effects of the burial environment, but which will also be capable of recording any subtle alterations that may occur in low temperature heated bone. One way to do this may be to look at subtle microstructural changes in the collagen component of cooked bone; this approach has formed the basis of my PhD research.

Part 2 Developing methodologies

Chapter 3 Methods and method optimization

This chapter is divided into two parts. In the first part the materials and methods that were used throughout the thesis are outlined. The second part describes a series of experiments that were conducted at the beginning of this PhD research in order to optimize sample preparation by addressing the concerns discussed below.

3.1 Introduction

The main techniques that were utilized during this research were (i) transmission electron microscopy (TEM) and (ii) differential scanning calorimetry (DSC). With the exception of my earlier (MSc) dissertation there has only been one previous study which has attempted to conduct TEM analysis on isolated and unfixed collagen fibrils from thermally altered bone, in that case fish bones (Richter, 1986). DSC has also only been used once to detect damage within low temperature cooked bone (Nielsen-Marsh et al., 2000a). An early part of my research was therefore concerned with developing suitable approaches for the preparation and analysis of a range of forensic and archaeological mammalian bones. The challenge was to find ways to optimize the extraction of the collagen fibrils, and to minimize artefacts introduced during sample preparation, so that accurate and reproducible results could be obtained. One important area was identified that needed particular consideration: the demineralization of samples for both DSC and TEM analysis.

3.1.1 Demineralization for TEM and DSC analysis

There are two methods commonly used for bone demineralization; dissolution in acid, mainly Hydrochloric acid (HCl) (e.g. DeNiro and Weiner, 1988a; Masters, 1987; Schoeninger et al., 1989), or chelation of calcium ions using Ethylenediaminetetraacetic acid (EDTA) (e.g. Glauert and Lewis, 1998; Hagelberg and Clegg, 1991). The two methods are fundamentally different; acid demineralization disrupts the crystalline lattice of the apatite whilst EDTA chelates surface calcium ions. The advantage of using HCl is that demineralization occurs more rapidly with this method than with EDTA. Secondly the HCl molecule is very small and can therefore is easily diffused within the bone matrix (Collins and Galley, 1998). As a consequence, the bone sample can have a small surface area (i.e. does not need to be broken into very small pieces)

and yet still be successfully demineralized. Demineralization in HCl however could result in additional hydrolysis of the peptide bonds in the α-chains of the collagen molecule (Freemantle, 1995). This in turn may cause a similar disruption to the collagen fibrils as that caused by heating. In order to minimize this undesired hydrolysis, it has been suggested that a dilute acid should be used and that demineralization should be carried out at 4°C (Collins and Galley, 1998).

A further consideration with using the HCl method is that immersion in acid causes collagen fibrils to swell (Haines, 1987). Collagen contains both positively and negatively charged groups. At the isoelectric point (just above neutral pH for native untreated collagen fibrils) collagen displays no overall net charge because the oppositely charged forces have been balanced out. If the pH is lowered by the addition of HCl the overall net charge on the collagen molecules becomes positive, because the carboxyl groups on the amino acid side chains become protonated. Changing the charge profile will cause repulsive effects between similarly charged groups on the amino acids of adjacent α-chains, which will push the collagen molecules apart. To regain a net neutral charge the positive charge must be balanced by negatively charged counter ions from the acid. As a result the collagen contains a higher concentration of charged ions within its structure compared to the surrounding solution. This causes an osmotic effect: to regain equilibrium there is an influx of water molecules into the collagen structure, which results in further swelling.

This phenomenon could be advantageous for visualizing damage along the collagen fibrils. Previous results (Koon et al., 2003) had suggested that the acid was not actually damaging the fibrils but rather visualizing microstructural damage caused by the heat treatment, by preferentially swelling these areas of microstructural damage. Unheated samples did not exhibit localized areas of swelling along the fibril after demineralization in HCl. However this observation was based on a small set of samples all from sheep metapodials and therefore warranted further investigation.

For the purpose of DSC analysis, swelling caused by acid demineralization could prove problematic. Nielsen-Marsh et al. (2000a) found that acid demineralization caused a reduction in the position of the DSC peaks and they were poorly resolved. The authors were unable to remove these effects and as a result they rejected this method for the preparation of demineralized bone collagen for DSC analysis. The effect of low pH on the thermal stability of leather has long been

known (Theis and Steinhardt Jr, 1942). It is thought that by swelling the collagen fibrils in acid, the distance between the α-chains is increased, which reduces inter-chain forces and as a result the shrinkage temperature of leather decreases (Haines, 1987). However this swelling can be suppressed at acidic pH by the addition of a high concentration of a neutral salt. The swelling of collagen as a function of pH and the effect of adding salt is illustrated in Figure 3.1. The possibility of counteracting the swelling was investigated by using high concentrations of sodium chloride in the demineralization solution and also by removing all of the acid from the sample prior to DSC analysis.

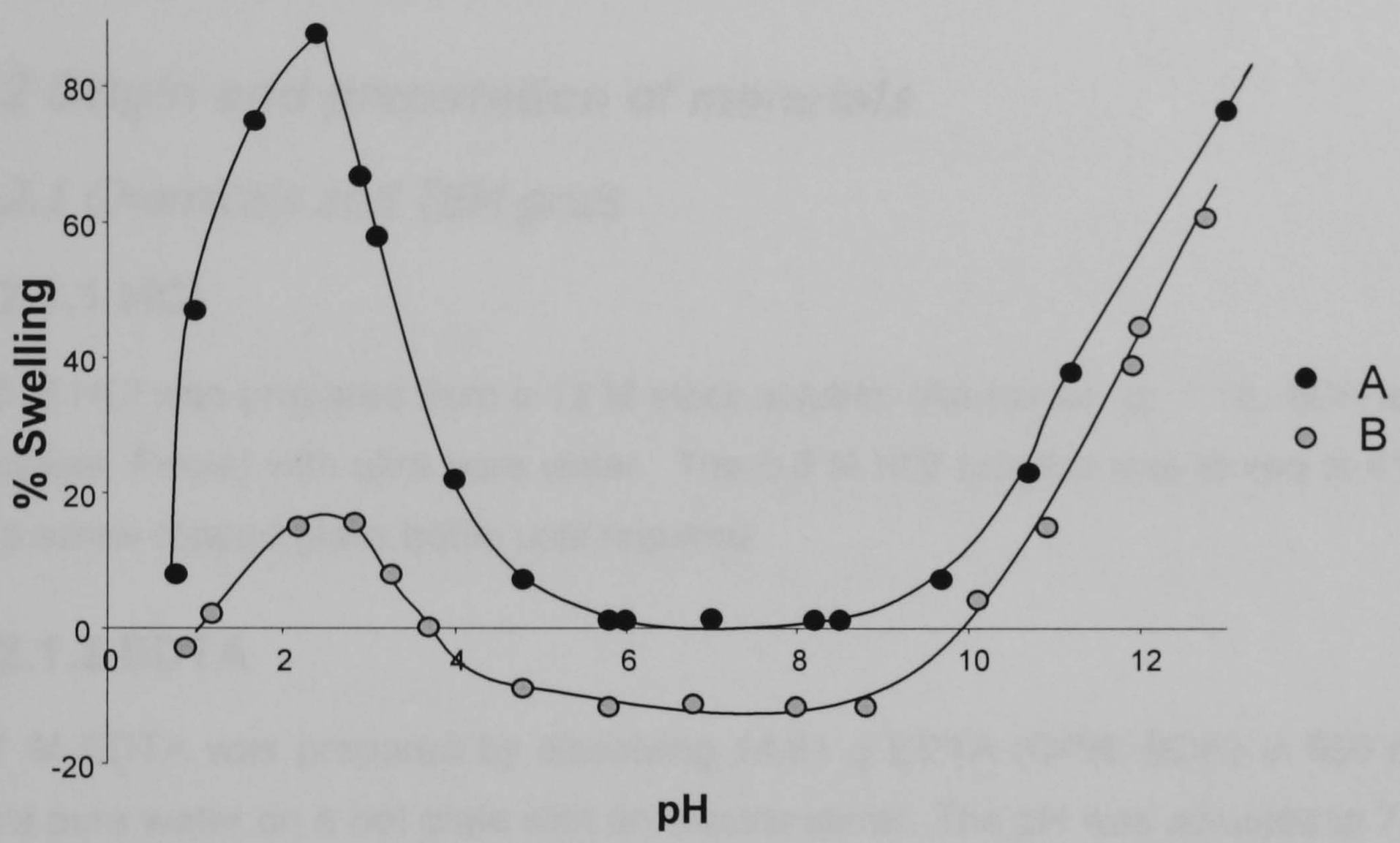


Figure 3.1 Image showing the swelling profile of collagen at different pH values, with and without added salt. A = no added salt B = 2.0N sodium chloride solution, adapted from Haines (1987), Figure 10, p: 4.

Demineralization with EDTA is a more gentle process thought to minimize damage to the protein (Collins and Galley, 1998). As such it is recommended for bone demineralization in preparation for TEM (Glauert and Lewis, 1998), and was also used in the previous TEM study on fish bone collagen (Richter, 1986). This approach was also used in the DSC study by Nielsen-Marsh et al. (2000a), where it was found to produce repeatable results on demineralized bone collagen. However this method is very slow and may not produce complete demineralization when using bone shards. In a recent study only 70% of the total calcium had been released from bone shards after nine days of EDTA demineralization (Collins and Galley, 1998). This slow rate has been attributed to

the size of EDTA molecule and the mechanism of removing the mineral, by chelation of Ca^{2+} from the surface of the apatite crystallites. By powdering the bone sample the available surface area is greatly increased and this can vastly improve the speed and effectiveness of demineralization with EDTA (Collins and Galley, 1998). However, potential mechanical damage caused during this process could be detrimental to the collagen fibrils (Semal and Orban, 1995). The length of time required for complete removal of the mineral from bone shards was investigated using both EDTA and HCl. Samples were also powdered (by freeze-drying and then spex-milling) to determine the extent of damage that this procedure actually causes to the collagen fibrils.

3.2 Origin and preparation of materials

3.2.1 Chemicals and TEM grids

3.2.1.1 HCl

0.6 M HCl was prepared from a 12 M stock solution (Aristar sp. gr. 1.18, BDH lab supplies, Poole) with ultra pure water. The 0.6 M HCl solution was stored at 4°C in a screw-topped glass bottle until required.

3.2.1.2 EDTA

0.1 M EDTA was prepared by dissolving 14.61 g EDTA (GPR, BDH) in 500 ml ultra pure water on a hot plate with an electric stirrer. The pH was adjusted to 7.4 using 0.5 N NaOH (Anala R, BDH). The 0.1 M EDTA solution was stored in a screw topped glass bottle until required.

3.2.1.3 Phosphate buffer (PBS)

31.2 g of Sodium dihydrogen phosphate (NaH_2PO_4, $2H_2O$) (GPR, BDH) was dissolved in 1 litre of distilled H_2O on a hotplate with an electric stirrer. A solution of disodium hydrogen phosphate (Na_2HPO_4, $12H_2O$) (analytical grade, VWR international) was also prepared by dissolving 28.39 g in 1 litre of ultra-pure water. 19.5 cm^3 of NaH_2PO_4 solution was then added to 30.5 cm^3 of Na_2HPO_4 solution and made up to 100 cm^3 with de-ionized H_2O to bring the buffer to pH 7. The PBS was stored in a screw topped glass bottle until required

3.2.1.4 Uranyl acetate

A 2% w/v solution of Uranyl acetate (Agar Scientific Ltd., Essex) was prepared with a 50/50% solution of ultra pure water and ethanol (absolute, analytical reagent grade, Fisher Scientific UK Ltd., Leicestershire). The solution was stored at 4°C in a screw-topped glass bottle sealed with parafilm. The bottle was wrapped in foil to protect the uranyl acetate from sunlight. A new solution was prepared each month.

3.2.1.5 Phosphotungstic acid solution

A 1% w/v solution of Phosphotungstic acid (Agar Scientific Ltd.) was prepared with ultra pure water. The pH of the solution was raised to pH 7 using 0.5 N sodium hydroxide. The solution was stored in a screw-topped glass bottle at 4°C. A new solution was prepared each month.

3.2.1.6 Glutaraldehyde

1% glutaraldehyde solution was made from 2 ml Glutaric dialdehyde 50% and 98 ml PBS. This preparation was carried out at the British Leather Sellers Centre, Northampton and the solution was used straight away, therefore not stored.

3.2.1.7 Formvar grids

The carbon coated copper formvar grids used for the TEM analysis were obtained from Agar Scientific. They were 3 mm in diameter and had a 300 mesh. This mesh size proved to be the best for this analysis because it meant that, in the majority of cases, the grid windows were big enough to visualize the full length of collagen fibrils without too much interference from the grid bars. At 200 mesh the windows were too large and the weight of the specimen on the grid often caused the windows to break. The grids were given a high tension (HT) discharge (1.5 kV and 30 A at $1x10^{-1}$ mbar) to make them hydrophilic before they were used.

3.2.2 Modern fresh bone

Only compact (not trabecular) bone was used in this thesis. Where possible samples were obtained from the mid-shaft of a femur or other long bone as this region often yields the best preserved compact bone in archaeological specimens and it is comparable with clinical literature.

3.2.2.1 Animal

Fresh animal long bones were obtained from a local butcher unless otherwise specified in the associated materials and methods section. Any adhered soft tissue was removed with a dissection knife. The articular ends were removed with a band saw leaving only the shaft. This portion was then sectioned longitudinally, and the exposed marrow was scooped out of the medullary cavity. The shaft sections were rinsed thoroughly under cold running water, patted dry with paper towels then placed between acid-free tissue paper and left to air-dry for 48 hours. Once dried the bones were stored at 4°C.

3.2.2.2 Human

A small number of non-infectious human forensic samples were used in this research. The samples were provided by the Ageing Research Group at Düsseldorf University, Germany. Ethical clearance for the use of this material was covered by the Ageing Research Group. The material was provided as one centimetre thick cross-sections of bone which had been removed from the mid-shaft of the right femur. This material was stored at 4°C.

3.2.3 Other collagenous materials

3.2.3.1 Achilles' tendon

Type I insoluble dried Bovine Achilles' tendon was obtained from Sigma and stored at 4°C. Prior to analysis the tendon was rehydrated overnight in PBS at 4°C.

3.2.3.2 Rat tail tendon

Rat tails were provided by Prof. Tim Wess, Cardiff School of Optometry and Vision Sciences, University of Cardiff. The rat tails were stored in a freezer. Prior to analysis the rat tails were defrosted and individual tendons were removed using a dissection knife and forceps. The tendons were washed with ultra pure water and then place in PBS and stored at 4°C until needed.

3.2.3.3 Forensic and archaeological material

Details pertaining to the origin and preparation of the forensic and archaeological samples obtained for this research can be found in the associated materials and methods sections (Chapters 6-8). Where the colour of a sample is described Munsell Soil Colour Chart codes (Munsell Soil Company Inc., 1994) have been

used to provide a more accurate description, e.g. 2.5YR 3/4 (after Nicholson, 1993; Shipman et al., 1984).

3.3 Methods

3.3.1 Shard preparation

It was decided not to use a hand-held electric cutting device (Dremal) as it produces a considerable amount of heat due to friction; which could mask any intended heat induced changes. Instead during the first year of this research a hacksaw was used to cut cross-sections from the bones. The majority of the experiments presented in the thesis were conducted during the second and third year of the research, during which time, following a move to York University, an Exakt water-cooled rotating saw was available, which became the method of choice. Two cuts approximately one centimetre apart were made into the anterior of the mid-shaft, using the water-cooled saw. A screwdriver was then wedged between one of the cuts in order to lever out the section. The cross-sections were then broken into smaller shards using a hammer. To ensure a degree of uniformity in the size of the shards they were put through a three millimetre sieve.

3.3.2 Demineralization

Whilst the bone samples were being demineralized they were kept at 4°C to hinder any microbial attack and in the case of the HCl treatment to minimize any destruction to the collagen by acid hydrolysis (Collins and Galley, 1998). The demineralization solutions were also constantly agitated to increase the rate and efficiency of demineralization.

3.3.2.1 HCl demineralization

60 mg of bone shards were placed in a 2.5 ml Eppindorf tube with 2 ml of cold 0.6 M HCl. The lid of the tube was closed and sealed with parafilm. Two of the Eppindorf tubes were then placed in a 25 ml Universal tube and the cap was screwed down. This Universal was then placed on a Roller Mixer SRT1 (Stuart Scientific) in a refrigerator. The HCl solution was changed after seven days then the sample was left to demineralize for a further seven days. After the period of demineralization, the acid was removed with a pipette and the demineralized shards were washed twice with ultra pure water then left in 2 ml PBS pH 7 overnight and stirred on the Roller Mixer. Afterwards the PBS solution was changed and the samples were stored at 4°C for up to 5 days for further analysis.

3.3.2.2 EDTA demineralization

60 mg of bone shards were placed in a 25 ml Universal tube with 15 ml of 0.1 M EDTA (pH 7.4). The lid of the tube was screwed down and sealed with parafilm .The Universal was then placed on a Roller Mixer in a refrigerator. The EDTA solution was changed after seven days then the sample was left to demineralize for a further seven days. After the period of demineralization, the EDTA was removed with a pipette and the demineralized shards were washed twice with ultra pure water then left in 2 ml PBS pH 7 overnight and stirred on the Roller Mixer. Afterwards the PBS solution was changed and the samples were stored at 4°C for up to 5 days for further analysis.

3.3.3 ICP-OES Elemental analysis

3.3.3.1 Function of ICP-OES

Elemental analysis was conducted on bone shards and demineralized bone collagen in order to determine the Ca content. This measurement was used to show the amount of mineral in the sample. The technique works as follows: the plasma coil heats Argon gas to over 1000°C. The Argon is promoted to an excited state and produces a 'flame'. The sample is introduced to the spectrometer in liquid form. It is mixed with Argon to form an aerosol. Small droplets of the aerosol are fed into the plasma 'flame'. The electrons in the elements of the sample move from their ground state to an excited state. As they reach the cooler parts of the flame however they revert to their ground state and in the process emit energy in the form of light. The frequency of the light emitted is recorded and produces a peak.

3.3.3.2 Sample preparation

All samples needed to be in liquid form for this analysis so in the first instance the samples were microwave acid digested. Each sample was placed in a tube with 5 ml nitric acid (Trace Analysis Grade, Fisher Scientific UK Ltd), heated up to 105°C in a microwave oven and kept at this temperature for a further 15 minutes. The sample was then left to cool to room temperature. The temperature of the solution was continuously monitored with a thermocouple. The sample was then diluted to 10 ml (if demineralized bone shards) or 50 ml (whole bone shards) with ultra pure water. 5 ml of the sample was then introduced to the spectrometer. Analysis was conducted on a Spectro Ciros^{CCD} ICP-OES. Ca has nine characteristic peaks; measurements were taken from the peak at 393.366 nm.

3.3.4 Transmission Electron Microscopy (TEM)

3.3.4.1 Function of TEM and specimen staining

The TEM is a high powered microscope with a magnification capability significantly greater than an optical microscope. This is because it uses a beam of electrons (instead of light) which allows for resolution down to a few nanometres (Glauert and Lewis, 1998). Figure 3.2 shows a schematic diagram of the TEM. Electrons are produced by an electron gun at the top of the microscope. These electrons are then accelerated through an anode plate and focused with a magnetic lens. The electron beam then passes through the specimen, electrons encounter atoms from the specimen and collide, thus scattering the beam. Electrons that are scattered by an angle of more than 0.5° are stopped by the objective aperture, situated below the specimen. The larger the amount and size of the atoms in the specimen, the greater the probability that the electrons in the beam will collide with one of them. Thus electrons will pass through less dense regions of the specimen more easily than more dense areas. The electrons that are able to pass through the specimen will then strike the phosphor image screen generating light, which produces the image. The dark areas observed in a TEM image therefore correspond to the dense regions where the electrons have been scattered and removed from the beam, where there are light areas they correspond to the less dense regions of the material which allow electrons to pass through (Grimstone, 1977).

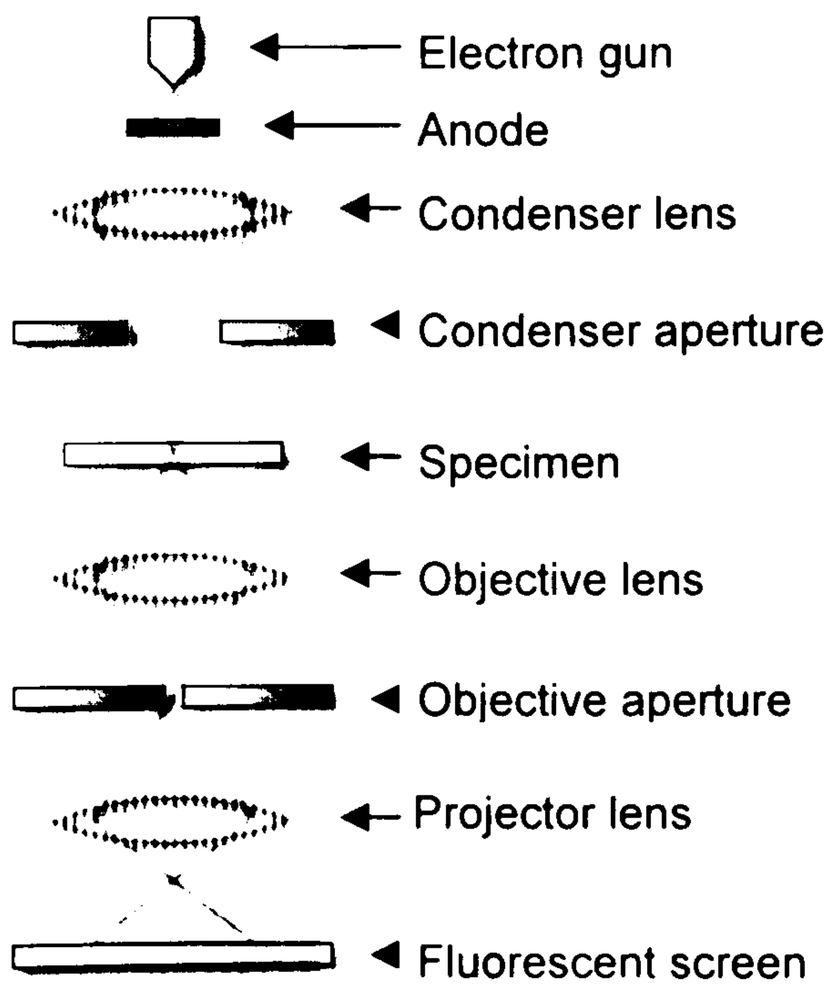


Figure 3.2 Schematic diagram of a Transmission Electron Microscope (adapted from http://invsee.asu.edu/ImageGallery/Schematic/Engineering/default.htm 9/01/06)

A collagen specimen is difficult to see under normal conditions in an electron image. This is because the atomic masses of the elements which make up collagen (mainly oxygen, carbon and nitrogen) are not high enough to produce powerful scattering of electrons. Thus in order to view collagen under the TEM, specimens must first be stained with heavy metals which will scatter the electron beam and enhance the contrast of the image. Two common methods for visualizing collagen under the TEM are by negative or positive staining. In the former the object remains unstained but is embedded in a dried film of heavy metal salt. The stain surrounds the molecules and penetrates into any open irregularities on the surface, such as the gap regions along the collagen fibril. With this technique the specimen appears light on a dark background. Positive staining involves treatment of the specimen with a chemical that increases the mass density. The sample is immersed in a heavy metal salt stain, but unlike in the case of negative staining, this is later washed away. Some of the heavy metal ions remain attached to charged groups on the surface of the collagen fibril. For example, phosphotungstate ions are negatively charged and so will react with the positively charged groups on the collagen. Uranyl acetate will react with negatively charged groups such as the carboxyl side chains of aspartic acid and glutamic acid residues. When positively-stained, the collagen fibrils appear dark against a light background.

Negative staining has advantages over the positive staining technique in that the preparation method is simpler and quicker. This technique was tested on unheated and heat-damaged collagen fibrils. Figure 3.3a and 3.3b both show examples of heat damaged collagen. In the first image the collagen fibril has been stained using a positive staining technique (the preparation method is described in the following section, 3.3.4.2). In the second image the collagen fibril has been stained using a negative staining technique (the preparation method is described in the figure legend). It has been suggested that measuring dimensions from negatively stained preparations can be problematic as 'edge detail' is obscured by the stain to a variable extent (Haschemeyer and Myers, 1972). It is therefore likely that any width measurements performed on negatively stained fibrils from digital images would not be accurate. Also it had proved difficult to distinguish heat damaged regions along the fibrils from areas where dried stain had accumulated, therefore this technique was rejected. This analysis did however show that damaged regions observed in heated samples are present

after both negative and positive staining treatment and are therefore not due to a reaction of the specimen to the positive staining treatment.

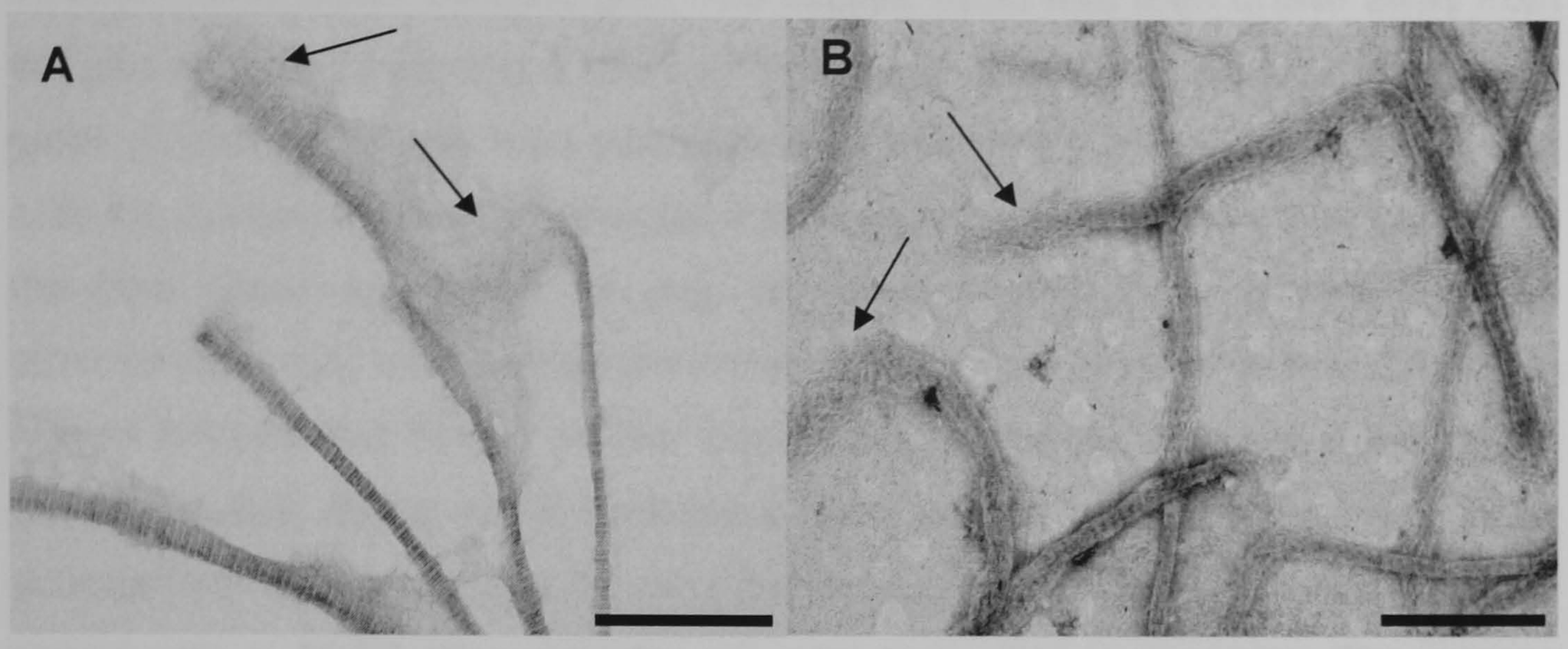


Figure 3.3 Examples of collagen fibrils exhibiting frayed ends due to heat damage (indicated by the arrows). The scale bars are 500 nm. The samples have been prepared using A) positive stain (see section 3.3.4.2 for preparation details) and B) negative stain. The negatively stained sample was prepared using 5 ml of 3% w/v Phosphotungstic acid adjusted to neutral pH. A PBS solution containing the previously homogenized sample was mixed with the stain in the same pipette. The solution was then dropped onto a grid and left for one minute before any excess liquid was wiped away.

For this research a positive staining technique was chosen. Phosphotungstic acid staining alone proved insufficient to visualize the damaged collagen fibrils therefore double staining with phosphotungstic acid and uranyl acetate was used as this has been shown to provide greater contrast (Puchinger et al., 2002).

3.3.4.2 TEM sample preparation

The demineralized bone shards were washed thoroughly with ultra-pure water then placed in a 25 ml Universal tube with 3 ml of a 1% phosphotungstic acid solution (pH 7). In order to liberate the individual collagen fibrils the solution was homogenized for three minutes using a homogenizer (Yellowline, DI25 basic). To maintain a low temperature the Universal tube was placed in an ice bath and homogenization was conducted in 30 second bursts with a 30 second intervals. (The head of the macerator was disassembled and cleaned thoroughly with ultra pure water before processing each new sample, to minimize cross contamination.) The resulting cloudy solution was centrifuged (U-32R centrifuge, Boeco, Germany) at 3000 g and 4°C for 15 minutes. Afterwards the supernatants were discarded and the remaining pellets were re-suspended in 1 ml of phosphotungstic acid and mixed using an Autovortex mixer (L46, Labinco). Two formvar grids were laid onto a Petri dish and a drop from the homogenizer

collagen fibril solution was pipetted on top. The grids were then left for five minutes in order for the collagen fibrils, which were suspended in the drop of solution, to fall down onto the grid. The excess liquid was then drawn away from the grid surface by placing a piece of filter paper at the very edge of the grid. A piece of filter paper was then placed onto another Petri dish and dampened with a 50:50 solution of an ethanol/water mix. A portion of dental wax was placed on the filter paper and a 0.5 ml drop of uranyl acetate (2% solution in 50:50 ethanol/water mix) was suspended on top. The formvar grids were then lifted with curved forceps and floated on the drop of uranyl acetate, specimen side down, for 30 minutes, during which time the system was covered to protect the uranyl acetate from the light. The grids were finally picked up with crossover forceps and rinsed with a 50:50 ethanol/water mix and then ultra pure water. The excess liquid was removed with filter paper and then the grids were left to air-dry. In the first year of this PhD research, TEM analysis was conducted on a Jeol 1200 EX TEM, however the majority of the analysis was conducted using a FEI Tecnai G2 Transmission Electron Microscope fitted with a CCD camera.

3.3.4.3 TEM operation

The following information relates to analysis that was carried out using the FEI Technai G^2 Transmission Electron Microscope. Once the microscope was set up (typically beam setting 100kV; aperture spot size of 1; focus step 3 or 4) and focused, a grid containing the sample was viewed at low magnification (x4,000). The grid was inspected to identify clean unbroken grid windows, using a tracker ball to roam over the grid surface. When a clear window was identified the magnification was increased to x10,000 to identify fibrils within the grid window. Once a fibril had been identified the magnification was increased to x30,000 - x 40,000. At this point the intensity was turned down and the CCD camera was inserted. The image of the fibril could then be viewed on a monitor, once the brightness and contrast had been turned up. The camera had a live feed, therefore the sample grid could be moved around and the magnification could be changed, and the updated image was shown on the monitor. In this way was possible to spend time looking over the entire fibril without introducing damage from the electron source (If the beam is at high intensity and is left on the fibril for too long the image can become grainy and usually the formvar on that window of the grid will melt). The fibril was then recorded (as explained below) and possibly photographed using the digital or plate camera. This procedure was carried out

for each fibril, and the focus had to be realigned after each change in magnification.

3.3.4.4 TEM analysis method

In order for comparisons to be made between samples, for each grid (2 grids per sample) the first 100 fibrils observed were counted and classified as unaltered or altered. The altered fibrils were classed as exhibiting one of two forms of damage; *Beaded*, where swollen areas appear at discrete positions along the fibril and at the ends; or *Dumbbell*, which consists of a short fragment of a fibril with swollen ends. The different forms of alteration as described below, and examples of each are shown in Figure 3.4.

Unaltered : Fibrils which have an equal diameter along the entire length of the fibril and show a continuous repeat pattern of banding, they are typically tens of microns in length (Figure 3.4A).

Beaded : Fibrils which display regions along the fibril where there has been a disruption to the banding pattern. In these regions there may be a faint remnant of banding, but usually the banding pattern has been completely lost; there is also an increased diameter in this disrupted region. They can be as small as five μm but are generally greater than 10 μm in length (Figures 3.4B).

Dumbbell : Fibrils which have a central fibrillar region between 500 nm and three microns in length where the banding is intact but the ends are observed as grey swollen regions with no evidence of banding (Figures 3.4C).

The *Dumbbell* appears to be a more advanced form of alteration and probably occurs through a parting of a *Beaded* fibril at the sites of damage. Figure 3.5 shows a close up of a *Beaded* region which looks about to break apart.

Only discrete fibrils were included in the analysis; any fibrils which were obscured by other overlying fibrils were not included. Each fibril was counted and classified then entered onto a recording sheet. Unless otherwise stated the values for the proportion of *Unaltered*, *Beaded* and *Dumbbell* recorded for each sample are given as a mean value. This value is the average from the two grids (100 fibrils per grid). If there was more than a 10% difference between the values obtained from the two grids a further 100 fibrils were counted and the mean was taken

from the two closest values, if there is still more than a 10% difference between the two closest values the sample was rejected.

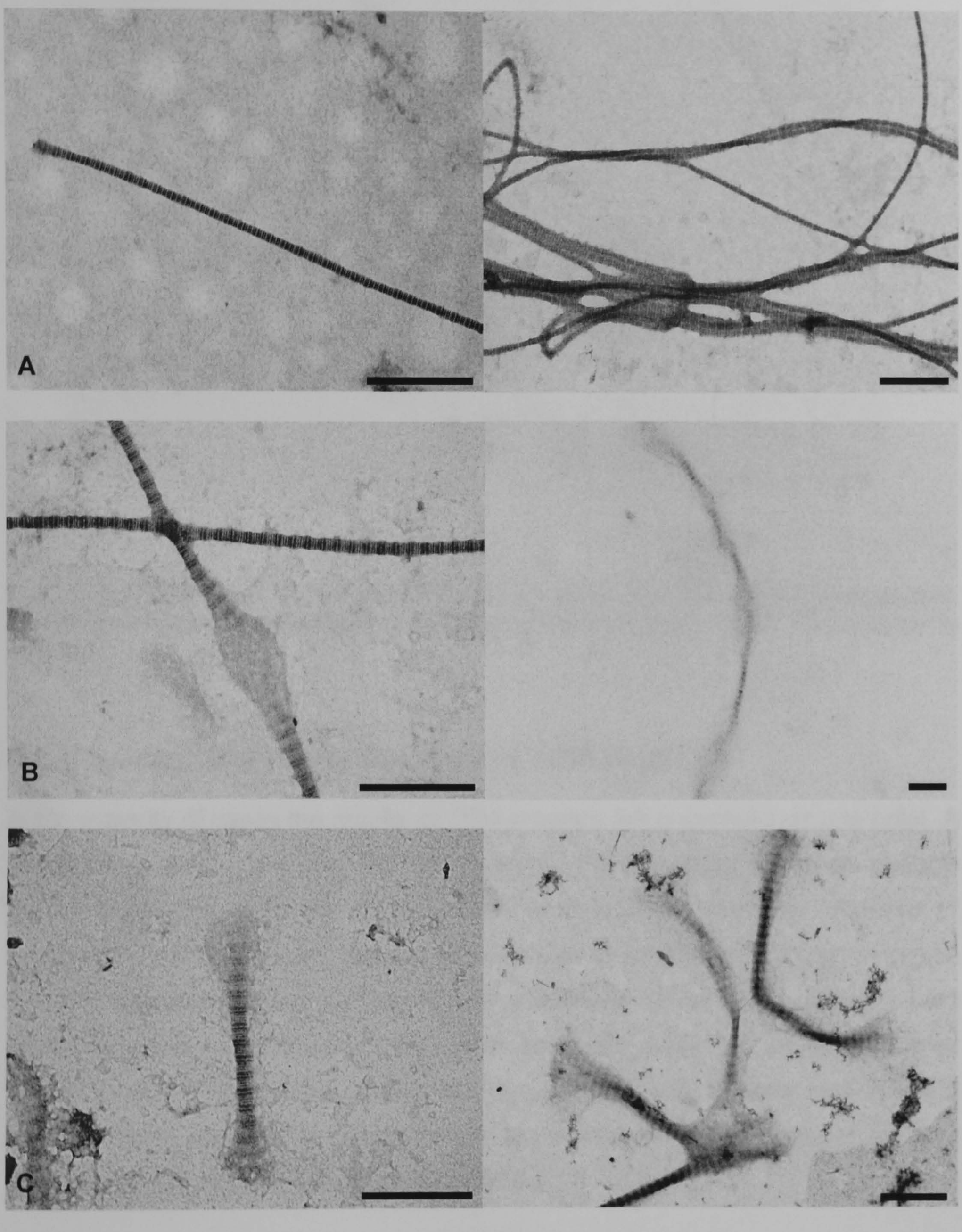


Figure 3.4 Shows three pairs of TEM images which are typical of the different forms of alteration observed in the collagen fibrils. A shows examples of *Unaltered* collagen fibrils, they have an uniform diameter along the length of the fibril and show a continuous repeat pattern of banding. B shows examples of *Beaded* fibrils, they have damaged '*Beaded*' regions along the length of the fibril and frayed ends. C shows examples of damaged collagen fibrils of a *Dumbbell* form. The banding is intact and clearly visible in the central fibrillar region but the ends are observed as grey swollen regions with no evidence of banding. The scale bars are 500 nm.

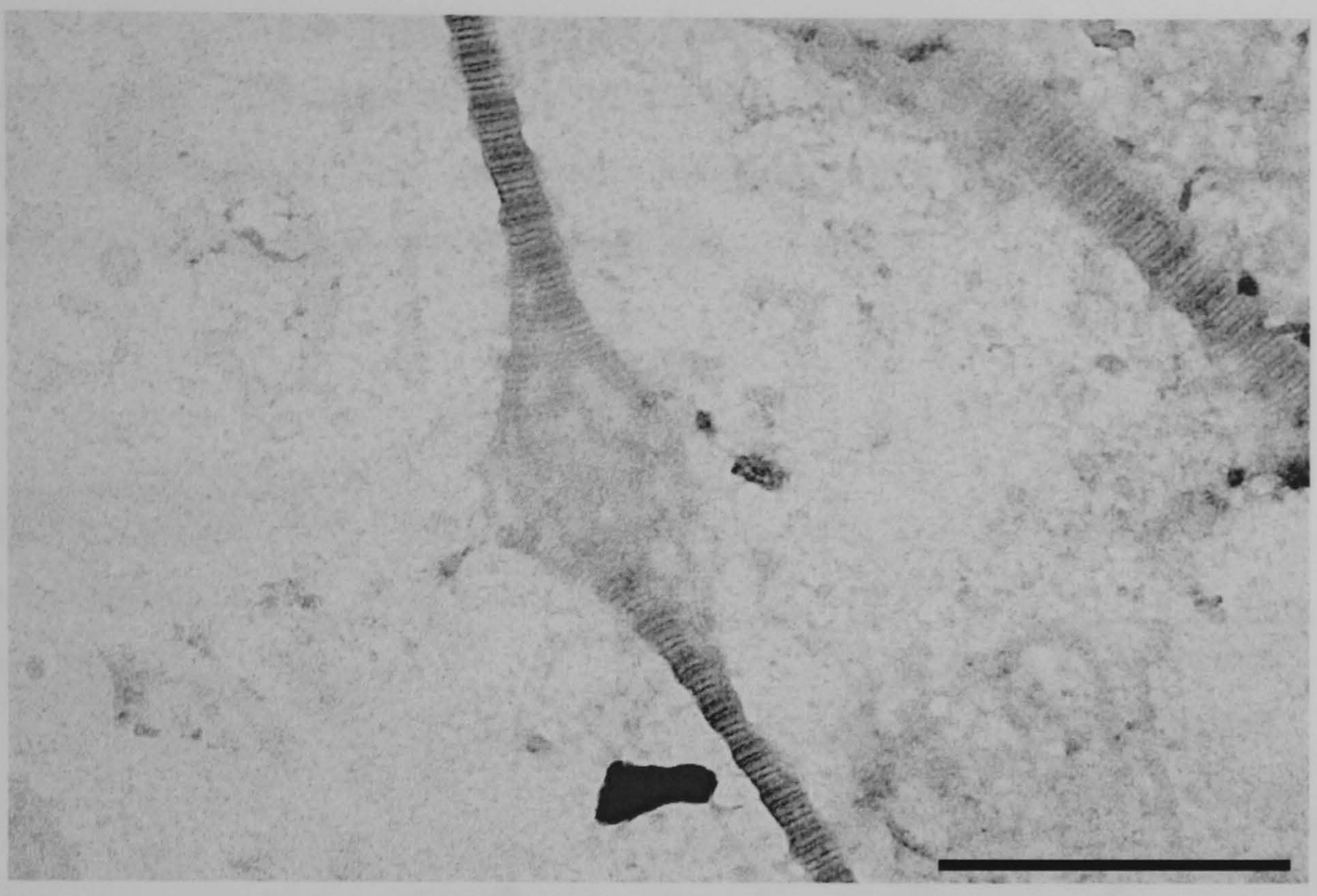


Figure 3.5 A TEM image showing a close up of a *Beaded* region on a heat damaged fibril. The *Beaded* region appears about to separate to form smaller fragments. The scale bar is 500 nm.

3.3.4.5 Graphical representation of TEM data

In the majority of cases the results of TEM analysis are presented on a ternplot. A ternplot is a way of showing the contributions of three components on a single graph; thus it is useful for showing the percentage of *Unaltered*, *Beaded* or *Dumbbell* observed in each sample. An example of a ternplot is shown in Figure 3.6. It has been set up so that a point in the left-hand corner of the triangle would represent a sample containing only *Unaltered* fibrils, a point in the upper corner would contain 100% *Beaded* fibrils and a point in the right-hand corner would contain 100% *Dumbbells*. Each point represents the mean value from two analyses of 100 fibrils unless otherwise stated.

3.3.4.6 Image capture method and measurements

Both a plate camera and a digital camera were used for this analysis. The photographs (taken using Kodak 4489 film) were much clearer than the digital images, but are more expensive and time consuming to produce, therefore in general digital images were taken using the CCD camera. Where measurements were taken of fibril dimensions an AnalySiS software program was used. The

software is linked to the TEM operating system therefore measurements could be obtained directly from digital images. Each periodicity value is based on the average measurement taken over 10 d-spacings. Each diameter value is based on the average of five diameter measurements taken along the fibril. Measurements are given to the nearest nm.

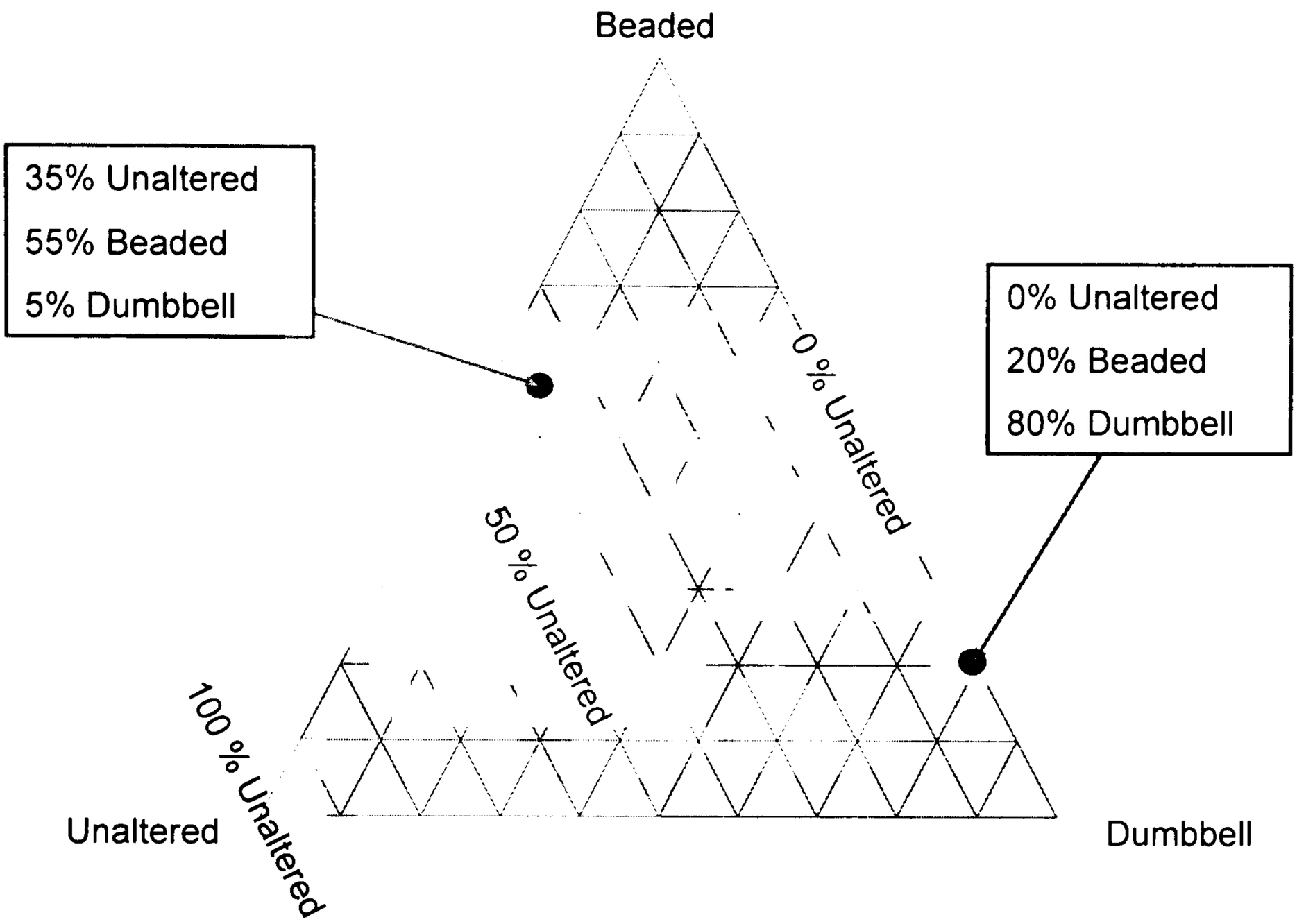


Figure 3.6 A diagram showing how the proportion of *Unaltered*, *Beaded* and *Dumbbell* type collagen fibrils, obtained from the TEM analysis, would be depicted on a ternplot as a single data point.

3.3.5 Differential Scanning Calorimetry (DSC)

3.3.5.1 Function of DSC

DSC measures the temperatures and heat flow associated with transitions in materials, as a function of time and temperature. The technique provides qualitative and quantitative information about physical and chemical changes that give out or take in heat. In basic terms two pans are heated at a constant rate, one pan contains the sample the other pan is empty and acts as a control. As the temperature increases the DSC measures the amount of energy required to keep both pans at the same temperature. If a transition occurs energy will be given out (exothermic) or taken in (endothermic) by the sample. Energy must then be supplied to one of the pans in order for the temperature of both pans to be made

the same once again. The energy that is supplied into the system is recorded and an enthalpy trace is produced. Collagenous materials are particularly suited to this technique because when heated collagen goes through a large endothermic transition. The temperature at which this peak takes place, sometimes referred to as the temperature of denaturation T_d, represents the unravelling of the collagen triple-helix to random coil gelatin.

3.3.5.2 DSC sample preparation

Demineralized or non-demineralized DSC samples were soaked in PBS (pH 7.0) for 24 hours prior to analysis to ensure that the collagen was fully hydrated. 10-20 mg of sample was then blotted dry with filter paper before being placed into the DSC pan. Depending on the heating regime aluminium (Al) or medium pressure steel pans were used (Mettler Toledo, Leicester, UK). The DSC pan and lid was first weighed without the sample then again once the sample was sealed into the pan.

3.3.5.3 DSC operation

The pans were heated using different temperature regimes between 15-195°C and a heating rate of 5°C min^{-1} in a DSC822e differential scanning calorimeter (Mettler Toledo, Leicester, UK) fitted with a nitrogen gas intracooler, using a sealed empty pan as a reference. All of the DSC analyses were run at the British Leather Sellers Centre, Northampton University

3.3.5.4 DSC analysis method

The onset temperature (the point at which the trace begins to deviate from the baseline), peak max (the apex of the peak), and peak end (the point at which the trace flattens out to the baseline after the peak), for each endotherm were measured using Mettler STARe integration software where possible, or else manually using a print out of the thermal scans. All samples were run at least in duplicate to ensure reproducibility.

3.4 Method Optimization

3.4.1 Determining the optimum length of time required for complete demineralization using EDTA and HCl

3.4.1.1 Materials

In this section two experiments are described. In the first experiment the aim was to determine the amount of time required to obtain fully demineralized collagen based on the macroscopic and microscopic appearance of the bone collagen. In the second experiment the efficiency of acid and EDTA demineralization approaches were compared using TEM analysis and % Ca determinations from four different modern compact bone specimens. Details of the bone samples used in these experiments are shown in Table 3.1.

Table 3.1 Details of the modern compact bone samples used for the demineralization experiments.

Species	Skeletal element	Age-at-death	Post-mortem history
bovine	femur	1.5 years	Obtained from a butcher, stored in a freezer for 4 years
bovine	femur	7 years	Obtained fresh from an abattoir
porcine	tibia	unknown	Obtained fresh from a butcher
human	femur	16 years	See section 3.2.2.2

3.4.1.2 Methods

Bone shards (≤ 3 mm largest dimension) were prepared from each sample using the method described in section 3.3.1.

Experiment 1

Only bone shards from the older cow (seven years old) were used in the first experiment. Seven sets of shards were demineralized using the HCl method described in section 3.3.2.1. There was one exception to the method; shards were removed after periods of 1, 3, 5, 7, 10, 14 and 21 days. The HCl demineralizing solution was changed every seven days. After the demineralization period, the appearance and texture of the bone shards was

noted. After the shards had been washed they were prepared for TEM analysis using the preparation method described in section 3.3.4.2.

Experiment 2

In the second experiment 12 sets of bone shards (60 mg) from each of the specimens were used. Two paired sets of shards for each specimen were demineralized in 0.6 M HCl and in 0.1 M EDTA using the standard methods (see section 3.3.2.1 and 3.3.2.2 respectively). One sample from each pair would be used to determine the amount of residual apatite in the insoluble fraction after demineralization and the appearance of the other sample would be determined by TEM. Each pair was removed after different durations. The acid demineralized samples were removed after 3, 5 and 14 days. EDTA is a slower process therefore longer demineralization periods were used; 7, 14 and 21 days. Demineralization solutions were changed every seven days. After each time interval a pair of shards from each specimen was removed. The demineralization solution (HCl or EDTA) was removed and the shards were washed twice with ultra pure water. 2 ml PBS pH 7 was added and the samples were left overnight at 4°C, and stirred on a Roller Mixer. The next day the PBS was removed from one of the pair of demineralized shards and these were rinsed again with ultra-pure water and then dried for 24 hours using a centrifugal evaporator. The shards were weighed and then prepared for Ca analysis (see section 3.3.3 for details). The second set of demineralized shards was prepared for TEM analysis.

3.4.1.3 Results and discussion

Experiment 1

Observations

Based on macroscopic appearance (see Table 3.2), the bone shards appeared to be only partially demineralized after one day of acid treatment. The shards could not be homogenized into solution and therefore could not be prepared for TEM analysis. By three days the shards had a rubbery texture and were semi-translucent, suggesting that the majority of the mineral had been removed. However, normally after homogenizing of a demineralized or non-mineralized collagen sample, the collagen is suspended within the stain and the solution appears cloudy. After the three day sample had been homogenized large sharp-edged fragments could be still be observed in the solution. This suggests that a large proportion of the collagen fibrils were not liberated into the solution and

therefore would not be transferred to the TEM grid. This could bias the TEM analysis by not providing a true representation of the sample. By five days the bone collagen shards could be homogenized without difficulty and appeared to be fully demineralized.

Table 3.2 General observations of the macroscopic and microscopic of bovine bone collagen at different periods of acid demineralization.

Duration of HCl demineralization (days)	**Macroscopic appearance**	**Microscopic appearance**
1	Opaque with a hard rubbery texture	Not observable
3	Semi-translucent and a stiff rubbery texture when squeezed with forceps	Some mineralized (?) fibrils are present; fibrils are mainly demineralized with varying degrees of swelling
5	Translucent and a stiff rubbery texture when squeezed with forceps	All fibrils appear to be demineralized; swelling is variable
7	Translucent; thicker shards still had a stiff rubbery texture but most of the shards were flexible when squeezed with forceps and tended to split into sheets.	All fibrils appear to be demineralized; approximately half of the observed fibrils exhibit some degree of swelling.
10	Translucent; thicker shards still had a stiff rubbery texture but most of the shards were flexible when squeezed with forceps and tended to split into sheets.	All fibrils appear to be demineralized; approximately 80% of the observed fibrils exhibit some degree of swelling
14	Translucent; thicker shards still had a stiff rubbery texture but most of the shards were flexible when squeezed with forceps and tended to split into sheets.	All fibrils appear to be demineralized; all fibrils are swollen and the swelling is uniform along the fibrils.
21	Translucent; all shards are very flexible when pressed with forceps.	All fibrils appear to be demineralized; all fibrils are swollen and the swelling is uniform along the fibrils.

TEM analysis

After three days of acid demineralization the collagen exhibited a mixture of damaged and undamaged fibrils, some of which were partially swollen. Of the

damaged fibrils; there was a larger proportion of *Dumbbells* than *Beaded* fibrils. Possibly this is a result of homogenizing collagen fibrils that still contained a portion of mineral which could have caused shearing. Also some of the observed fibrils appeared still to be mineralized. Figure 3.7 shows two examples of these; they are very dark (electron dense) and irregular 'fuzzy' edges. The fibrils bear a resemblance to the TEM image of mineralized bone collagen fibrils from Kadler (1994), shown in Figure 1.12.

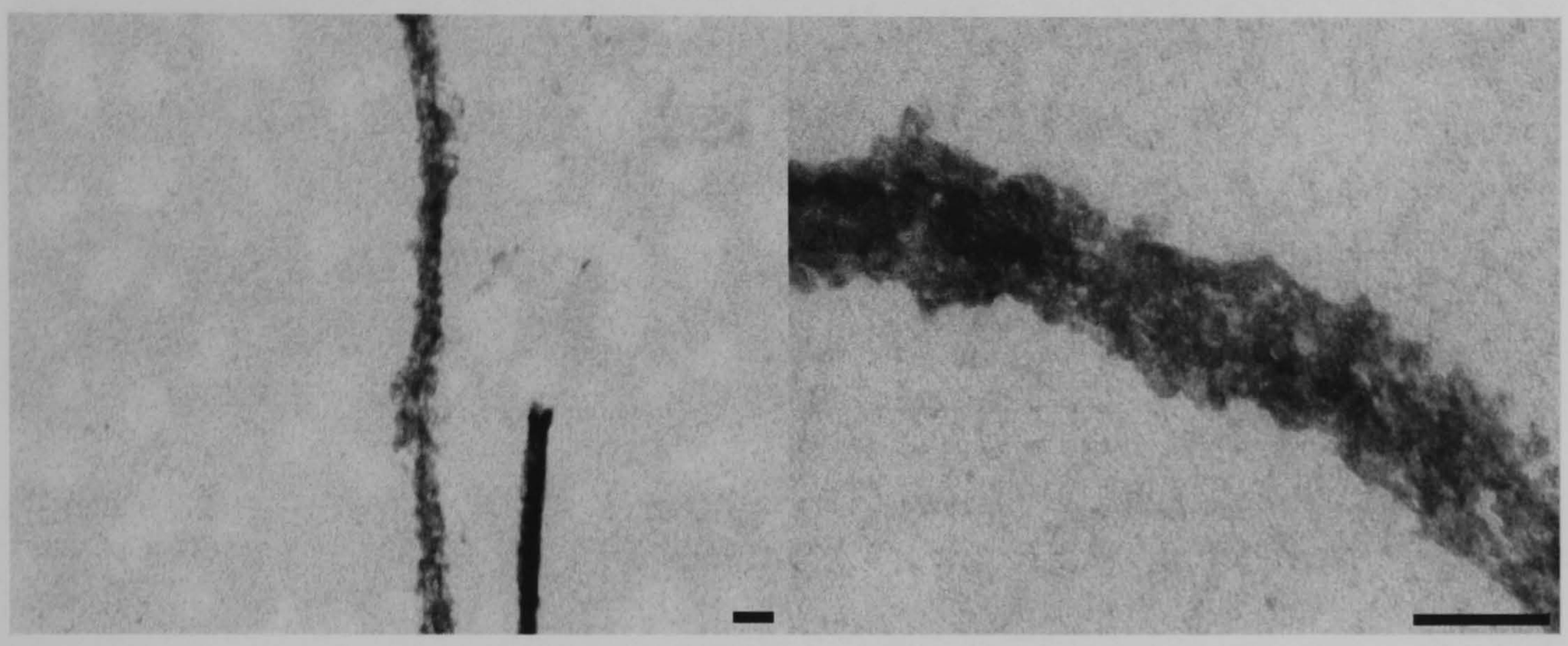


Figure 3.7 TEM images of probable mineralized collagen fibrils, taken from a bovine bone sample which had been demineralized in HCl for three days. The scale bars are 100 nm.

After both five days and 10 days of acid demineralization the collagen fibrils appeared to be fully demineralized but exhibited varying degrees of swelling; both between fibrils, and along the same fibril. This often made it difficult to distinguished *Beaded* alteration from simply uneven swelling. By fourteen days the swelling was much more uniform; over 90% of the fibrils were swollen. In these fibrils the diameter was approximately 2.5 times that of the non-swollen fibrils observed after three days of demineralization (Figure 3.8). The swollen fibrils had a uniform diameter along their length and appeared to be less electron dense than the non-swollen fibrils. There was no difference in the appearance of the collagen fibrils which had been demineralized in HCl for fourteen and twenty-one days.

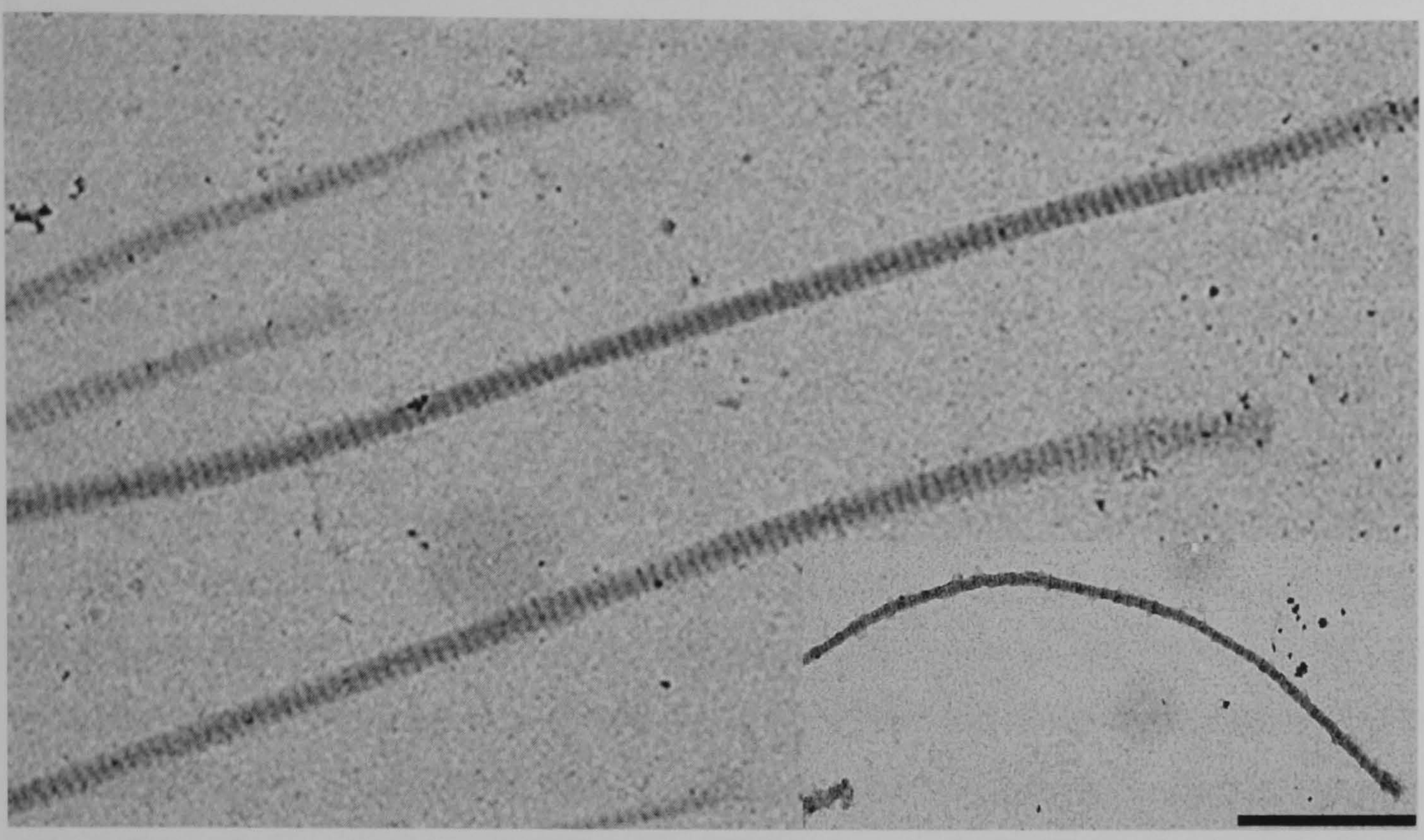


Figure 3.8 Two TEM images of undamaged fibrils showing the effects of acid swelling, the inset is a fibril is from the 3 day demineralized bovine bone sample, the swollen fibrils are from the 14 day sample. The scale bar is 1 μm and is the same for both images.

Experiment 2

The samples which had been demineralized in EDTA for seven days were not prepared for TEM analysis or Ca analysis because, based on the macroscopic appearance of the shards, they were not sufficiently demineralized. The shards were white, hard and could not be cut with a dissection knife. Also the young (18 month old) bovine bone proved problematic to prepare because all of the acid demineralized samples from this specimen were extremely degraded. After just three days of demineralization there was very little collagen present on the grid. There was some amorphous grey material, which was most probably gelatin, the remaining collagen fibrils were mainly in a *Beaded* or *Dumbbell* state and all were swollen. After EDTA demineralization of this sample, there were again very few fibrils observed on the grid and all of the fibrils were in a *Beaded* or *Dumbbell* state. As a result the young bovine bone specimen had to be removed from the study. It is not clear why the collagen from this specimen was so degraded.

Calcium determinations

The elemental analysis of the calcium content showed that in the HCl demineralized samples all but approximately 5% of the Ca was removed after just three days of treatment but this residual Ca remained within the sample even

after 14 days. In the EDTA treated samples less than 0.1% Ca was present after 14 days. Interestingly there appears to be a correlation between the residual Ca found in the samples after demineralization and the theoretical amount of Ca which binds to the two most abundant proteins in bone; collagen and osteocalcin (OC). The bar chart in Figure 3.9 shows the % of Ca remaining in the samples and the predicted Ca bound to the collagen and osteocalcin. This predicted value is based on the amount of carboxyl groups (Asp and Glu, Gla) available to bind the Ca. In this calculation it is assumed that two carboxylic acid groups are required to bind a single divalent cation (Ca^{2+}) except in the case of Gla, a dicarboxy residue. Estimates were made of the number of Asp (88) and Glu (154) in type I bovine collagen and osteocalcin (three Gla, five Asp and two Glu) (based on *Swiss-Prot*) assuming that all the Ca bind to the two most abundant proteins and there is no preferential loss of collagen over osteocalcin. Based on this calculation, it is suggested that with EDTA demineralization all of the Ca is removed from the collagen and all but the dicarboxy groups of osteocalcin are stripped of Ca; whereas HCl treatment does not remove all of the Ca from the Collagen.

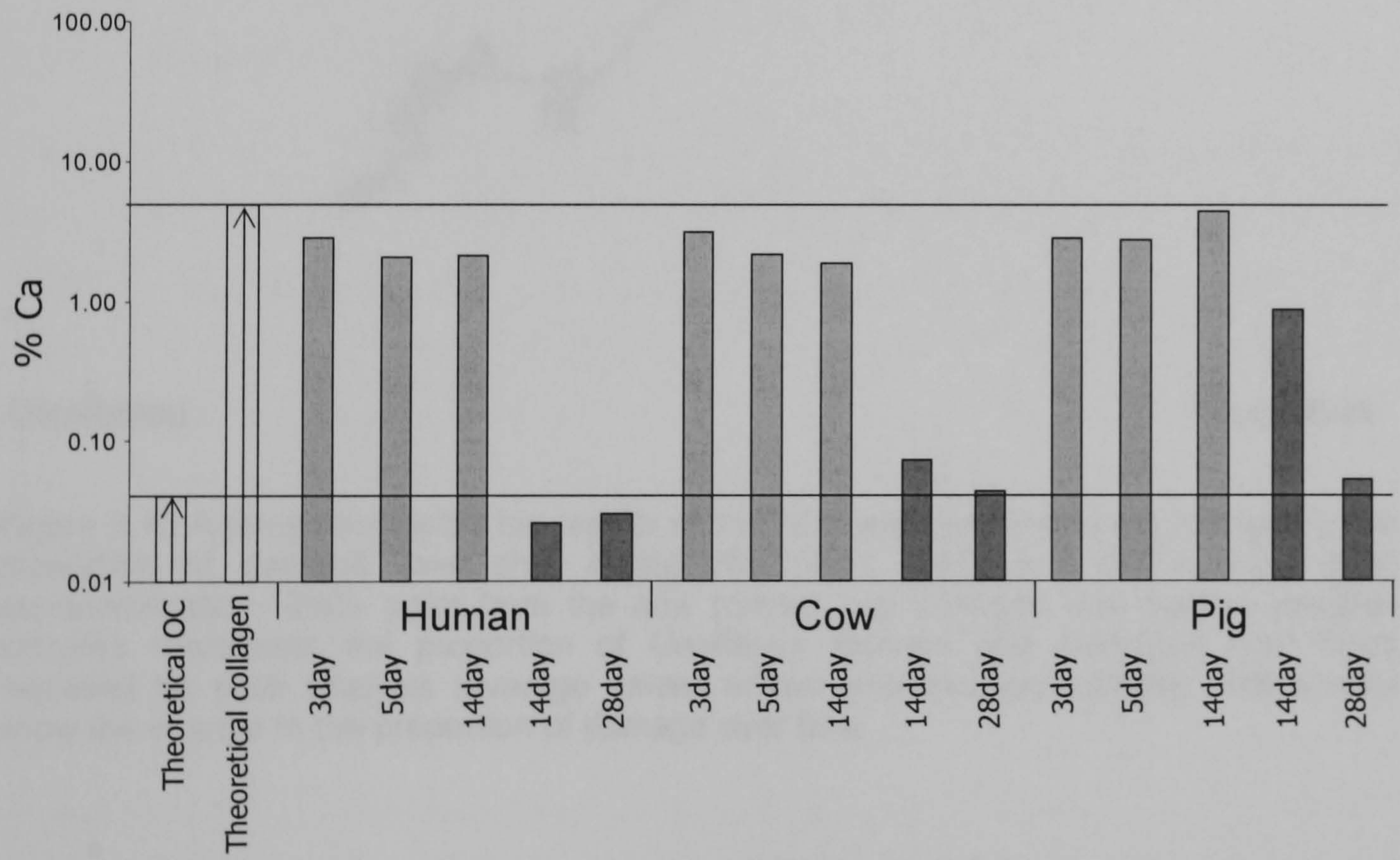


Figure 3.9 bar chart showing the % Ca remaining after demineralization in 0.6 M HCl (light grey) or 0.1 M EDTA (dark grey) for different durations. The lines represent the theoretical amount of Ca which binds to OC and collagen.

TEM analysis

Figure 3.10 is a ternplot showing the results of the TEM analysis in which the HCl and EDTA demineralization methods were compared. The arrows on the ternplot show the change in the proportion of *Unaltered*, *Beaded* and *Dumbbell* type fibrils observed over time; they point from the five to the 14 day HCl demineralized samples, and the 14 to 21 day EDTA demineralized samples (the three day HCl demineralized samples were not included because they appeared to still be partially mineralized).

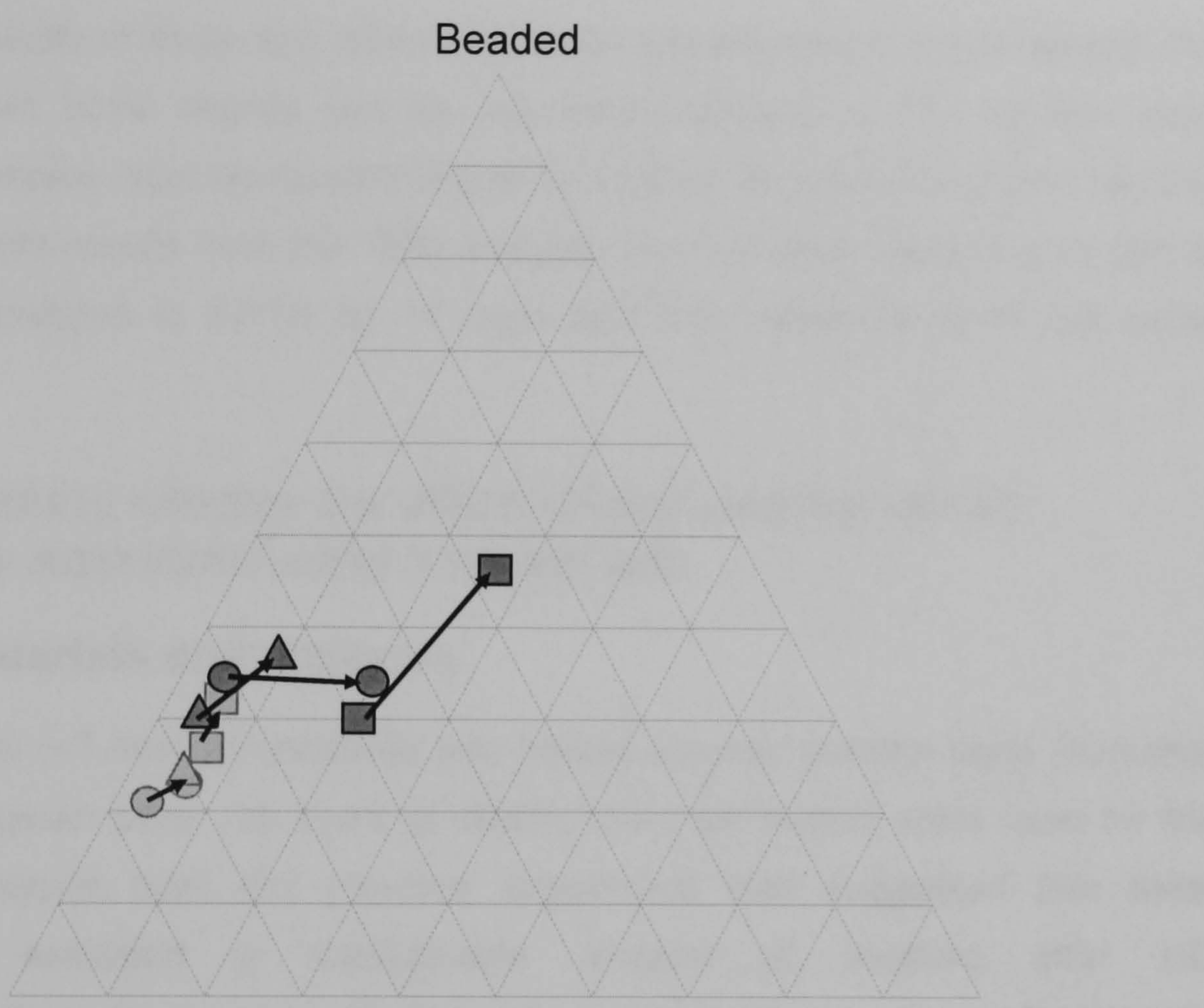


Figure 3.10 A ternplot showing the results of the TEM analysis comparing changes in the proportion of damage over time during HCl (dark grey) and EDTA (light grey) demineralization. Each point from the cow (circle), pig (triangle) and human (square) samples represents the proportion of *Unaltered*, *Beaded* and *Dumbbell* type fibrils recorded for each analysis (average values of two analyses per sample). The arrows show the change in the proportion of damage over time.

Those which had been demineralized in EDTA exhibited the least amount of damage. There was also almost no difference between samples which had been treated with EDTA for 14 and 21 days, and the fibrils did not exhibit any swelling. By comparison, and as expected, the HCl demineralized samples exhibited significant swelling. The porcine and bovine samples which had been demineralized in HCl for five days displayed a mixture of swollen and non-swollen

fibrils and the degree of swelling often varied along the same fibril. In the young human sample all of the fibrils were uniformally swollen after just five days. In the porcine and bovine samples over 90% of the fibrils exhibited uniform swelling after 14 days of acid demineralization. The other 10% were completely non-swollen. In all of the acid treated samples there was an increase in the proportion of *Beaded* and *Dumbbell* type fibrils between five and 14 days of treatment and this was most dramatic in the young human bone sample.

3.4.1.4 Summary

From the results of these two demineralization experiments it would appear that small modern bone shards can be fully demineralized in HCl by five days, however samples must be demineralized for 14 days to produce uniform swelling and consistent results from the TEM analysis. On the other hand shards can be fully demineralized in EDTA by 14 days and this treatment does not cause swelling.

3.4.2 Assessing whether the effects of acid swelling can be sufficiently suppressed using a neutral salt

3.4.2.1 Materials and methods

Small shards (<3 mm) of unheated and heated modern porcine bone (mid-shaft tibia) and human bone (16 years at death, mid-shaft femur) were used for this analysis. Results from the previous experiment had suggested that these specimens exhibited a considerable amount of swelling after HCl demineralization. To prepare the heated samples, 60 mg of shards for each sample were placed in a glass tube and heated on a heating block (Grant UBD) for one hour at 80°C. After the heating period the tube was quenched in an ice bath and the sample was then stored at 4°C prior to analysis. The samples were demineralized in 0.6 M HCl for 14 days using the standard method described in section 3.3.2.1, with the following modification; the 0.6 M HCl solution was prepared using 2 M sodium chloride (Analytical reagent grade, Fisher Scientific UK Ltd.) rather than ultra-pure water. Samples of the heated and unheated porcine and human bones were also demineralized using the standard HCl demineralization technique (without salt). Once the samples had been demineralized they were prepared for TEM analysis (see section 3.3.4.2 for preparation method).

3.4.2.2 Results and discussion

The samples which had been acid demineralized in the presence of salt exhibited less swelling. In general, samples which had been treated with salt displayed – except in damaged regions – a mixture of completely non-swollen and partially swollen fibrils (Figure 3.11). The results of the TEM analysis are presented in a stacked bar chart in Figure 3.12. By comparing the paired samples (with and without salt) it can be seen that samples with added salt displayed more *Unaltered* fibrils and fewer *Dumbbells*.

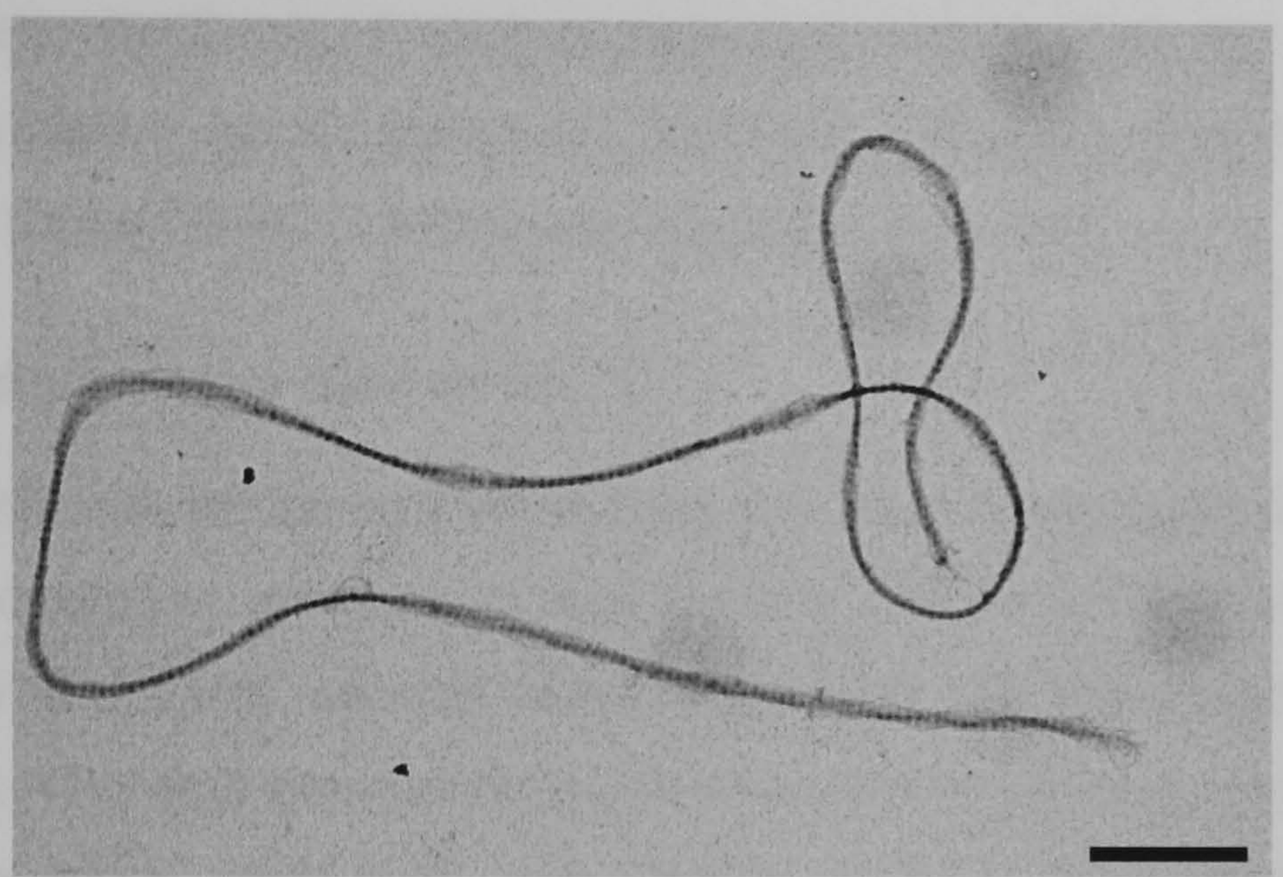


Figure 3.11 TEM image showing a partially swollen fibril from the pig bone sample. The sample had been demineralized in HCl with salt added to the demineralization solution. The scale bar is 1µm.

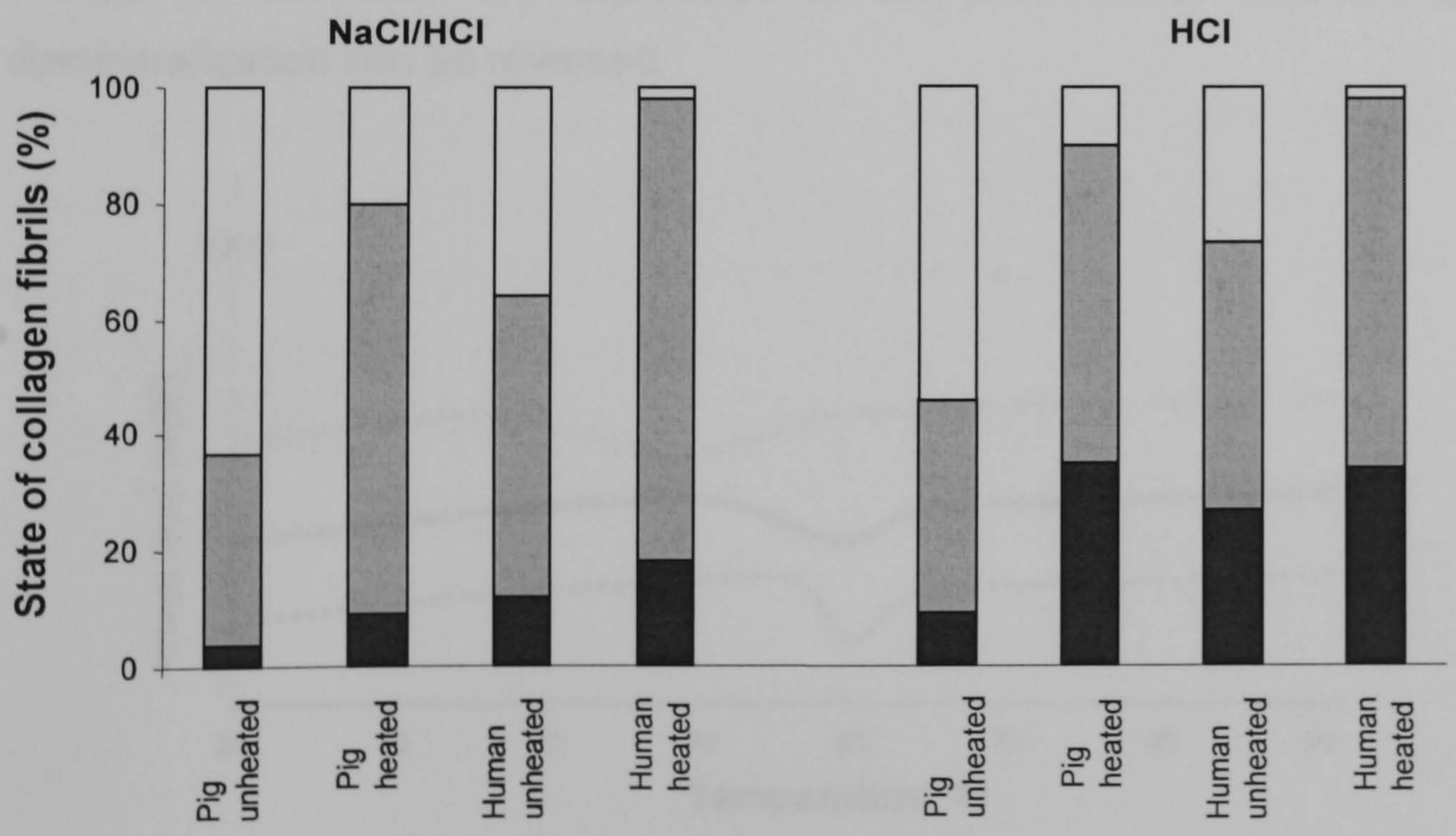


Figure 3.12 A stacked bar chart comparing the proportion of *Unaltered* (white), *Beaded* (grey) and *Dumbbell* (black) type fibrils recorded from unheated and heated bone samples after demineralization in HCl with (left) and without (right) added salt (the values are the average from two analyses per sample).

3.4.3 Preparing samples for DSC analysis

3.4.3.1 Materials and methods

Small shards (<3 mm) of unheated modern bovine bone (mid-shaft femur) were used for this analysis. Two samples were prepared using the HCl demineralization method and one sample using EDTA (see sections 3.3.2.1 and 3.3.2.2 respectively). Prior to DSC analysis one of the HCl demineralized samples was placed in PBS and stirred on a Roller Mixer for 24 hours. The other two samples were simply rinsed with de-ionized water (following Nielsen-Marsh et al. (2000a)). 10-20 mg of each sample was then blotted dry with filter paper before being sealed into an aluminium DSC pan. The pans were heated using the temperature range 15-95°C and a heating rate of 5°C min^{-1}.

3.4.3.2 Results and discussion

The HCl demineralized sample which had only been rinsed prior to DSC analysis, showed a broad peak with a peak max at 49°C. This is 11°C lower than the peak observed for the same sample which had been demineralized in EDTA (see Figure 3.13). The acid demineralized sample which had been left in PBS for 24 hours displayed a peak that was slightly broader than that of the EDTA demineralized sample but it did occur at close to the same temperature. This means that simply by placing an acid demineralized bone collagen sample in pH 7 PBS for 24 hours the depression of the endothermic transition by acid demineralization can be reversed.

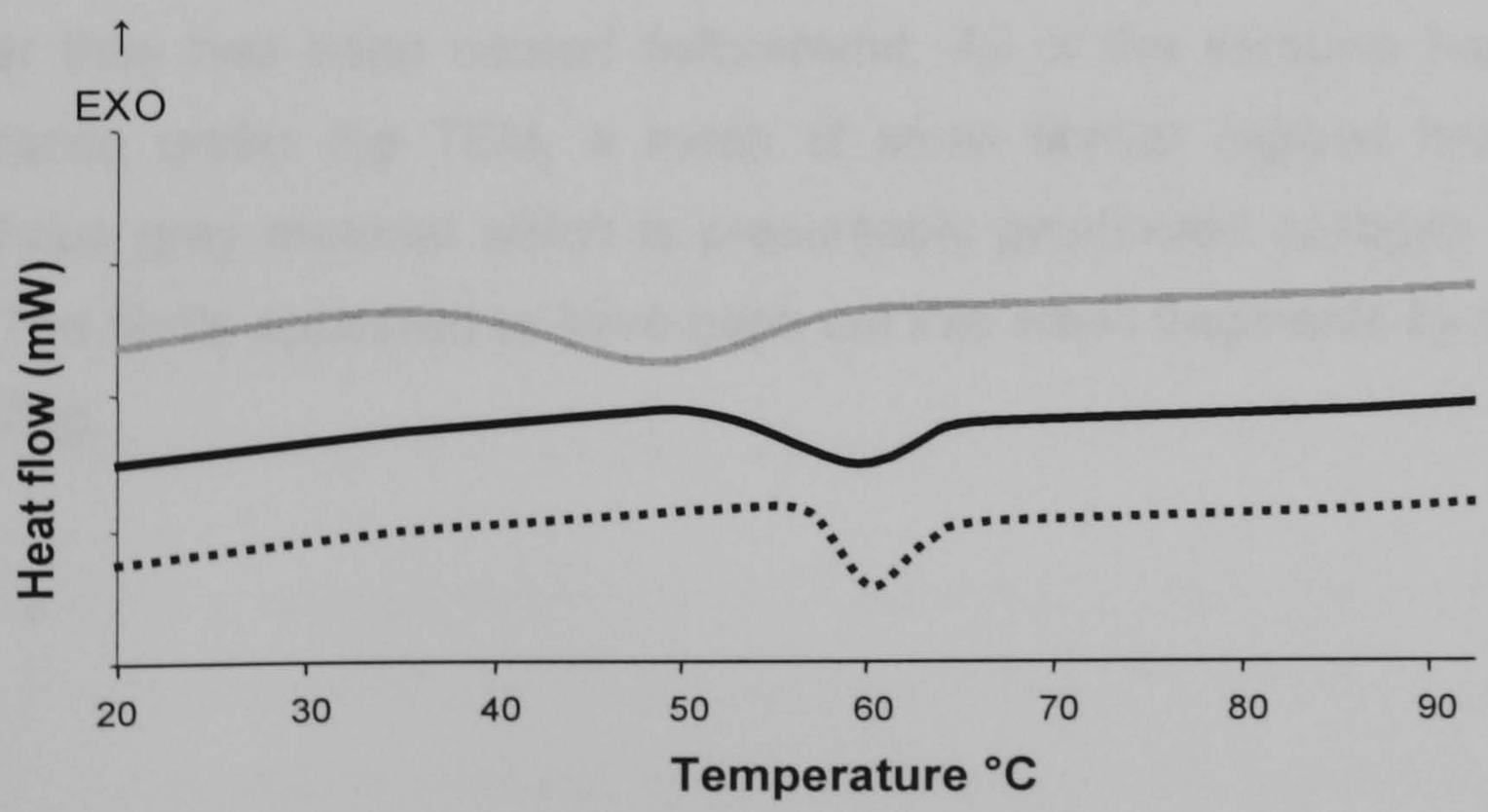


Figure 3.13 A combined DSC trace showing the modern bovine bone which as been demineralized with A) HCl followed by a rinse with deionized water (grey line) B) HCl followed by a rinse with deionized water and 24 hours in PBS (black line) C) EDTA followed by a rinse with deionized water (dotted line). The DSC traces are to the same scale but have been offset along the y-axis so that they can be viewed more easily.

3.4.4 Assessing the viability of powdering bone samples to speed up EDTA demineralization

3.4.4.1 Materials and methods

Both a heated and an unheated bone sample were used for the experiment to determine if bone that has already been thermally altered would be more susceptible to mechanical damage induced by powdering. Large shards (<1 cm and >3 cm) of unheated and heated modern bovine bone (mid-shaft femur) were used for the analysis. The heated shards had previously been placed in a glass tube and heated on a heating stage for one hour at 80°C then stored at 4°C prior to analysis. Bone shards from the specimens were broken into smaller dimensions (<3 mm) using a hammer, then a 60 mg sample of the shards was placed into small metal spex-mill tubes. The samples were then pre-cooled in liquid N_2 for five minutes, and then pulverized for two minutes before cooling for a further two minutes. This cycle was repeated three times. Following this procedure the resulting powders were removed and placed in universal tubes with 15 ml 0.1 M EDTA and demineralized for 48 hours (based on Collins and Galley's (1998) figures for complete removal of Ca from powdered bone). After demineralization the samples were prepared for TEM analysis using the method described in section 3.3.4.2. This procedure was carried out in duplicate per sample.

3.4.4.2 Results and discussion

This procedure caused significant alteration to the collagen fibrils regardless of whether they had been heated beforehand. All of the samples had the same appearance under the TEM; a mesh of small fibrillar regions held within an amorphous grey material which is presumably gelatinized collagen (see Figure 3.14). The fibrils appeared to have been cut into small fragments by the action of powdering.

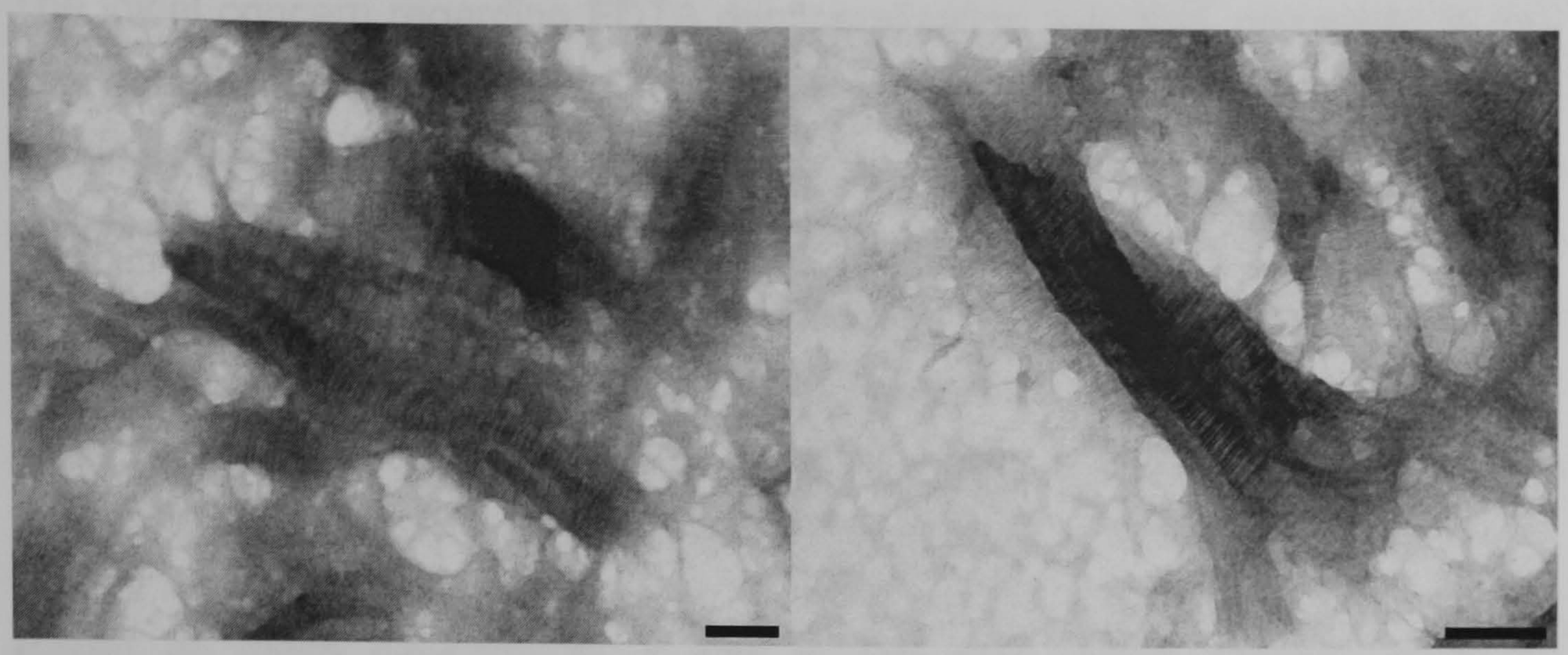


Figure 3.14 TEM images of collagen from bovine bone which, prior to demineralization, had been powdered under liquid nitrogen using a spex-mill. The scale bars are 200 nm.

3.5 Conclusions from the method optimization experiments

The aim of these experiments was to address the concerns outlined at the beginning of the chapter and find the best way to prepare demineralized bone collagen samples for TEM and DSC analysis. The demineralization experiments confirmed that HCl removes mineral more rapidly than EDTA; the majority of the mineral was removed after three days by acid treatment, but bone shards were still highly mineralized after seven days of EDTA demineralization. However, treatment with HCl caused the fibrils to swell. The uneven swelling along fibrils made it difficult to identify damaged fibrils. As a result it was found that samples would have to be demineralized in HCl for 14 days until the swelling within the collagen fibrils was uniform. Introducing salt into the HCl demineralization solution did reduce the swelling but was not completely successful, resulting in uneven swelling along the fibrils. As a consequence of these problems, EDTA treatment proved to be the better method for sample preparation for TEM analysis. The advantage of EDTA was that the fibrils were not swollen and displayed less damage than after HCl treatment.

Powdering bone to improve the speed of EDTA demineralization was not a good option. Powdering introduced visible damage to the collagen fibrils, yielding short fibril fragments and amorphous gelatinous material; this damage could explain the dramatic increase in the solubility of demineralized powders (14%) compared to shards (2%) that was observed by Collins and Galley (1998).

The initial concern regarding EDTA demineralization was that, compared to HCl demineralization, it is a very slow process and may not provide complete demineralization. Incomplete demineralization is not desirable as it may either (i) obscure or hide damage; or (ii) create damage; by shearing fibrils during homogenization. However the demineralization experiments revealed that even though EDTA demineralization takes 14 days, a similar time is required for HCl in order to reduce the inconsistencies seen after shorter demineralization times, and *more* Ca (99.9%) was removed after EDTA treatment.

Based on these experiments, it was evident that usable collagen could be produced from bone shards demineralized for 14 days with either HCl or EDTA. However for examining heat damaged fibrils under the TEM, EDTA would be the method of choice as damage was clearly visible and altered and unaltered fibrils were easily distinguishable. HCl swelling has the advantage that differences in the stability of collagen fibrils between samples can be identified. For instance the young human samples exhibited a greater degree of swelling and a greater proportion of *Beaded* and *Dumbbell* altered fibrils than the porcine and older cow samples after HCl treatment. For DSC analysis again it was decided that both methods of demineralization could be used, but it is necessary to raise the HCl treated collagen to neutral pH, by placing rinsed collagen samples in PBS for 24 hours prior to analysis.

Chapter 4 An examination of the denaturation transitions in collagen at the fibrillar level

4.1 Introduction

Collagen is the most abundant protein in the animal kingdom and has been exploited for its material properties since antiquity. The material properties and their changes as a consequence of modification (e.g. tanning, aging and glycation) and deterioration (e.g. exposure of historic parchments to acidic pollution), have resulted in a range of analytical approaches and terminologies.

The most common investigations of the material properties of collagen have been conducted by **leather chemists**. Principally their interests relate to the ways in which leather procurement and treatment will influence the physical and material properties of the product. Chief amongst these investigations has been the way in which tanning enhances the hydrothermal stability of collagen by increasing the denaturation temperature. Typically in these studies the temperature of a fully hydrated collagen specimen is raised and the change in the enthalpy of the sample is monitored using DSC; denaturation is assumed to occur at a peak in the enthalpy trace (e.g. Bosch et al. (2002), Chahine (2000) and Kronick and Buechler (1986)). Also at the same temperature there is a macroscopic shrinkage observable by using a microscope and hot table or by using hydrothermal isometric tension, in which one monitors the force needed to maintain a tissue at a fixed dimension; denaturation is assumed to occur when there is a sudden increase in the force (Haines, 1987).

Biologists have also often concentrated on processes which alter the physical properties of collagen; for instance as a result of age related changes. With age there is an increase in both enzymatic and non-enzymatic cross-links. The effects of these cross-links on the material properties of biological materials have been subject to much investigation (again using techniques such as DSC (e.g. Flandin et al. (1984) and Mentink et al. (2002)), and hydrothermal isometric tension (e.g. Lee et al. (1995) and Zioupos et al. (1999)). At a molecular level age related changes have also been investigated in the amino acid profile (i.e. the extent of amino acid racemization as a function of biological age and remodelling in bone, e.g. Gineyts et al. (2000) and Ritz-Timme and Collins (2002)).

The principal collagen based material studied by **conservation scientists** has been parchment (prepared from skin). In this instance the main concern has been with the deterioration of collagen as a consequence of time and pollution. Again this has been studied both at a material level (by examining the decline in both shrinkage and endothermic transition temperature, e.g. Larsen et al. (2002) and Chahine (2000)). But also at a molecular level by monitoring triple helical collapse (i.e. by Raman spectroscopy, e.g. Garp et al. (2002) or x-ray diffraction, e.g. Wess and Nielsen, (2002)). **Archaeologists** are also interested in collagen degradation but in this case the aim is to obtain an uncontaminated sample of ancient bone collagen for either ^{14}C dating or stable isotope analysis (Ambrose, 1990; DeNiro and Weiner, 1988a; DeNiro and Weiner, 1988b; Hedges and Law, 1989). In this field research has focused upon understanding collagen decay as a means of improving the isolation of a consistent collagen fraction (e.g. by gelatinization and ultra-filtration). Studies have tended to concentrate on the molecular basis of decay of ancient collagen, adopting a polymer degradation model, originally devised for the preparation of **biomaterials** (Collins et al., 1995).

It is perhaps not surprising that, because collagen degradation and stabilization has been studied by leather chemists at a material level, by archaeologists at a molecular level and by biologists and conservation biologists at both levels, there is confusion over terminology. This has resulted in terminology that fails to differentiate the hierarchical scaling of Type I collagen, from the triple helix, to the fibril and fibre. Terms such as *gelatinization* and *melting* are used to describe the deterioration of acid degraded parchment, the molecular unpacking of soluble collagen and the endotherm observed in fibrillar collagen. The bottom-up focus on the triple-helix and the top-down focus on material properties have also meant that the study of collagen degradation at the level of the collagen fibril has been largely neglected. It is important to consider this level of hierarchy considering the major collagen type found in mammalian tendon, skin and bone is a fibrillar form.

The purpose of this chapter is to gain a clearer understanding of the thermal transitions observed in type I collagen, not only in terms of physical changes as detected by DSC but also to examine microscopic changes in fibril packing during the endotherm, using TEM. DSC is the main technique which has been used to explore the endotherm in collagen and TEM is commonly used to visualize this protein but these two approaches have not previously been used together to

systematically investigate collagen transitions. The chapter is set out as a series of experiments in which the endothermic transitions in tendon, mineralized bone and demineralized bone are investigated. By using both non-mineralized and mineralized collagen over a range of temperatures the aim was to produce a more fully integrated picture of the process of collagen collapse as a result of thermal alteration. These experiments are preceded by a brief overview of the current understanding of heat denatured collagen.

The results of this analysis, although incomplete, have suggested that the terminologies which have previously been used to describe the degradation of fibrillar collagen require refinement.

4.2 Collagen thermal-denaturation

The denaturation of collagen was initially believed to be an equilibrium process in which collagen could be transformed to a native or denatured state by varying the temperature (Flory and Garrett, 1958; Privalov et al., 1979). This view is supported by Beier and Engel's (1966) paper, which reported that 'reformed collagen' could be achieved when gelatinized calfskin collagen was cooled to 24°C. The reformed product had a very similar thermal stability and was highly cooperative and helical as shown by enzyme specific attack of non-helical regions. More recently there has been a debate as to whether collagen denaturation is an endothermic transition resulting from a reversible melting of crystalline regions (Engel and Bachinger, 2000), or in fact a highly temperature dependent, irreversible rate process. The latter view is based on calorimetric studies, which show that the temperature of denaturation is dependent on scan rate (Miles et al., 1995).

The mechanism behind the thermal denaturation of collagen is usually considered at the level of the collagen molecule. Studies from protein chemistry show moderate heating can result in a local unfolding of polypeptide chains, which regain native structure after cooling. This unfolding may be due to the breaking of a small number of consecutive hydrogen bonds (more correctly termed coulombic interactions) that can reform (zipper-like). More severe heating results in the helix to coil transition which is thought to occur through the breaking of longer sequences of hydrogen bonds (Wright and Humphrey, 2002). In collagen this transition is believed to represent the transformation of the triple helical collagen molecule to a random coil gelatin (Flory and Garrett, 1958). This

is detected in collagenous material as a large endothermic transition that is highly temperature sensitive (Ea ~500 kJ mol^{-1}; Miles et al., 1995) and occurs over a narrow temperature range; suggesting that the unfolding is highly cooperative. Miles and Bailey (2001) proposed that the unfolding of the triple helix does not occur at random regions along the triple helix but is initiated in a specific region which is more thermally labile (see section 1.3.3).

For mammalian collagen molecules in solution, this endothermic transition occurs at close to body temperature (37°C; Leikina et al., 2002). For materials such as skin, tendon and demineralized bone collagen, where the collagen molecules are packed into fibrils there is no endotherm at this temperature, but there is a transition at 60-65°C (Miles et al., 1995). This endotherm is assumed to represent the same physical process (unravelling of triple helices), only shifted to a higher temperature as a consequence of a stabilization mechanism involving the fibril structure (Miles and Ghelashvili, 1999) (see section 1.3.7).

In this chapter three separate studies are described, which investigate the endothermic transition in (i) tendon, (ii) mineralized collagen and (iii) demineralized collagen. The results from each study are discussed individually at the end of each section; the implications of all the results are then considered together in a discussion of alternative views of the denaturation of collagen at the end of the chapter.

4.3 Endothermic transition in tendon

The collagen endotherm is usually studied using tendon, therefore in the first combined DSC and TEM investigation bovine Achilles' tendon was used. This tendon is easy to prepare for analysis and when stained produces clear TEM images. In order to make a direct comparison between this commonly recorded endotherm and the observed damage to the collagen fibril structure, the DSC was used as an 'oven' and programmed to increase the temperature at a consistent rate (5°C min^{-1}) but then stopped at key points along the thermal transition; samples were then examined by TEM. The combined DSC and TEM approach was subsequently repeated on rat tail tendon in order to determine if the previous results were a property of tendon in general or specific to bovine Achilles' tendon. To corroborate the TEM analysis small angle x-ray diffraction (SAXS) was also conducted on the Achilles' tendon.

4.3.1 Materials and methods

The Type I insoluble bovine Achilles' tendon and the rat tail tendon were placed in PBS and stored at 4°C overnight prior to DSC analysis (further information detailing the origin and preparation of these materials can be found in section 3.2.3).

A 20-40 mg sample of tendon was placed in an Al pan and run on the DSC using the temperature range 15-95°C and a scan rate of 5°C per minute. This experiment was repeated twice and the position of the peak was identified by calculating the average of the onset temperature, peak maximum and peak end. Four methods were then set up on the DSC; heating up to (i) ten degrees before the transition, (ii) the transition onset, (iii) the peak maximum and (iv) just after the transition, as illustrated in Figure 4.1; each method was run in duplicate. After each run had finished the pans were immediately quenched in an ice bath in order to cool them down as quickly as possible. The samples were then stored (still within the pans) in a refrigerator until they could be processed. Prior to preparation for SAXS and TEM analysis the samples were removed from the Al pans by breaking the seal with scissors and their appearance was noted. The sample preparation for TEM analysis is described in section 3.3.4.2. The SAXS analysis was carried out on beamline 2.1 at the SRS facility at Daresbury, using a 4 m sample-to-detector distance. The samples were prepared and run by Prof Wess and Dr Goh, University of Cardiff using the methodology described in Wess and Orgel (2000)).

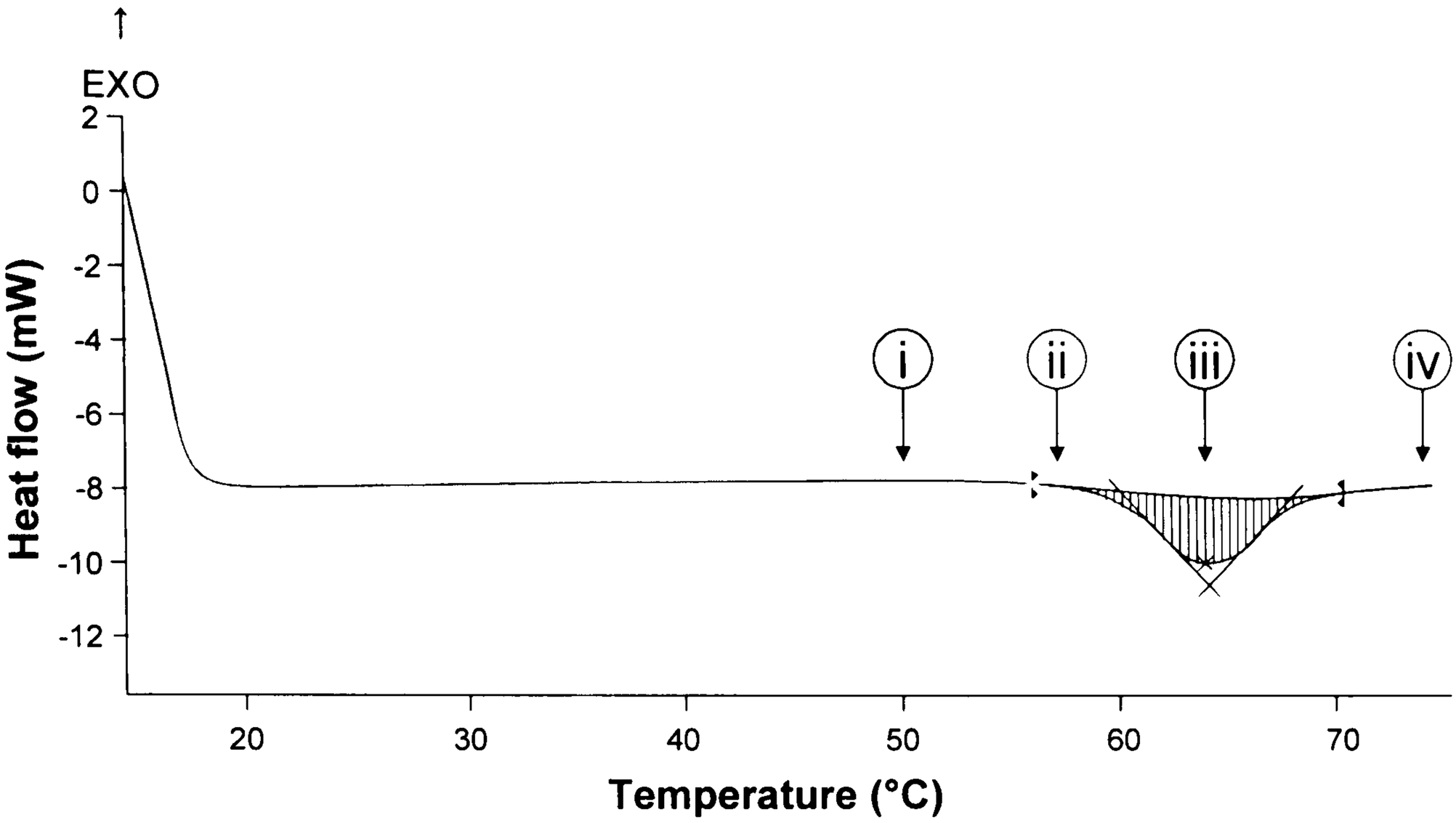


Figure 4.1 A DSC trace from bovine Achilles' tendon showing the points at which samples were analysed by TEM.

4.3.2 Results and Discussion

Both types of tendon behaved in a similar way in these experiments. The endotherm consisted of a single sharp peak. In the case of the bovine Achilles' tendon the onset values were 60.19°C, 59.67 and the peak maximum 63.89°C, 64.05. The rat tail tendon displayed onset values of 59.45, 60.50 and peak maximum values at 63.75, 64.02.

For both types of tendon those samples heated to ten degrees before the transition or those heated to the onset appeared macroscopically unchanged. However samples which had been heated to the peak maximum and to beyond the transition had changed to a clear rubbery mass.

The samples were observed at a microscopic level by TEM. Examples of the appearance of the collagen at different points in the transition are shown in Figures 4.2, 4.3, 4.4 and 4.5. At 10°C below the transition the bovine Achilles' tendon consisted of multiple fibrils of different diameters, all of which appeared unaltered (see Figure 4.2). By 60°C small areas of alteration were evident, where the periodicity of the fibril was lost. By the time the sample had reached the peak maximum the collagen fibrils showed larger regions of damage where the

periodic banding was completely absent, whilst in other regions along the same fibrils the banding was unaltered (see Figure 4.3). In these damaged regions there was at least a two-fold increase in diameter. Beyond the transition (75°C) this damage had spread to the entire fibril and what remained to be observed were no longer tight fibrils with a clearly defined banding pattern but 'strings' of electron dense material (see Figure 4.4). This same collapse of the fibril structure was observed in rat tail tendon (see Figures 4.5).

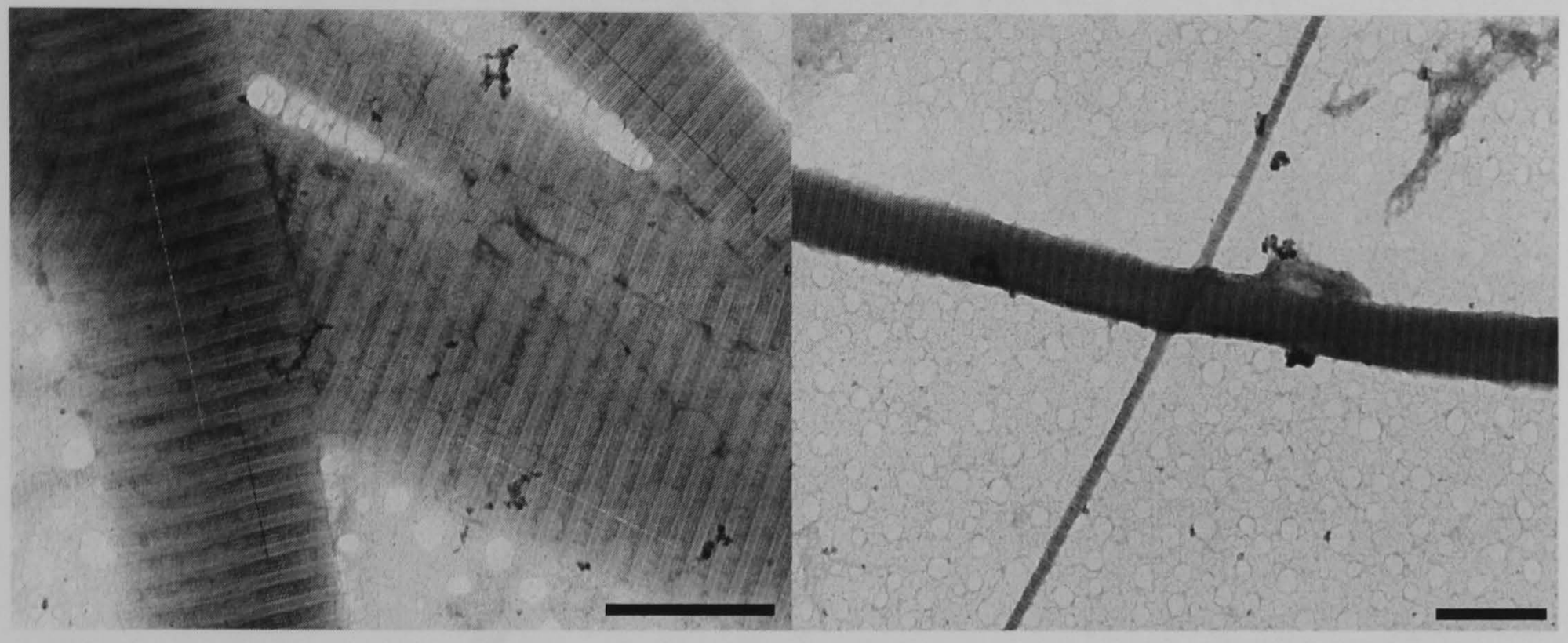


Figure 4.2 TEM images of bovine Achilles' tendon which had been heated to 10°C before the endothermic transition. The scales bars are 500 nm.

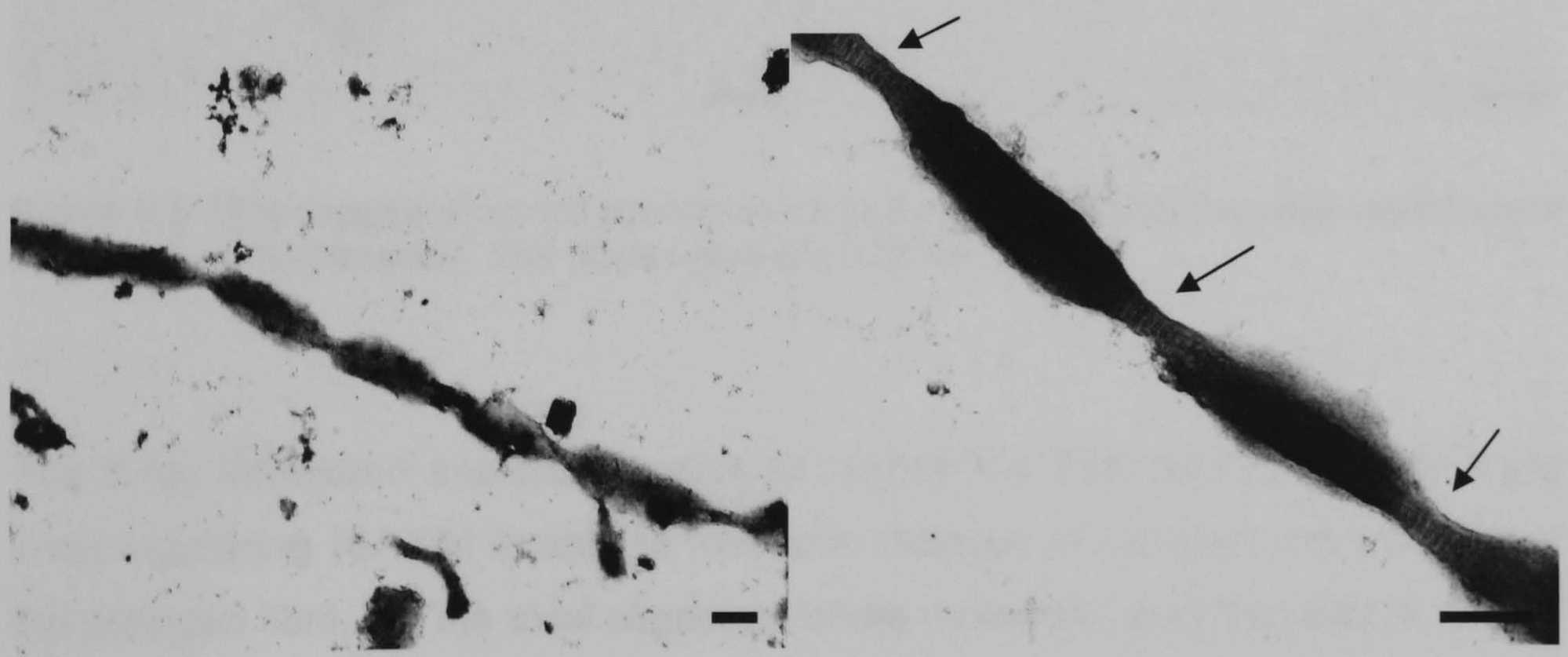


Figure 4.3 TEM images of bovine Achilles' tendon which had been heated to the peak maximum of the endothermic transition. The arrows point to regions where the banding pattern is still intact. The scales bars are 200 nm.

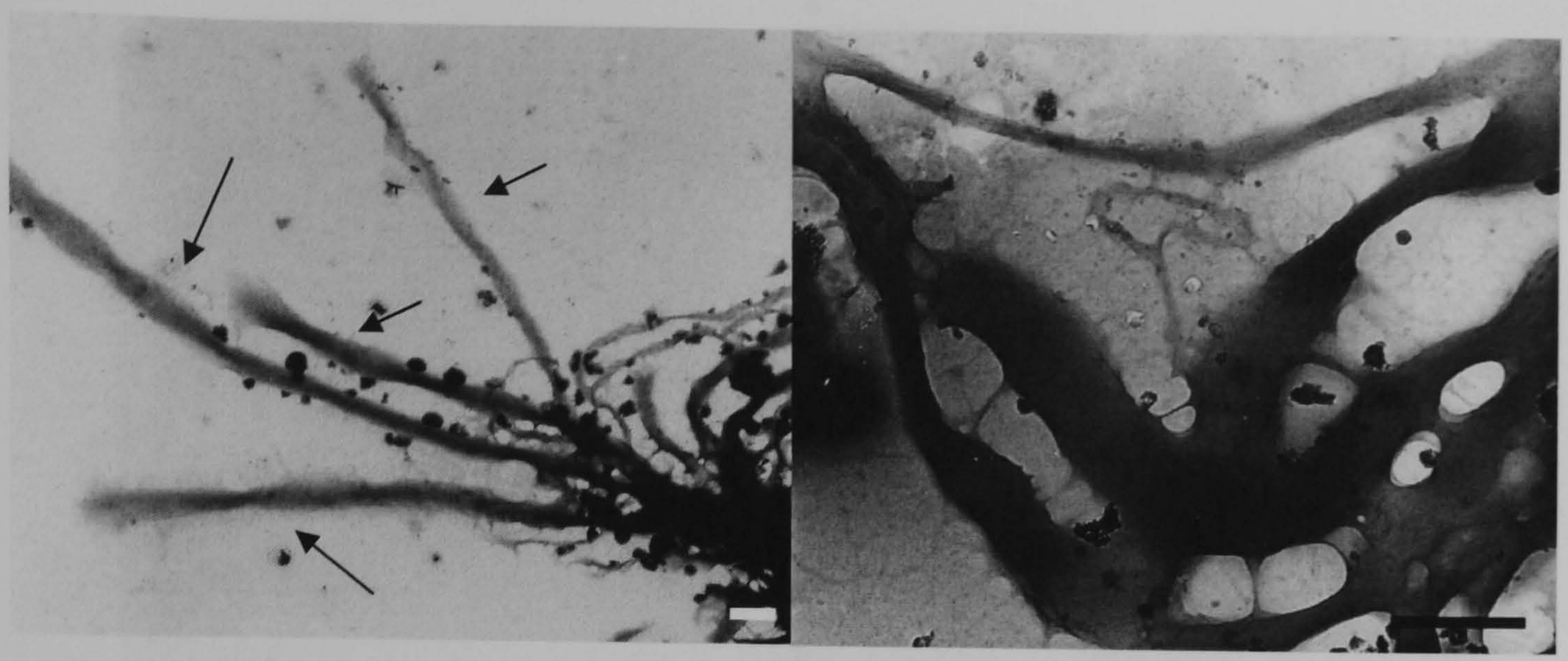


Figure 4.4 TEM images of bovine Achilles' tendon which had been heated to beyond the endothermic transition. The arrows show the string-like structures. The scales bars are 500 nm.

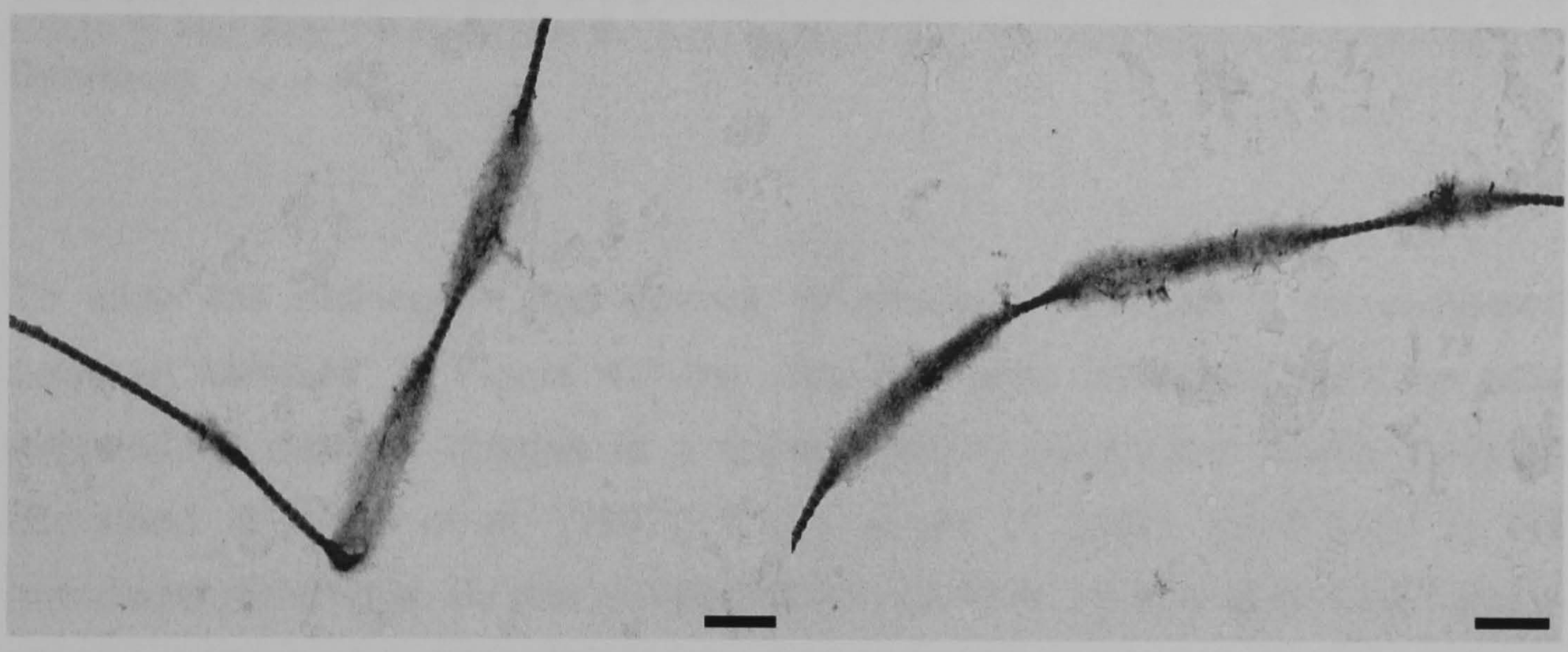


Figure 4.5 TEM images of rat tail tendon which had been heated to the peak maximum of the endothermic transition. The scales bars are 500 nm.

The X-ray diffraction analysis appears to confirm the TEM analysis. Small angle x-ray scattering (SAXS) is able to measure changes in the axial organization of the collagen fibril, i.e. the axial alignment of the molecules and the relative ratio of the gap / overlap period (Orgel et al., 2000). Figure 4.6 shows the diffraction images taken from samples which had been heated to before and after the endotherm. The meridional Bragg reflections produced by an axial alignment of the molecules can be seen as concentric rings in image 4.6A. A loss of the meridional diffraction pattern as seen in image 4.6.B indicates that the axial contrast is lost.

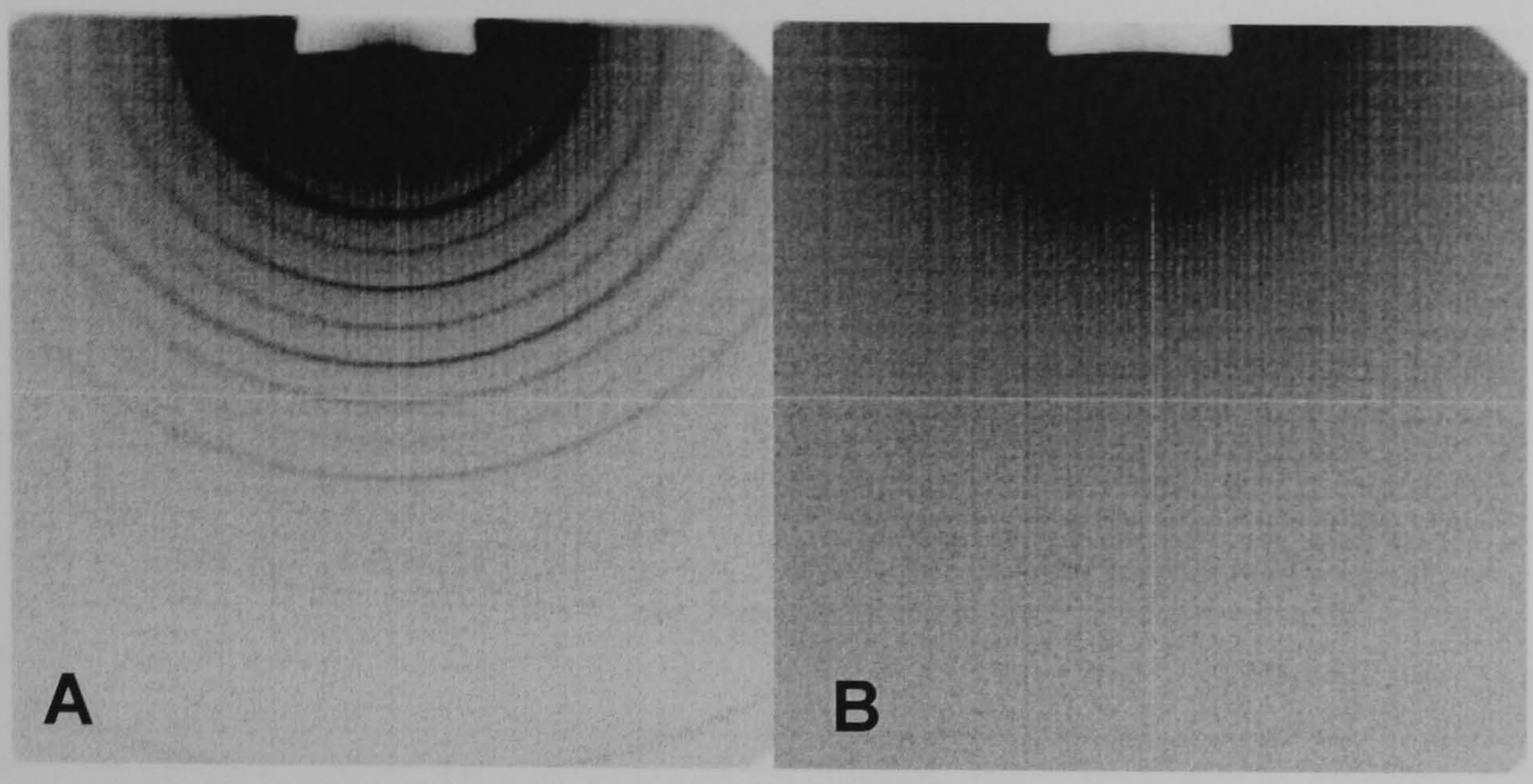


Figure 4.6 X-ray diffraction images from bovine Achilles' tendon samples heated to before (A) and after (B) the endothermic transition. The diffraction peaks seen in image A are caused by the axial periodicity of the collagen molecules within fibrils. It can be seen from image B that after the transition the axial order is lost. Samples were run on the SRS at Daresbury.

To allow the distribution and spacing of diffraction features to be compared between samples, in Figure 4.7 the data has been converted from the two-dimensional detector images to a linear intensity distribution (using methods described in Wess et al. (1997)). The series of peaks correspond to the meridional reflections. By first comparing the plot from the control and the sample which was heated to 60°C (the onset of the transition) it is clear that there is no change in the intensity and position of the peaks. There is a broad peak, after the 12^{th} meridional diffraction in the control sample. This is unrelated to the collagen periodicity (it does not lie on the collagen meridional series) but may be caused by lipid (Kennedy et al., 2003). The sample heated to 64°C (peak max) shows a slight peak at the third meridional diffraction peak but no further peaks and the sample heated to 75°C (after the transition) shows no peaks at all, only background scatter. This suggests when the samples were heated to temperatures before the transition there were no structural changes in the axial structure. However when samples were heated to beyond 60°C the quarter-stagger alignment of molecules in the axial plan of the collagen fibrils was lost.

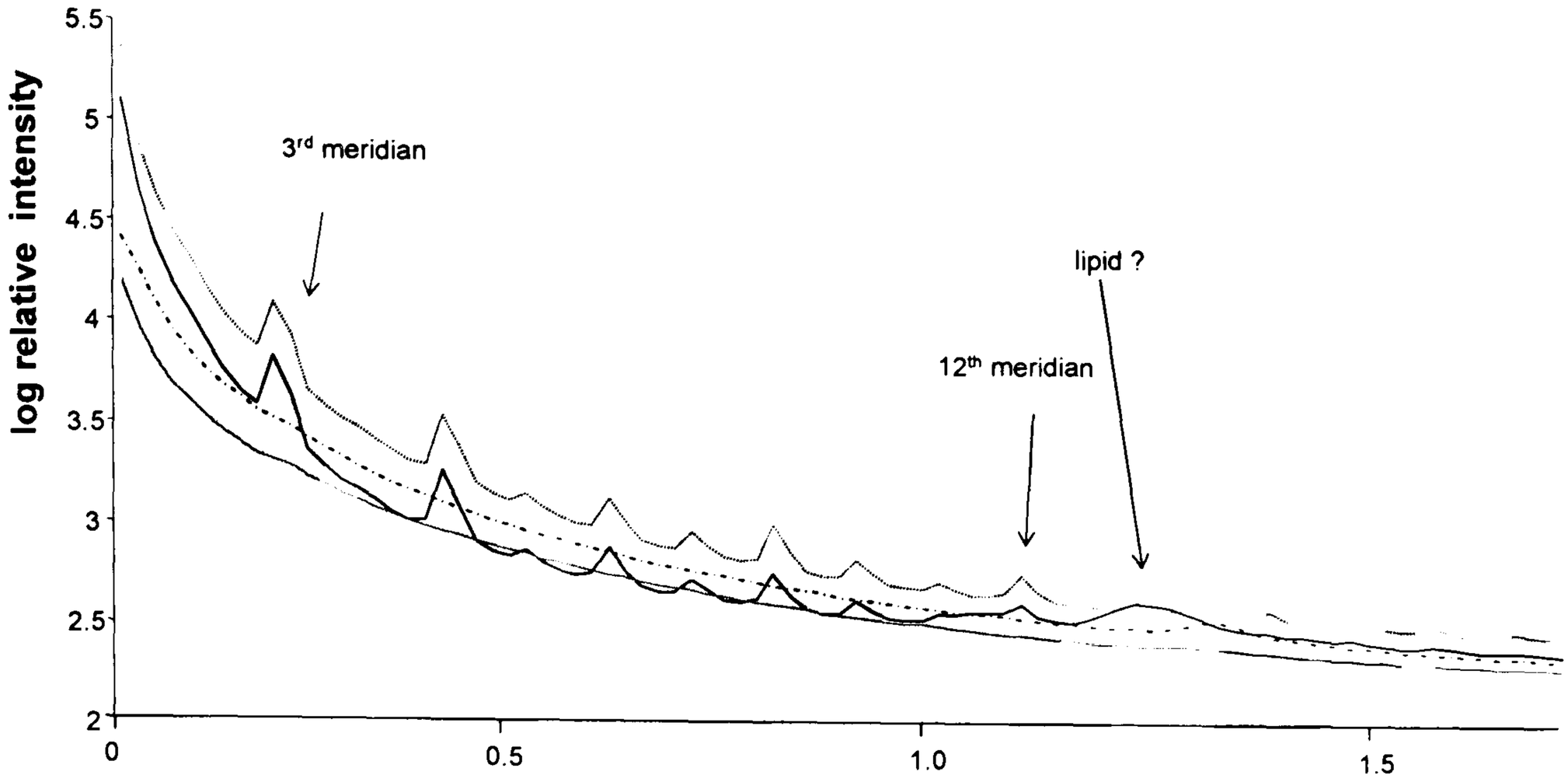


Figure 4.7 X-ray profiles of the Achilles' tendon diffraction data shown on a linear plot of log intensity against distance (from the centre of the diffraction image). The intensity distribution relates to the structure of the tendon's axial periodicity. Note that the control (solid black line) and the sample heated to 60°C (dotted line) display meridional peaks, but the axial periodicity is lost in the samples heated to 64°C (solid grey line) and 75°C (dot and dash line).

The TEM analysis suggested that as tendon goes through the endothermic transition the fibril structure collapses leaving behind electron-dense string-like structures. This unpacking of fibril structure was found to occur in both bovine Achilles' tendon and rat tail tendon. The loss of axial order is confirmed by the SAXS analysis and shows that the observed morphological changes are not simply an artefact of sample preparation. This is further supported by Spadaro and Becker (1972), who heated bovine Achilles' tendon in a water bath at temperatures ranging from 40-90°C for an hour each time. They observed amorphous areas at localized regions along the fibril and at the ends. Like the results of this study, at higher temperatures this "melting" spread to the entire fibril, causing a loss of banding and leaving it expanded with a substantial increase in diameter. The TEM images from Spadaro and Becker (1972) study suggest that the string-like structures persist even after the tendon has been heated for a prolonged period beyond the transition (one hour at 90°C). They also fixed a sample of tendon in formaldehyde (10% buffered formalin) after heating at 70°C for 1hr and observed the same alteration under TEM as the unfixed samples. This provides further evidence that the fibril collapse is not an

artefact of sample preparation or due to dehydration as a consequence of the TEM analysis.

Snowden and Weidemann (1978) also used TEM to look at the hydrothermal stability of tendon, in this instance to examine factors affecting the toughness of meat. They heated the tendinous sheath of the longissimus dorsi muscle from mature sheep and observed amorphous "melted" areas at the ends of the fibrils which progressed along the fibril at higher temperatures or after prolonged exposure. However they were also able to observe progressive changes occurring during the "melting" of a fibril viewed in transverse section. These images suggest that the unpacking is initiated at the edge of the fibrils and progresses through to the middle in a spiral-like pattern see Figure 4. 8.

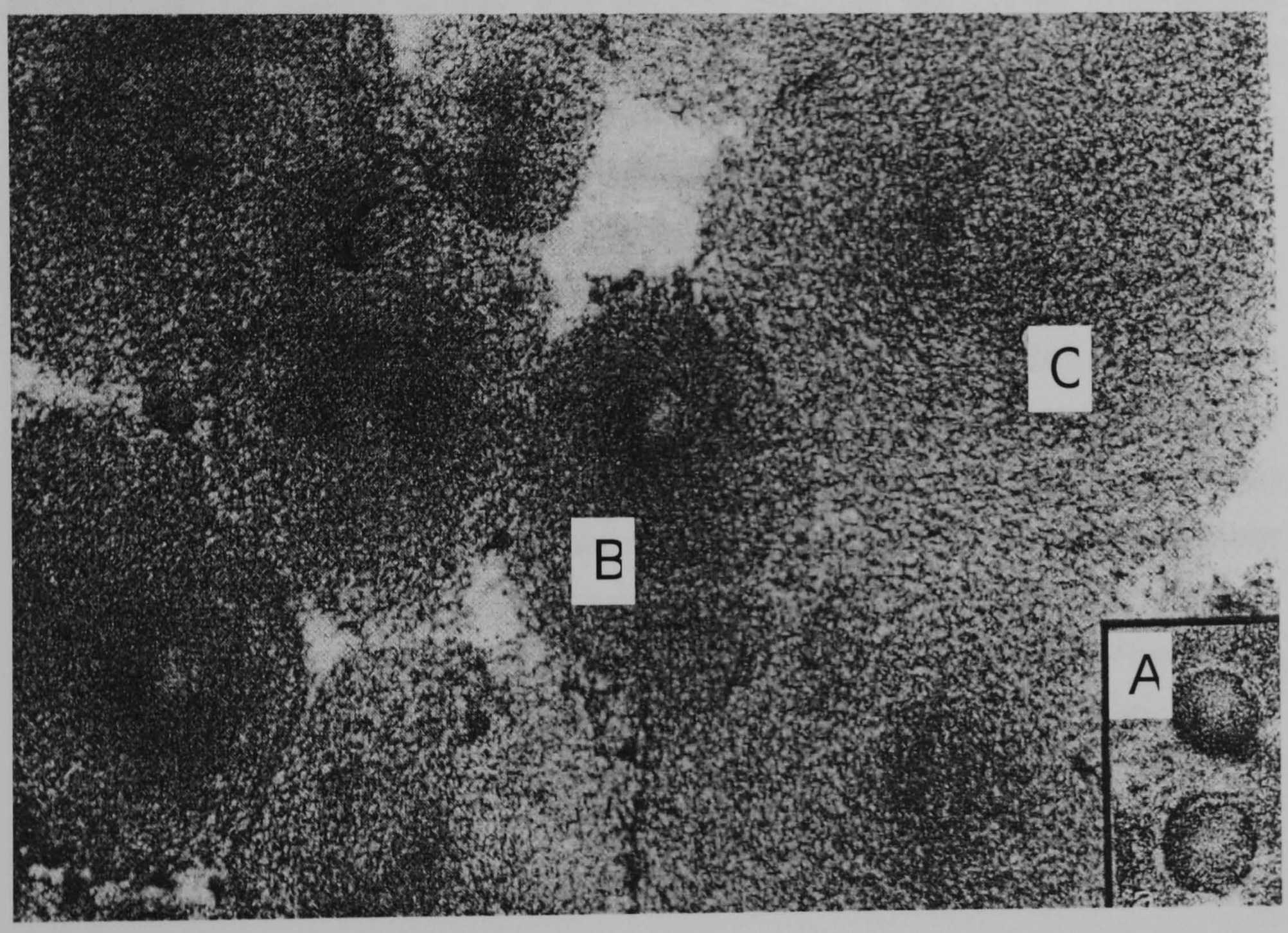


Figure 4.8 TEM images showing cross-sections through collagen fibrils in various stages of melting. The fibrils appear to progress from A-C through a spiral structure which initiates at the outer edge of the fibril. From Snowden and Weidemann (1978), Figure 6, p10.

The TEM data from these studies and the analysis described in this chapter give an indication of the stabilizing ability of the fibril. The images from both rat tail and bovine Achilles' tendon show that damage can occur in localized areas along the collagen fibril whilst the rest of the fibril remains relatively intact. This indicates an

ability of the collagen fibril to tolerate damage until eventual catastrophic failure. Indeed studies of degraded parchment have shown that collagen fibrils can sustain considerable oxidative and hydrolytic damage without converting to gelatin (Garp et al., 2002). Wang et al. (2002a) have also shown that the collagen network in demineralized bone can maintain its structural integrity even when large amounts of collagen molecules have been damaged, through heat induced unwinding or enzymatic cleavage.

Surprisingly even after the fibril has undergone extensive alteration and no longer shows the quarter stagger alignment of the collagen molecules, some degree of organization still exists in the form of 'strings' of electron dense material. The string-like structures suggest that part of the original fibril organization is retained even though the quarter-stagger arrangement has been lost. As the endothermic transition takes place the hydrogen bonds between α-chains are ruptured causing the triple helices to unravel, and the loss of helical order in both rat tail tendon and bovine Achilles' tendon, after the transition temperature, has been shown by x-ray diffraction (Bonar and Glimcher, 1970). However the collagen fibril structure is also stabilized by covalent cross-links which are more thermally stable than hydrogen bonds and will not rupture during this transition (for a review of covalent cross-links see Bailey (2001) and Knott and Bailey, (1998), also see section 1.3.6). The string-like structures may therefore represent denatured collagen molecules held within a network of covalent cross-links. The behaviour of bovine Achilles' tendon in dilute acetic acid may provide further evidence for this intermediate state between unaltered fibrils and soluble gelatin. The tendon has been found to swell in dilute acetic acid to an almost gel-like state without converting to soluble gelatin (Glimcher and Katz, 1965).

4.4 Endothermic transition in mineralized tissue

If packing within the fibril stabilizes the triple helices, and cross-links seem to allow some organization to be retained even within the collapsed fibril, what role does mineral play in stabilization? In the next series of experiments the role played by the mineral in protecting collagen fibrils from thermal alteration was examined. In the first instance a sample of bone (mineralized collagen) was heated to 85°C (a temperature beyond the transition observed in tendon) and then demineralized and examined by TEM. In the second experiment heating was continued until an endothermic transition was finally observed.

4.4.1 Materials and methods

The samples were fresh bovine, mid-shaft femur, compact bone shards (for details of shard preparation see section 3.3.1).

For the first experiment 20-40 mg of the shards were sealed into an Al DSC pan. The sample was then run using the heating range of 15-85°C at a scan rate of 5°C per minute. After the run the pan was quenched in an ice bath. The samples were then removed from the pans and demineralized using the EDTA demineralization method. The samples were finally prepared for TEM analysis (for further details of the demineralization method and details of the TEM preparation method see sections 3.3.2.2 and 3.3.4.2 respectively). For the high temperature experiment a medium pressure steel pan had to be used. Again 20-40 mg of the bone shards were sealed into a pan but this time the heating range was 15-195°C at 5°C per minute. Both experiments conducted in duplicate.

4.4.2 Results and Discussion

The DSC analysis showed that the endotherm which is present in tendon at around 65°C is *not* observed in mineralized bone. Further when the bone samples were heated on the DSC to beyond this temperature and then demineralized and viewed under the TEM, the collagen fibrillar structure remained intact and indistinguishable from unheated demineralised bone collagen. The DSC analysis from the second experiment, where the bone shards were heated to 195°C, showed that there is a large endothermic transition with onset values at 147°C and peak max values at 161°C, 160°C (an example of a DSC trace from mineralised bone collagen showing this transition can be seen in Figure 5.12). This finding was previously noted by Kronick and Cooke (1996) who recorded a peak maximum at 155°C in bone. The authors suggest that collagen in bone melts at a higher temperature because the physical constraints of the mineral prevent the collagen molecules from denaturing at a lower temperature.

Clearly, the mineral plays an important role in protecting bone collagen from thermal alteration, stabilizing the collagen to temperatures beyond that which can be achieved by conventional tanning agents. Chrome sulphate, the most efficient tanning agent used in the leather industry, increases the temperature of endothermic transition in hide to 100°C-120°C (Covington, 2001).

4.5 Endothermic transitions in demineralized collagen

In the following set of experiments the endothermic transition in demineralized bone collagen was investigated. In the first experiment, the aim was to determine if the high temperature transition that is observed in mineralized collagen is also present when the mineral has been removed. The aim of the second experiment was to determine if the same fibril collapse that is seen in tendon collagen also occurs in demineralized bone collagen.

4.5.1 Materials and methods

The bone samples (bovine, mid-shaft femur) were the same as those used in the previous section except in this case they were demineralized in 0.1M EDTA for 14 days prior to analysis. After demineralization the shards were placed in PBS on a rocker-roller overnight. Before being weighed in the DSC pan each sample was rinsed with PBS and blotted dry with filter paper. In the first experiment the sample was run in duplicate using medium pressure steel pans and a heating range of 15-195°C at 5°C per minute. For the second experiment the same procedure was used as is outlined in section 4.3; where samples were run to various points over the low temperature endothermic transition.

4.5.2 Results and discussion

Experiment 1

Figure 4.9 shows a DSC trace of demineralized bovine bone collagen. There are two endothermic transitions present; the first appears at a similar position as is seen in tendon and skin collagen (Miles et al., 1995; Flandin et al., 1984). Onset values of 57°C and peak maximum values at 61°C, 62°C were recorded for this transition. This peak is slightly broader than was seen in the tendon collagen (see section 4.3), suggesting more heterogeneity in the stability of bone collagen molecules. There is also a second high temperature peak. This peak appears to be about the same size as the first transition but onset values of 150°C, 149°C and peak maximum values at 159°C were recorded. This peak overlaps with the high temperature transition observed in mineralized bone, which raises the question; are both of these high temperature transitions the same event, or are they representing two separate events which just happen to occur at the same temperature? This issue is discussed in section 4.6.2. A second peak at a similar high temperature has previously been observed by Green (2005) in bovine hide powder, a collagenous material which is not mineralized. This shows that the

peak in the demineralized bone collagen is not simply due to incomplete demineralization.

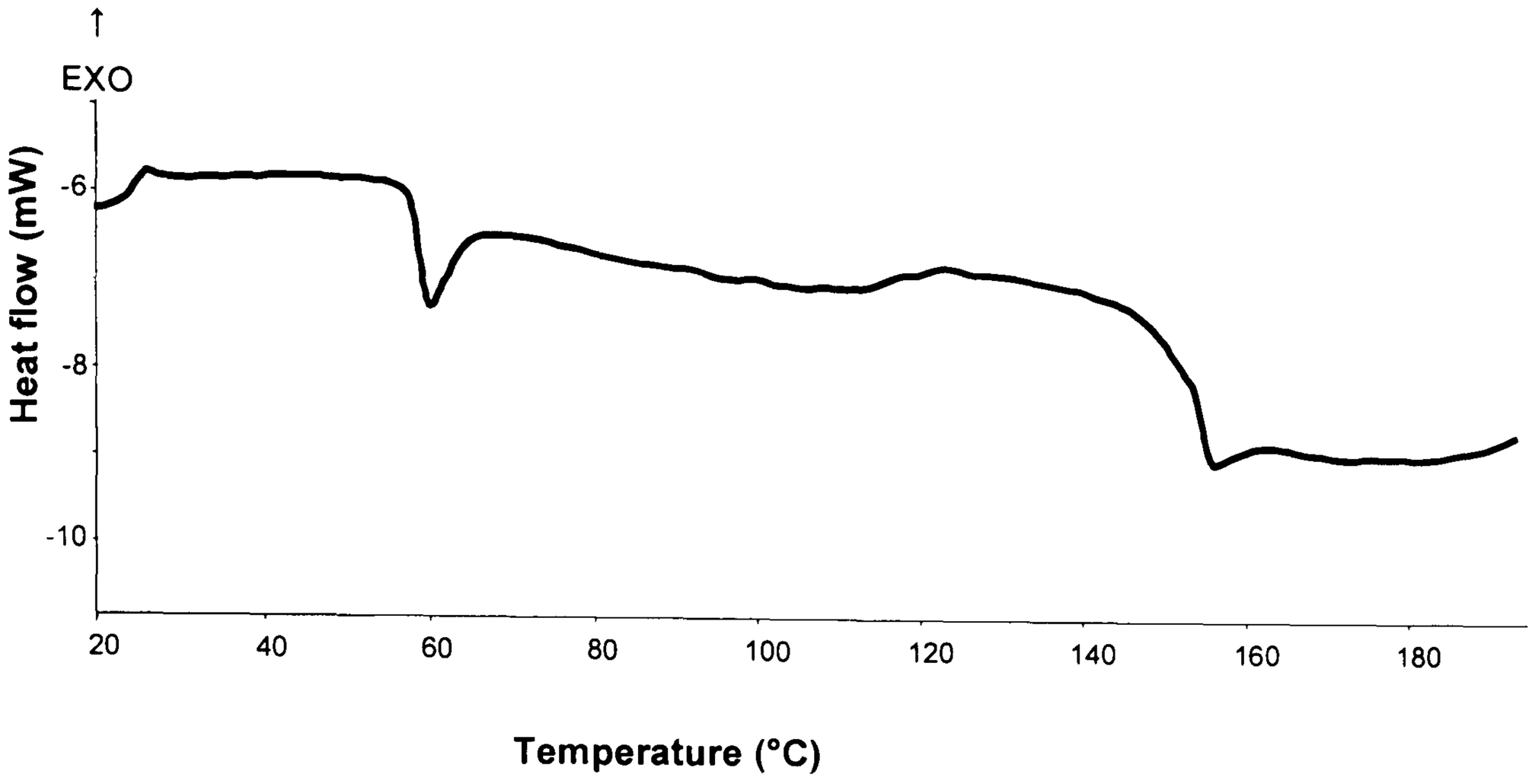


Figure 4.9 DSC trace from demineralized bovine bone

Experiment 2

Examination of the first transition in demineralized bone showed that just before the transition (55°C), the collagen fibrils appeared mainly unaltered with a d-spacing of 64 nm +/- 0.5nm. (to the nearest nm, based on the average value from 30 fibrils, +/- S.D.) (see Figure 4.10) . There were however some fibrils which displayed small areas of alteration along the fibril and in the ends. At the mid-point of the transition (60°C) the collagen was almost completely swollen and staining was weak. There were almost no unaltered collagen fibrils and the majority of collagen was in short fragments (see Figure 4.11). After the transition the collagen was still swollen and faint, and was almost exclusively found clumped together (see Figure 4.12). In the centre of the clump it is difficult to make out any banding, however at the edges there did appear to be collagen with regions of banding with a d-spacing of 63 nm +/- 0.4nm. (to the nearest nm, based on the average value from 30 fibrils, +/- S.D.).

The demineralized bone and tendon collagen appear to behave differently over the low temperature (60-65°C), transition event. In both bovine Achilles' tendon and rat tail tendon the unpacking of the fibril seems to spread along the fibril until the banding has been completely lost, but at the same time a string-like structure

is still preserved, indicating some lateral order. By contrast in demineralized bone collagen the fibrils seem to separate into short fragments which lose their fibril structure and become clumped together as an amorphous mass lacking any lateral order but still with apparent periodicity within the clump.

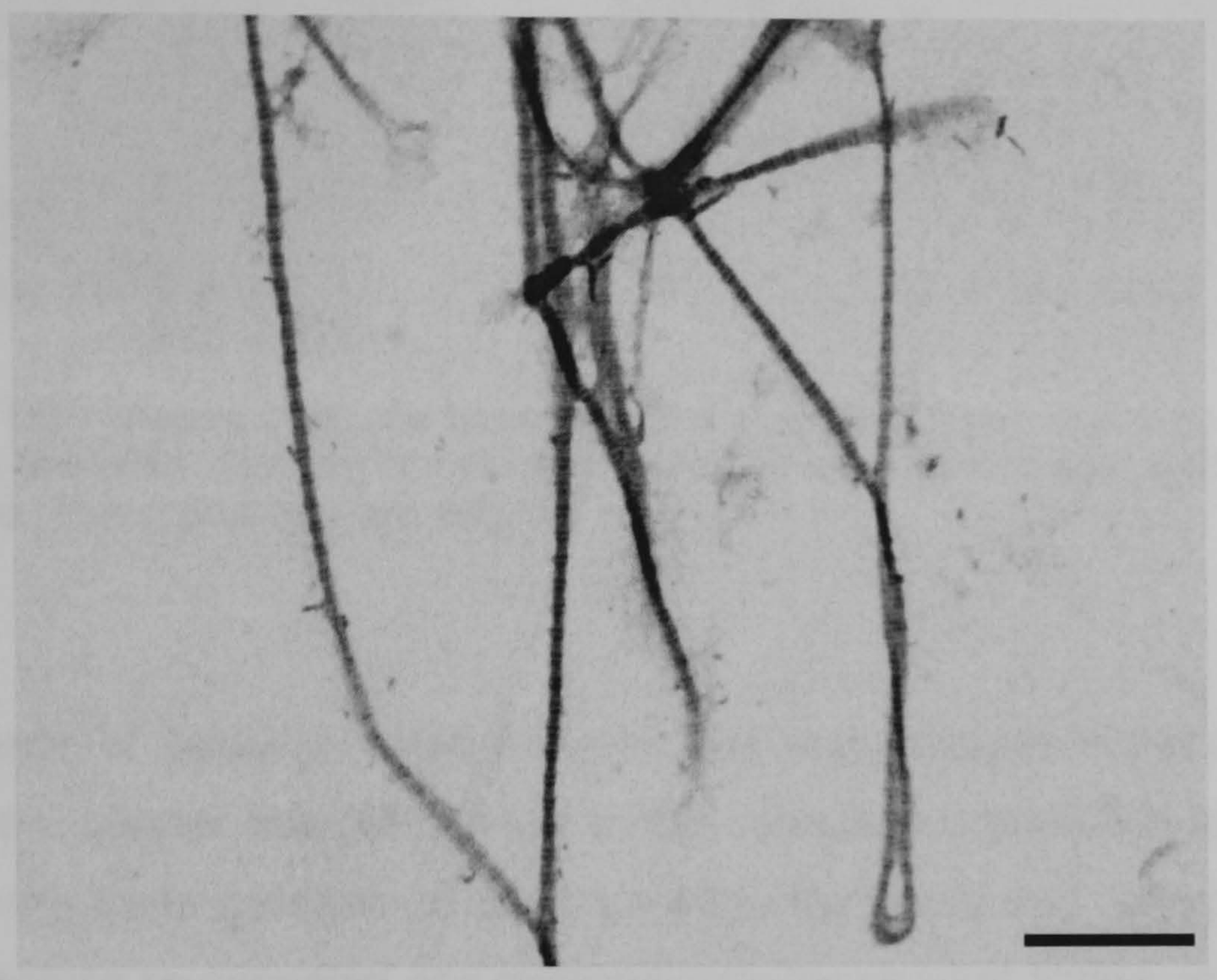


Figure 4.10 TEM image of bovine bone collagen which had been heated to 10°C before the endothermic transition. The scales bars are 500 nm.

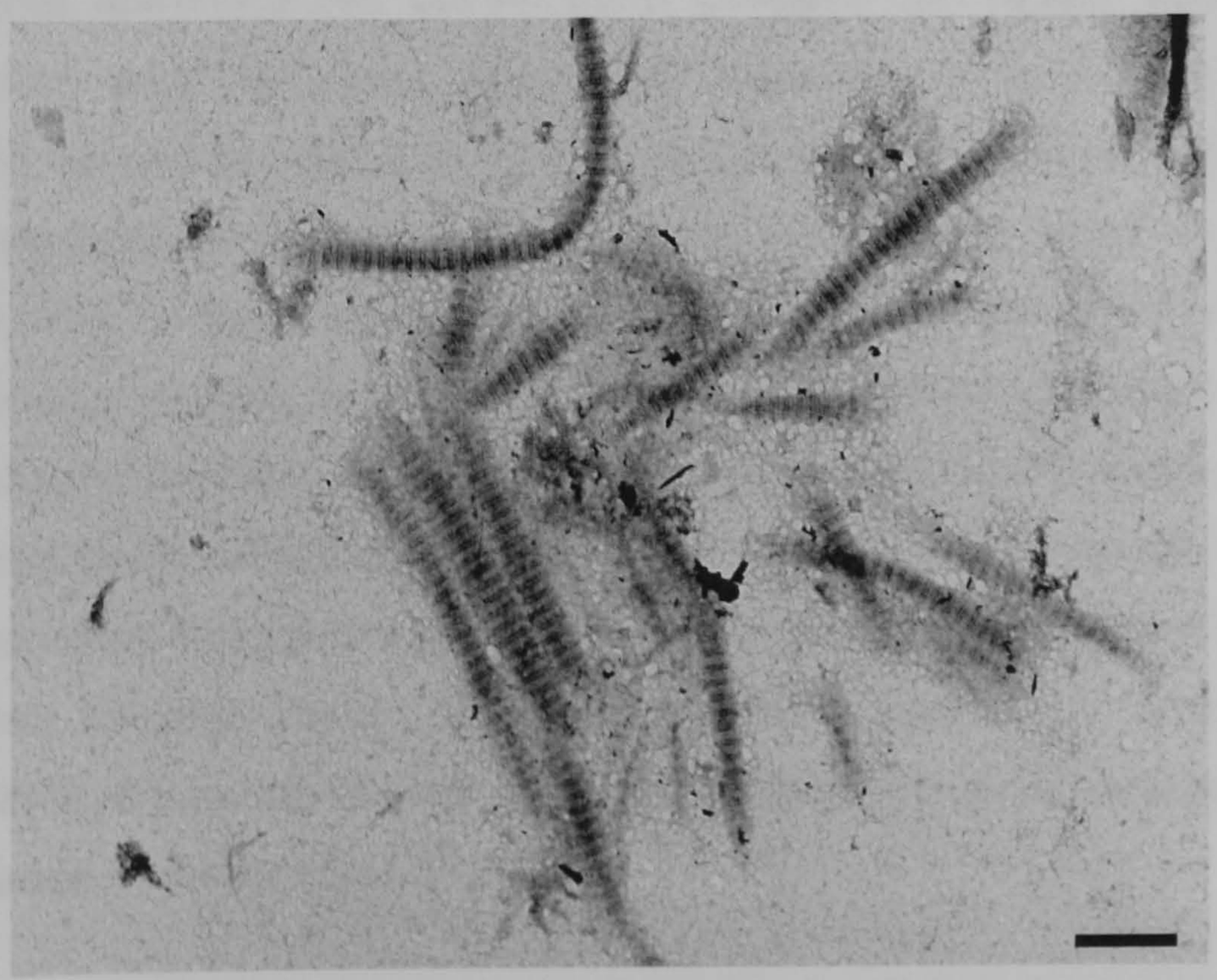


Figure 4.11 TEM image of bovine bone collagen which had been heated to the peak maximum of the endothermic transition. The scales bars are 500 nm.

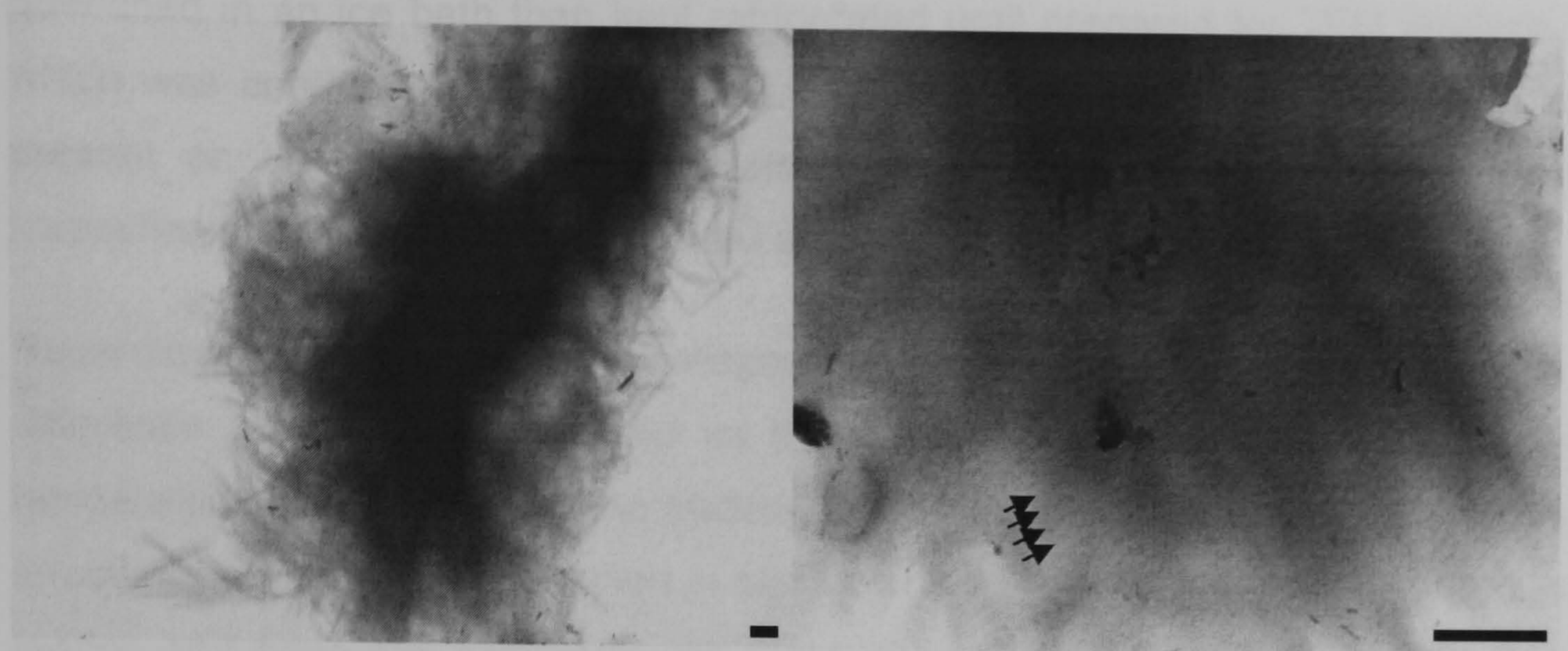


Figure 4.12 TEM images of bovine bone collagen which had been heated to beyond the endothermic transition. Banding is clearly visible in the right-hand image, and is indicated by the arrows. The scales bars are 500 nm.

The presence of banding could indicate that the collagen molecules are in register in the quarter stagger alignment. One possible explanation for apparent banding in the bone collagen is that on cooling the denatured molecules in the collagen became re-annealed and realigned into these short fibrillar regions. This could have occurred between the DSC which was conducted at Northampton and when the sample was transported back to York and resuspended in PBS before it was prepared for TEM analysis. There is evidence from wide and low-angle X-ray diffraction that demineralized bovine bone collagen can regain both its short-range (helical) and long-range (staggered alignment of molecules) order on cooling if the sample is heated to 5°C above the transition (Bonar and Glimcher, 1970). Interestingly in the same study, rat tail tendon was found to recover its helical order but no long range order. This is consistent with the TEM analysis discussed in this chapter; both the bovine Achilles' and rat tail tendon had been heated to beyond the transition and subsequently treated in the same way as the demineralized bone collagen and yet neither showed any evidence of banding which would have indicated a recovery of long range order. On the other hand the perceived banding could simply be an artefact caused by parallel aligned fibrils lying in layers on top of each other and producing interference patterns with the appearance of d-spacing.

To test the possibility that the demineralized bone collagen had reannealed two further bone collagen samples were tested. A 62 year old human and a porcine bone were EDTA demineralized then heated on a heating stage in PBS to 80ºC (beyond the first endothermic transition) for 30 minutes. The samples were then

quenched in an ice bath then kept refrigerated until prepared for TEM analysis, which was conducted within 24 hours. Again in both cases the collagen was present on the grid as clouds of electron dense material but under close inspection clear banding was observed around the edges.

Regardless of whether the bone collagen had become renatured there was still a difference between the behaviour of bone and tendon collagen over the low temperature transition. After the transition tendon collagen produces string-like structures which are not observed in bone collagen. The type I collagen found in tendon and bone has essentially the same molecular structure and composition (Glimcher and Katz, 1965; Mechanic et al., 1985). Once collagen molecules have aligned into collagen fibrils however, they become covalently cross-linked and the composition and distribution of these cross-links differs between tissues (see review in Bailey (2001) and Knott and Bailey (1998)). The covalent cross-linking provides the collagen fibril with stability and tensile strength (Bailey, 2001; van den Nieuwendijk et al., 1999). The differences in the appearance of the collagen after the transition may be linked to the different strains put upon these collagens because of their different functions and physiological position in the body. Tendons attach muscle to bone therefore they need to have high tensile strength to allow movement. In bone the properties of the collagen are dependent not only on its structural integrity but also on the presence of mineral and the interaction between the mineral crystals and the collagen molecules. One could postulate that as bone collagen will always be protected by mineral it is less important that the fibril structure is held together whereas in tendon the structural integrity is more important.

In bone the mineral may protect the fibril structure but it may also hinder or preclude the formation of a multi-functional cross-link network. This would impact upon the structural integrity and physical properties of the collagen fibril once the mineral has been removed. One property of demineralized bone collagen is that it is resistant to swelling in dilute acid and this has been commonly attributed to extensive cross-linking (Carmichael et al., 1977; Kronick and Cooke, 1996). However Glimcher and Katz (1965) looked at differences in the properties of bovine Achilles' tendon and demineralized 10-14-week-old chicken bone collagen and found that whilst the bone collagen was more resistant to swelling, this was not related to covalent cross-linking.

Pyridinolines are tri-functional non-reducible cross-links which have been found in both tendon and bone collagen (Mechanic et al., 1985). However there is a paucity of these mature cross-links in bone, with only one per five collagen molecules (Knott and Bailey, 1998). Banes et al. (1983) showed evidence, from collagen tryptic peptides obtained from compact bone, that pyridinoline cross-links are present in the non-mineralized compartment of bone and not in the mineralized compartment. This led the authors to speculate that the presence or absence of multi-functional cross-links may dictate whether or not mineralization occurs, or that the mineral may preclude formation of the cross-links. They suggest that in non-mineralized collagen the tri-functional cross-links hold the molecules at a shorter distance than the bi-functional cross-links of mineralized collagen in bone, and thereby physically preclude the formation of hydroxyapatite crystallites. Conversely once bone collagen is mineralized the apatite does not allow close enough location of the molecules to form multifunctional cross-links (Mechanic et al., 1985). It has been suggested however that the deficit in pyridinoline cross-links in bone can be accounted for by a novel tri-functional pyrrole cross-link (Knott et al., 1995). Its precise structure and position in the collagen fibre is not known (Knott et al., 1995) but a correlation of the pyrrole cross-links with bone strength has been found (Bailey, 2001). As well as these enzymatic cross-links there is also a secondary cross-linking reaction with glucose or its metabolites. These advanced glycation end products (AGEs) have been shown to increase with age in bone collagen (Li et al., 2003; Wang et al., 2002b) and are thought to be responsible for the increased stiffening which occurs in collagenous tissues in old age (Bailey, 2001).

The TEM analysis however showed that after heating to 80°C the 62 year-old human bone collagen (presumably with a high quantity of these non-enzymatic cross-links) fell apart in the same way as the other bone collagen samples (porcine and bovine). This suggests that if there were a greater quantity of cross-links in the human bone sample there were not enough or they were not in the right position to prevent fibril collapse after the transition.

The presence of mineral crystals within and between the collagen fibrils may hinder the formation of cross-links. In mineralized collagen plate-like minerals are present, which reside mainly in the gap regions between collagen molecules (Weiner and Traub, 1986) (see section 1.3.8 for further information). Using TEM analysis of sheep and human bone, Cressey and Cressey (2003) recently found

that as well as these plate-like minerals which run in parallel along the collagen fibrils, there are also vast thin sheets of mineral which are perpendicular to the longitudinal dimension of the fibrils and interconnect the parallel aligned mineral crystals. At this point it is not clear how these mineral 'box-constructions' interact with the collagen fibrils in bone but their presence may prevent the formation of a continuous cross-linked network.

Either or both the lack of multifunctional cross-links in bone compared to tendon, and a discontinuity in the network of cross-links because of obstructions from the mineral may explain the TEM analysis. This would account for short fragments seen in the bone collagen compared to the long string-like structures observed in the tendon collagen during the thermal transition. It may also explain the observation that bovine Achilles' tendon can re-elongate after thermal shrinkage where as demineralized bone collagen can not (Glimcher and Katz, 1965).

4.6 Implications

4.6.1 Integration of terms – role of the collagen fibril

As collagen-based materials are heated they go through a transformation. This can be observed as a large endothermic transition thought to represent the breaking of hydrogen bonds between the individual α-chains of the triple helix and its subsequent transformation to a random coil gelatin. At the same time there is also a macroscopic shrinkage of the tissue. The temperature at which this transition takes place has variously been described as the; *shrinkage temperature* (T_s), *denaturation temperature* (T_d) or *melting temperature* (T_m). The results of the analysis undertaken in this chapter show that there is also a collapse of the fibril structure during this transition; although the extent of the collapse appears to differ between tissues and appears to be more severe in the demineralized bone collagen. Therefore denaturation becomes less a property of individual triple helices and more that of the fibril. This difference may also have implications for the extent to which renaturation can occur. There is evidence from wide and low-angle x-ray diffraction analysis that demineralized bovine bone collagen can regain both its short-range (helical) and long-range (staggered alignment of molecules) order on cooling, if the sample is heated to 5°C above the transition, bovine Achilles' tendon and rat tail tendon however only recover helical order and *not* long range order (Bonar and Glimcher, 1970). This suggests that if the fibril structure is only partially collapsed the full reversibility of the

transition is prevented. From the above, it is clear that when dealing with fibrillar collagen both shrinkage and denaturation are valid terms but melting is not, as it suggests a phase transition in which the collagen can be transformed between native and denatured states by heating and then cooling.

4.6.2 What is the high temperature transition?

The higher temperature endotherm has been given much less consideration in the literature. The DSC results from this study showed that the transition which occurs at around 65°C in non-mineralized collagen is absent in mineralized tissue; which displays an endotherm at 150°C but nothing below this. This value is considerably lower than the typical temperature for pyrolysis of the organic content within bone (Shipman et al., 1984); the endotherm does not therefore represent combustion. Also when bone collagen is heated without its mineral component a peak appears at around 60°C. The question is whether the endothermic transition observed in mineralized tissue is the same unpacking event which occurs in tendon and demineralized bone at around 60-65°C, only shifted up to a higher temperature due to the physical constraints of the mineral; as implied by Kronick and Cooke (1996)?

There is evidence in favour of this explanation, see section 1.3.9. Whilst it is entirely conceivable that the presence of mineral is stabilizing the bone collagen in this way, the results presented in this chapter show that there are three problems with the idea that both the low and high temperature transitions represent the same event; (i) when the mineral is removed there is a peak at 60°C but also still a peak at 150°C, (ii) as the fibril collapse has already occurred at 60°C in the demineralized bone this high temperature transition must represent a different process; (iii) if the mineral is stabilizing the collagen molecules by physically constraining them from unfolding, what happens at 150°C to allow space for the unpacking of the fibril and denaturation of the collagen molecules to take place? X-ray diffraction and infra-red studies have shown no detectable change in the mineral component of bone until it has been heated to much higher temperatures (Holden et al., 1995a; Roberts et al., 2002).

An alternative explanation for the high temperature peaks seen in both mineralized and demineralized collagen is that they are caused by hydrolysis of the polypeptide chains. Heating experiments conducted by Collins (*pers. comm.*) have shown that at this temperature hydrolytic damage leading to gelatinization

would occur in less than a minute. To determine if the transition represents gelatinization through hydrolysis it would be necessary to examine the state of the collagen from before and after the high temperature transition by TEM. This was attempted several times but has thus far proved impossible. The Al pans burst at around 120°C, due to the evaporation of water. A further attempt was made with mineralized collagen by piercing the Al pan, in the hope that whilst some water may be lost there would still be enough within the mineral matrix to prevent the collagen from dehydrating, however this too proved unsuccessful. Medium pressure steel pans could be heated to beyond the transition but could not be reopened to obtain the sample. Finally resealable gold pans were used, but these could not be sufficiently sealed. A further way to test this would be to look at the activation energies of the transitions. The activation energy for the low temperature transition (*fibril unpacking*) is 518kj per mol (Miles et al., 1995) whereas for gelatinization through hydrolysis the activation energy is much smaller at 170kj per mol (Collins unpublished data).

Chapter 5 Heating experiments

5.1 Introduction

A key aim of this research was to develop a technique which could be used to identify cooked bone. When a fleshed bone is roasted or boiled the temperature of the bone is unlikely to exceed 100°C; therefore the technique needed to be able to identify thermal alteration at these mild temperatures. It has been suggested that bone collagen is protected from heat damage due to the presence of mineral (Covington, 2001; Kronick and Cooke, 1996; Nielsen-Marsh et al., 2000a). Indeed there is no observable transition in bone below 100°C (endothermic transition at 155°C for bovine bone; Kronick and Cooke (1996); also see Chapter 4). However, TEM data from heated sheep bone (Koon et al., 2003) and fish bone (Richter, 1986) have shown that microstructural damage *can* occur within mineralized collagen fibrils at temperatures below 100°C.

This chapter describes a series of heating experiments that were carried out on mineralized and demineralized bone collagen. Samples of bone were heated under the mild heating regimes that would be associated with cooking. Four separate heating studies are included in this chapter; the potential use of (i) DSC, (ii) TEM as techniques to identify cooking, and the variability in the thermal stability of mammal bone collagen between (iii) different species and due to (iv) age-at-death. Each of these studies is reported separately; an unusual 'flattening' of partially degraded fibrils is also reported. The final part of the chapter considers the implications of these studies for our understanding of the thermal deterioration of collagen and the roles that mineral and cross-links play in stabilizing bone collagen fibrils.

5.2 DSC as a technique to identify cooking

In this set of experiments preheated bone samples were analysed by DSC in both a mineralized and demineralized state. The aim was to determine to what extent microstructural damage caused by a previous low temperature heating event would affect the stability of the bone collagen; detected as a change in the size, shape and position of the endotherm. From this analysis it would be possible to determine if DSC could be used to identify cooked bone.

5.2.1 Materials and methods

Modern, mid-shaft femur, bovine bone was used for this experiment. Small shards were prepared using the method described in section 3.3.1. Prior to the DSC analysis the bone shards were heated in a heating block. For each heating regime 180 mg of shards were placed in a glass vial which was then inserted into the heating block and heated as indicated in Table 5.1. After each heat treatment the glass vials containing the samples were quenched in an ice bath. Samples were analysed on the DSC either in a mineralized state or with the mineral removed. For the mineralized samples preheated shards were transferred to Eppindorf tubes and stored at 4°C until the DSC analysis. To produce the demineralized samples, shards were treated with HCl (section 3.3.2.1) and prepared for DSC analysis as described in section 3.3.5.2. The demineralized samples were run in aluminium pans at a scan rate of 5°C a minute and a heating range of 15-85°C. The mineralized samples were run in medium pressure steel pans at a scan rate of 5°C a minute and a heating range of 15-195°C; all scans were run in duplicate.

Table 5.1 Details of the heat treatment that was applied to bone samples prior to DSC analysis. The table also shows which of the samples were analysed in a mineralized and/or demineralized state.

Duration (hours)	Temperature (°C)	Demineralized	Mineralized
0	unheated	yes	yes
0.5	60	yes	no
1	60	yes	no
1.5	60	yes	yes
2	60	yes	no
3	60	yes	yes
0.5	100	yes	no
1	100	yes	no
3	100	yes	yes
6	100	no	yes
9	100	yes	yes

5.2.2 Results and discussion

In Figures 5.1 and 5.2 the *onset* (the point at which the DSC trace begins to deviate from the baseline at the start of the endothermic transition), *peak max* (maximum of the peak) and *peak end* (the point at which the DSC trace rejoins the baseline at the end of the endothermic transition) values have been plotted for the mineralized and demineralized samples respectively. The mineralized sample that had been heated for nine hours was not plotted on the graph

because it was not possible to obtain a reproducible scan. This sample was run six times and in most cases a very broad and shallow peak was observed that began between 110-140°C and ended between 170-190°C. The mineralized samples heated for three hours at 60°C and 100°C exhibited a slight increase in the temperature of the endotherm, compared to the unheated sample and the sample heated at 60°C for one hour 30 minutes (Figure 5.1). This apparent stabilization could be the result of dehydration, which is known to increase the denaturation temperature of collagen (Miles and Ghelashvili, 1999; Trebacz and Wojtowicz, 2005). Dehydration may have occurred because the bone shards were not heated in water; however the sample which was heated at 100°C for six hours did not display an increased denaturation temperature.

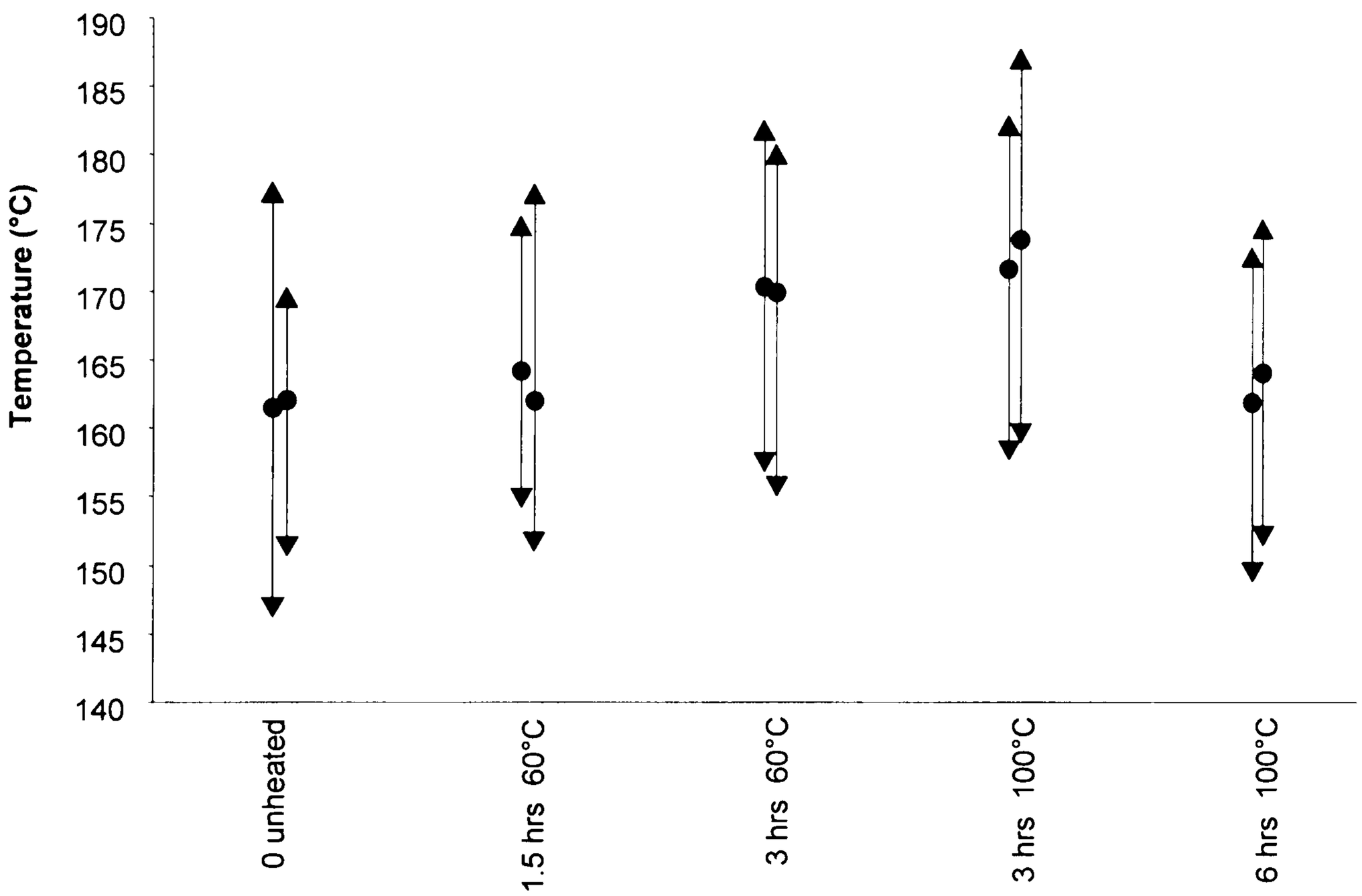


Figure 5.1 A plot showing the DSC values obtained from mineralized bovine bone which had been heated at 60°C and 100°C for different durations. The onset (▼) peak max (●) and peak end (▲) values from each DSC scan are shown.

For most of the demineralized samples the endotherm occurred at the same temperature as the unheated control sample (average peak max value; 60.6°C) (Figure 5.2). Two exceptions were noted, the sample heated at 60°C for three hours showed a slight increase, and the sample heated at 100°C for nine hours, displayed a large decrease in the temperature of the endotherm.

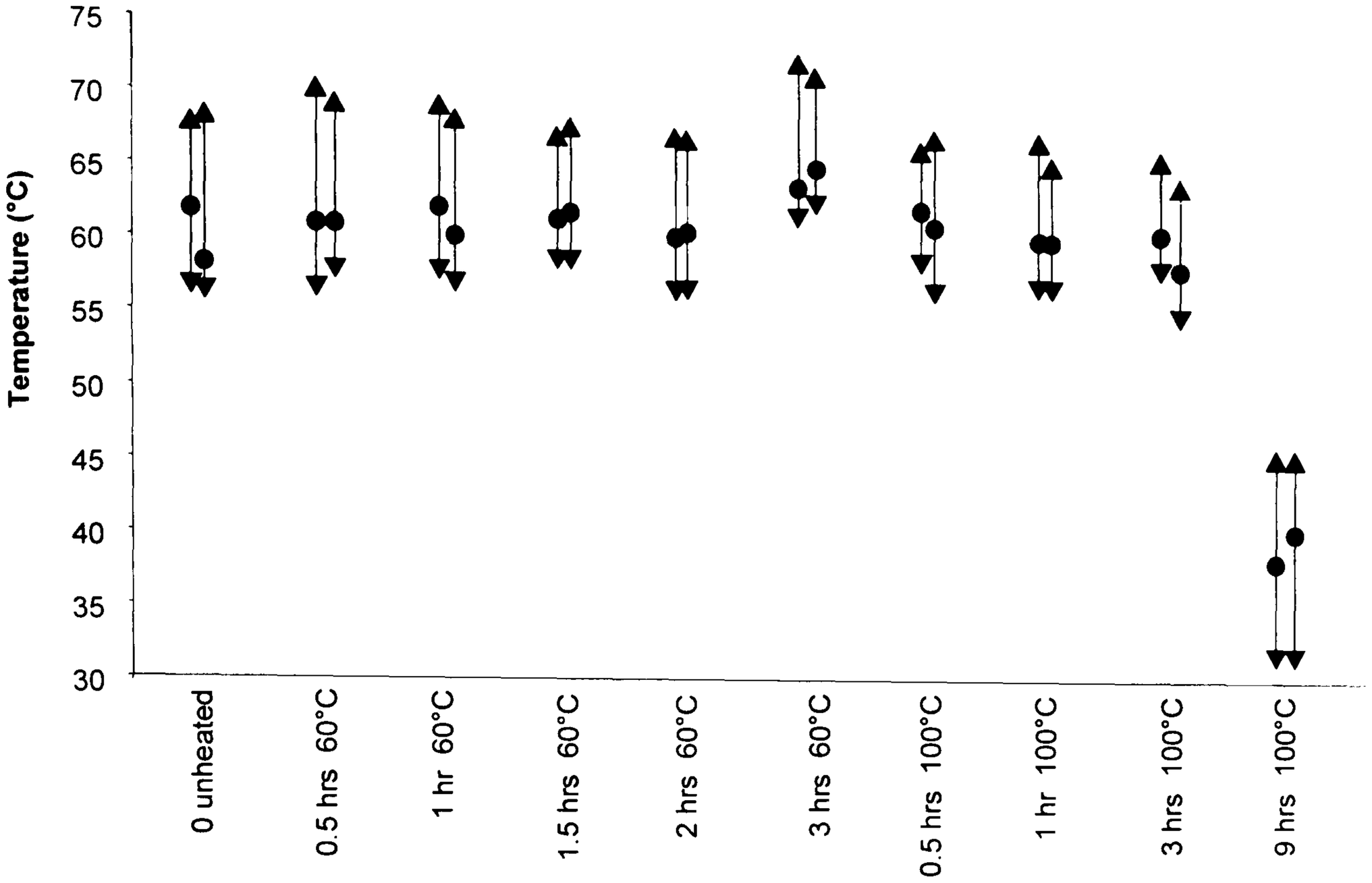


Figure 5.2 A plot showing the DSC values obtained from demineralized bovine bone which had been heated at 60°C and 100°C for different durations. The onset (▼) peak max (●) and peak end (▲) values from each DSC scan are shown.

As well as measuring the position of the peak, the enthalpy of the transition can also be measured from the area under the peak. It was predicted that if the samples were damaged by heat, less energy would be required for the collagen to denature and therefore the size of the endotherm would be reduced. The enthalpy values (ΔH) for these samples were measured using Mettler STARe integration software and were based on the weight of the mineralized sample. An assumption was made that as all the samples were from the same mid-shaft femur, the collagen content would be comparable, and therefore the ΔH would be comparable between samples. The values for all of the mineralized samples were very similar, ranging between 3.26 - 8.08 J g^{-1} and showed no correlation with temperature or duration of heating. The ΔH values for the demineralized samples were very similar, though slightly lower, ranging between 1.21 - 4.94 J g^{-1} and again showed no correlation with temperature or duration of heating. The results of this analysis are consistent with values observed by Nielsen-Marsh et al. (2000a); their modern cooked sample had a peak max of 61.7°C and ΔH of 3.15 J g^{-1}. However, the modern unheated bovine sample, analysed by Nielsen-Marsh et al. (2000a) had a peak max at 68°C, higher than the value obtained in this experiment for the unheated sample and in fact higher any of the values obtained for untreated demineralized bone collagen in my research.

For both the mineralized and demineralized samples, only those which had been extensively heated (nine hours at 100°C) showed a considerable change in the endothermic transition. After this heating regime the endotherms observed by DSC were broader and shifted to a lower temperature than that of the unheated control samples. This indicates that the collagen within these samples consisted of molecules in different states of degradation. However in all of the other heated samples the endotherm was not affected. It would appear that when collagen is mineralized any microstructural damage incurred during mild heat treatment is insufficient to destabilize the molecular structure enough to affect either the temperature or size of the denaturation endotherm.

This means that DSC would not be a useful technique to identify bone heating under normal domestic heating regimes.

5.3 TEM as a technique to identify cooking

In this set of experiments TEM analysis was conducted on a series of bovine bone samples which had previously been heated to different temperatures and durations. The aim was to encompass the full range of deterioration that could be observed in collagen fibrils using TEM. This would allow identification of the minimum heat exposure required to cause observable damage to the collagen fibril, right through to the point at which full degradation occurs and there are no recognisable collagen fibrils remaining; thereby identifying the limits of the TEM technique to detect cooked bone. These heating experiments also examined the sensitivity and reproducibility of this technique and provided a more detailed analysis of the early stages for collagen degradation than my previous research on sheep bone collagen (Koon et al., 2003).

5.3.1 Materials and Methods

The bone samples used in this group of heating experiments came from modern bovine bone (mid-shaft femur). A mid-shaft section was broken into medium sized shards (typical dimension 5 mm) for the heating experiments. Shards were used instead of whole bone or large sections of bone because the aim of these experiments was not to replicate cooking practise but to determine the amount of alteration that is caused by each of the temperature regimes. Shards have the advantage of a larger surface area and therefore more rapid and homogeneous heat transfer. The heating regimes for this set of experiments are displayed in Table 5.2. For each heating experiment 180 mg of bone shards were placed in a glass vial and then heated in a pre-heated heating block or oven (Binder) for a

designated time and temperature. Samples which were heated above 100°C had to be heated in an oven. After each heat treatment the vials containing the samples were quenched in an ice bath to rapidly reduce the temperature. The shards were then demineralized using the EDTA method described in section 3.3.2.2. Once demineralized the samples were washed several times with ultra pure water and placed in PBS at 4°C until they were prepared for TEM analysis. The TEM sample preparation method is described in section 3.3.4.2. The method of data collection and analysis is also explained in Chapter 3 in section 3.3.4.4.

Table 5.2 Details of the heating regimes that were used for the TEM analysis. * The asterisks denote heating experiments which were replicated. Samples shaded in grey were not included in the ternplot in Figure 5.3.

Duration (hours)	Temperature (°C)
0	unheated
1	50*
1	70*
1	80
1	100*
3	100*
6	100*
9	100
12	100
1	140
1	170

5.3.2 Results and discussion

The results from the heating experiments are displayed in Figure 5.3 on a ternplot. The results show that with increasing temperature and duration of heating there is a progression in both the amount and extent of altered fibrils. The pattern of deterioration which was observed in the collagen fibrils is described below in section 5.3.2.1. The duplicate heating experiments on bovine bone produced consistent results. This shows that the amount of alteration observed in the collagen fibrils of each sample is directly related to the temperature at which the bone has been heated and the duration that it has been heated for, rather than simply an artefact of sample preparation. These results also suggest that the TEM analysis method gives a good indication of the general extent of damage to the collagen fibrils when heated under these specific regimes; this is discussed further in section 5.3.2.2.

The results for three of the heating experiments are not included on the ternplot; these were the bovine bone samples which had been heated for nine and twelve

hours at 100°C and one hour at 170°C. The majority of the material that could be observed from these samples did not show any banding. A few *Dumbbell* and *Beaded* type fibrils could be seen in the samples heated at 100°C but in general the only material present on the grids was amorphous and electron dense, presumably gelatin.

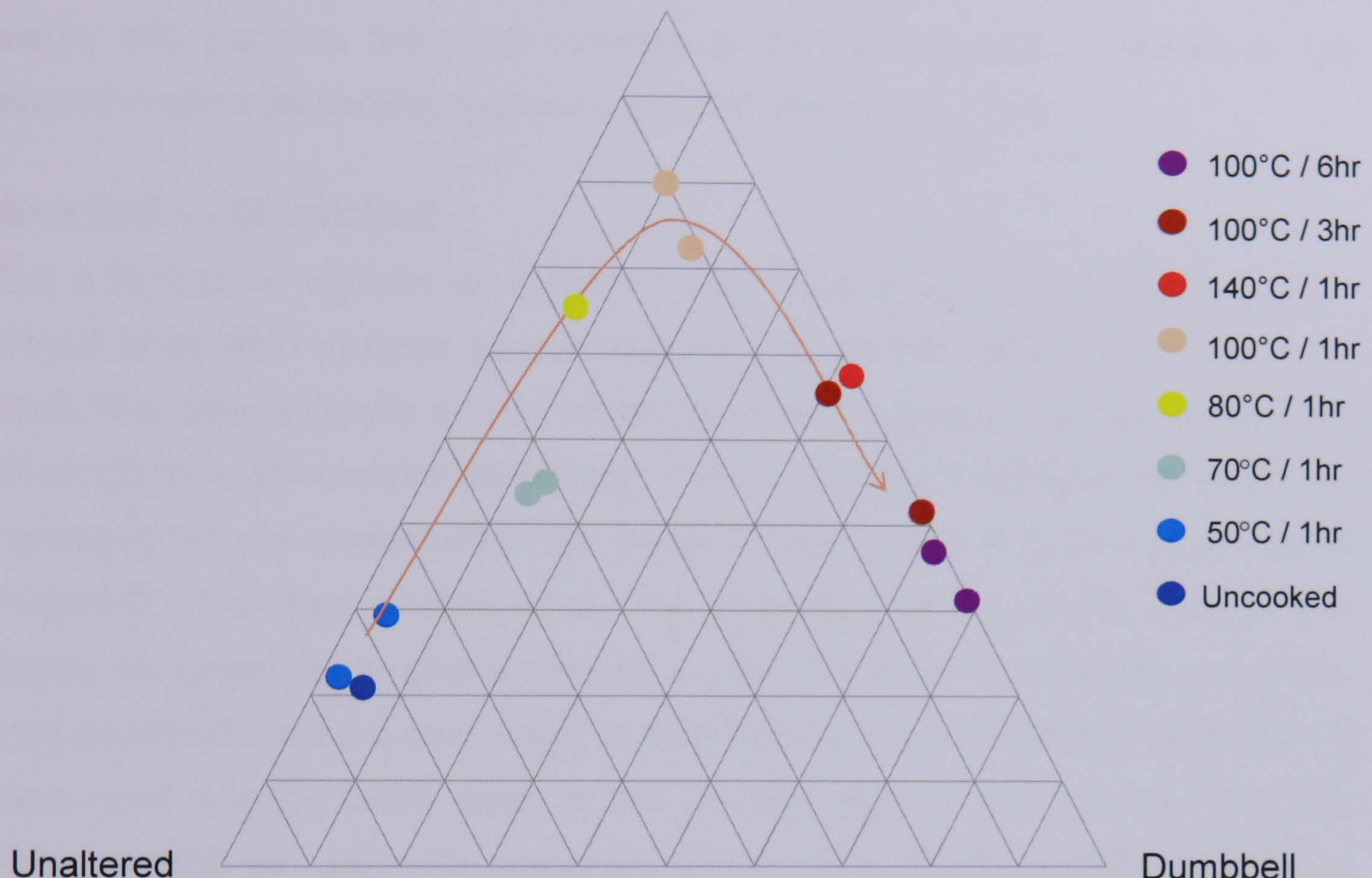


Figure 5.3 A ternplot showing the results of heating experiments conducted on mid-shaft femur bovine bone. Each point represents the proportion of *Unaltered*, *Beaded* and *Dumbbell* type fibrils record from each sample (average values from two analyses). Different coloured points represent different heating regimes. The arrow follows the trend in alteration from the least extreme through to the most extreme heating regime.

5.3.2.1 Pattern of fibril degradation

From Figure 5.3 it can be seen that the initial heat-induced change to the collagen is an increase in the proportion of fibrils exhibiting *Beaded* alteration, with little change in the amount of *Dumbbells*. It is not until most of the collagen fibrils are in a *Beaded* state that many *Dumbbell* fragments start to appear. By the time the bone has been heated at 100°C for three hours there are almost no *Unaltered* fibrils observed, and most of the collagen displays either *Beaded* or *Dumbbell* type alteration. Between three hours at 100°C and six hours at 100°C there is only a small increase in the proportion of *Dumbbells*. To explain this apparent slowing down in the rate of damage incurred by the collagen fibrils one

must take into account what actually has to happen to produce *Beaded* and *Dumbbell* alteration.

Unaltered → Beaded

For a fibril to go from *Unaltered* to *Beaded* there only needs to be a disruption to the packing of the fibril. It may exhibit a frayed end or a small region of disruption along the fibril. One would imagine that this type of damage can occur quite easily and explains the rapid increase in *Beaded* alteration between bones heated under mild heating regimes from 50°C through to 100°C.

Beaded → Dumbbell

For a fibril to be classed as *Beaded* it may have a frayed end (Figure 5.4) or frayed ends AND multiple *Beaded* regions (Figure 5.5) along the length of the fibril. The latter is clearly a more advanced state of alteration but yet for the sake of simplicity is still classed as *Beaded.* This means that a fibril can become more damaged but yet remain within the *Beaded* classification. A *Dumbbell* is a small fragment which has swollen ends. The proportion of *Dumbbells* increases at higher temperatures or after prolonged exposure to heat. It is therefore assumed that *Dumbbells* are the next stage of deterioration; and for a *Dumbbell* to form a fibril must actually break apart at the *Beaded* regions (see Figure 3.5). One *Beaded* fibril will generate many *Dumbbells*. The *Dumbbell* then appears to shorten by wastage from either end until all fibril packing is lost. Once the periodic banding pattern which allows the collagen to be definitively identified has been lost, the state of the collagen can no longer be detected using this TEM method.

These different factors explain why there is not the same rapid conversion of *Beaded* to *Dumbbell* as from *Unaltered* to *Beaded*, and why bone can be heated at 100°C for six hours and still retain 30% of the fibrils in a *Beaded* state. As a consequence the changing percentages of *Unaltered*, *Beaded* and *Dumbbell* fibrils do NOT represent a linear scale of decay, but rather the changing proportions record an estimate of the extent of deterioration within the bone.

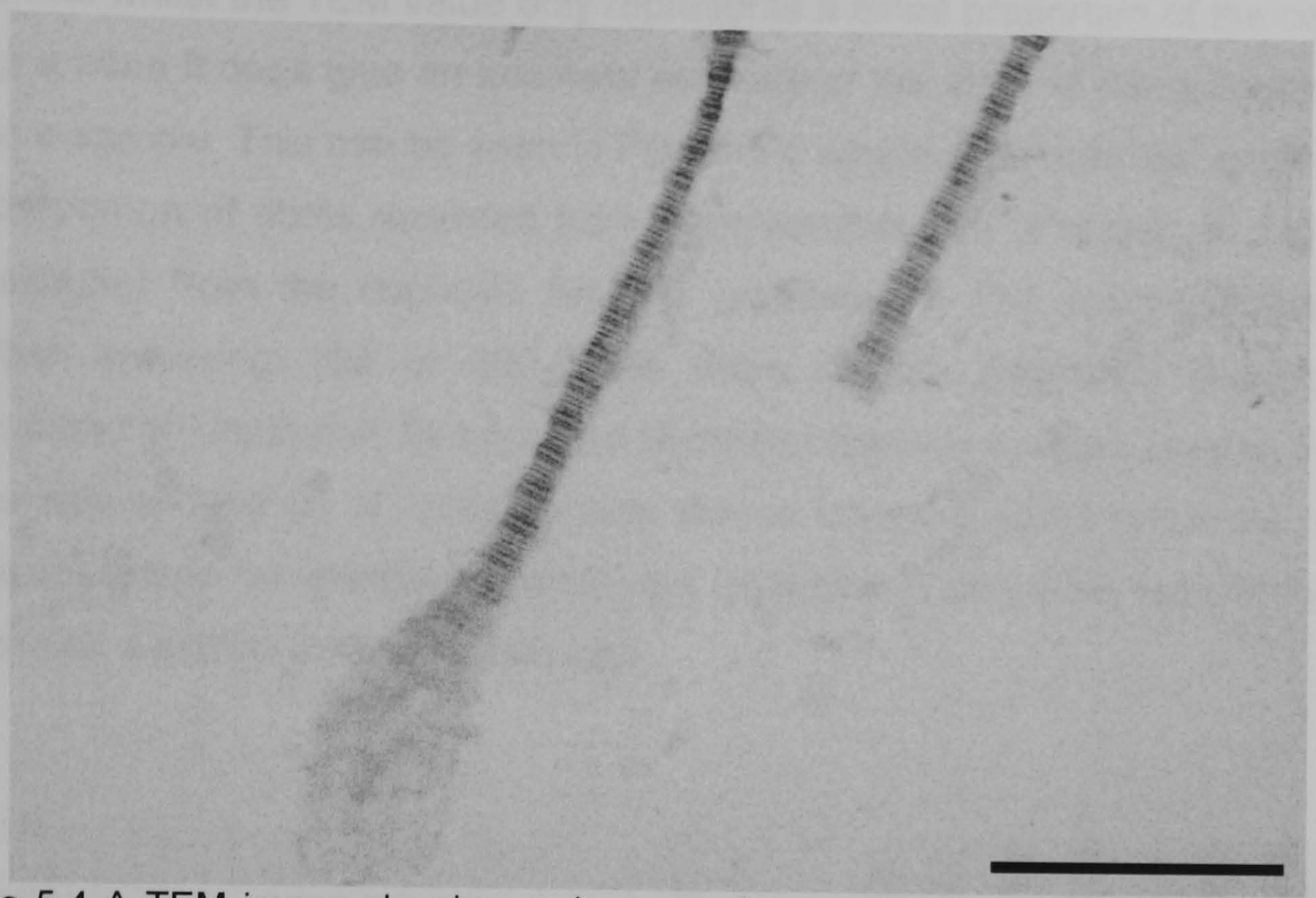


Figure 5.4 A TEM image showing a close up of the ends of two collagen fibrils, one of which exhibits heat damage in the form of a frayed end. The scale bar is 500 nm.

Figure 5.5 A TEM image showing a heat damaged collagen fibril with regions of *Beaded* alteration. The scale bar is 500 nm.

5.3.2.2 Replication

This TEM approach is only able to look at a very small representation of the collagen fibrils within the entire sample. It contains collagen from the outer and inner surface, and the middle of the bone, and will contain newly formed collagen as well as older collagen. However the replications from the heating experiments

show that whilst the TEM value only represents a small proportion of the collagen within a bone it does give an accurate estimate of the state of the collagen fibrils within a sample. This can be seen in Figure 5.6 where a stacked bar chart shows the proportion of fibrils recorded from each sample (two analyses of 100 fibrils per sample) from the duplicate heating experiments. The chart indicates that whether analysing 100 or 200 fibrils there is little difference between the proportions of *Unaltered*, *Beaded* and *Dumbbell* fibrils in a single sample. Further if two sets (A and B) of modern bone shards from the same bone are heated under the same temperature regime, but heated and prepared separately, they will exhibit a similar amount of damage.

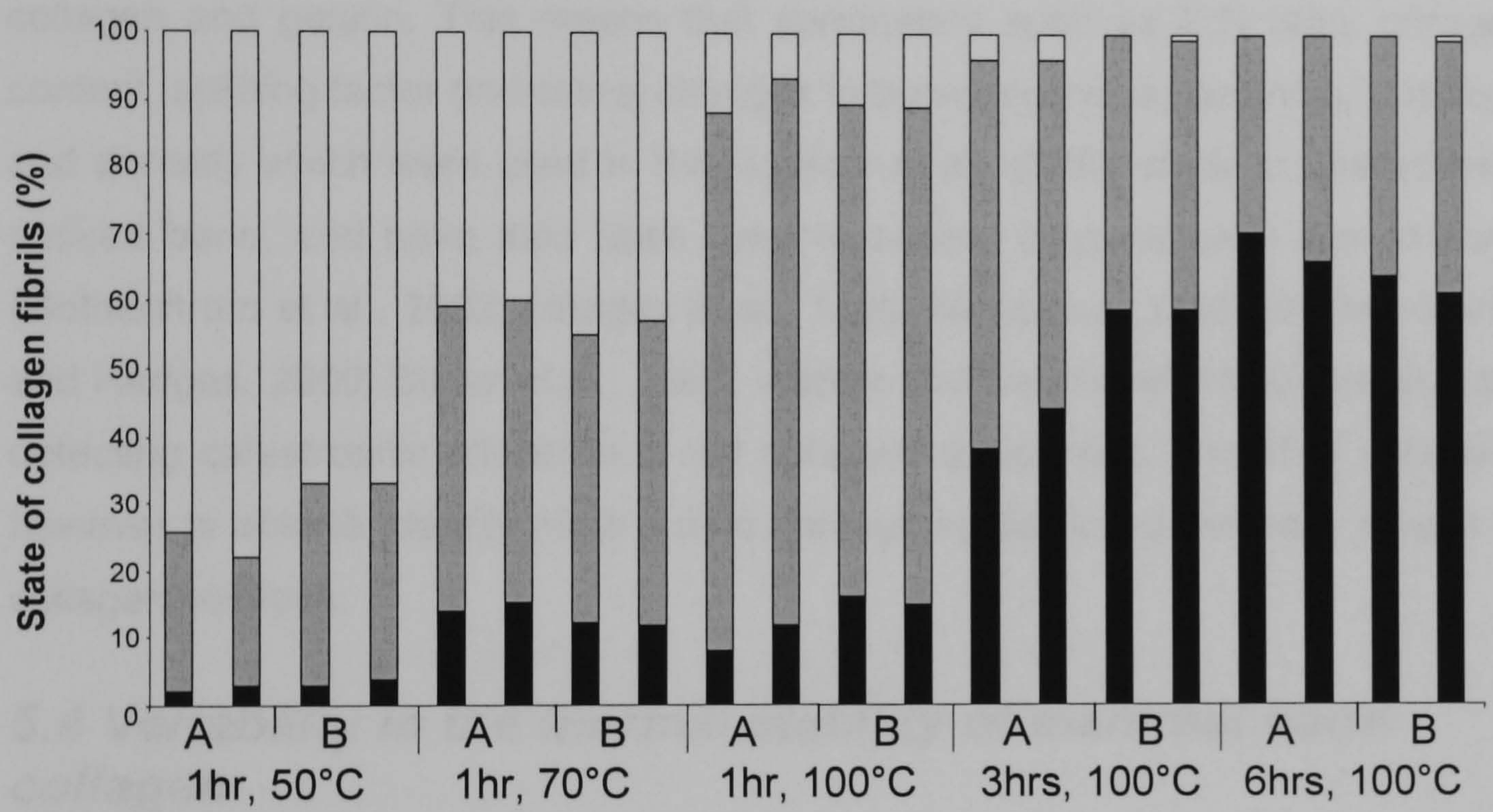


Figure 5.6 A stacked bar chart showing the proportion of *Unaltered* (white), *Beaded* (grey) and *Dumbbell* (black) type fibrils recorded from each analysis (two per sample) from the duplicate heating experiments (A and B).

5.3.2.3 Limits of TEM approach and comparisons with other heating studies

The morphological changes observed in this study are similar to those observed by Richter (1986), however these results and those from my previous heating experiments (Koon et al., 2003) suggest that the rate of degradation in experimentally heated mammal bone are somewhat slower than that of fish (*Pleuronectes platessa or Plaice*). In this study 10% of the fibrils were *Unaltered* and most were in a *Beaded* state after being heated at 100°C for 60 minutes. However, Richter (1986) found that at 100°C after 30 minutes all the collagen

had 'melted'. This is anticipated due to the lower thermal stability of fish bone collagen (Woodhead-Galloway, 1980).

The TEM analysis from the bovine specimen suggests that mammal bone heated between 70°C and 140°C for at least one hour can be detected using this technique. This level of heat treatment is very mild and is not currently detectable by other methods. Roberts et al. (2002) state that more than nine hours of boiling would be needed to show any physical or chemical alteration in bone. However after just one hour at 100°C the TEM approach shows that almost all of the observed collagen fibrils are thermally altered. By nine hours at 100°C the TEM (and DSC) analysis showed that the collagen is already extremely degraded; when removed from the mineral it is only observed at small fragments of fibrillar collagen and gelatin. This means that parameters such as C:N ratio, nitrogen content, splitting factor (indicating changes in bone mineral crystallinity), histology and porosity which were used in the Roberts et al. (2002) study to characterize cooked bone, and have also been used to assess diagenetically altered bone (Gotherstrom et al., 2002; Hedges et al., 1995; Nicholson, 1998; Nielsen-Marsh and Hedges, 2000; Stiner et al., 1995; Weiner and Bar-Yosef, 1990) are actually detecting catastrophic alteration to the collagen component. The TEM approach however is able to identify more subtle damage by detecting the early stages of collagen collapse.

5.4 Variability in the thermal stability of mammal bone collagen

Studies of bone diagenesis commonly compare mammal bone from archaeological sites without making any discrimination between species (except human). Thereby implying an assumption that there is little difference in the structure and composition, and in the rates of degradation in the burial environment, between bones from different mammalian species (Jans et al., 2004; Nielsen-Marsh and Hedges, 2000; Trueman and Martill, 2002). It has been found, however, that bovine bone collagen gelatinizes faster than human bone collagen when bone shards are boiled at 95°C (Dobberstein, unpublished data). If bone collagens from different mammal species exhibit different degrees of stability, as suggested by these results on gelatinization rates, this could bias the survivorship of different species within the burial environment and also complicate the use of the TEM technique to identify cooked bone.

In this set of heating experiments the aim was to determine whether mammalian bone from common domestic species would exhibit a similar degree of thermal alteration when heated at 80°C for one hour. This regime was chosen because the previous section (5.3) had shown that whilst only mild, this temperature would produce a mixture of *Unaltered*, *Beaded* and *Dumbbell* type alteration. A more extreme regime would cause all of the samples to display a large proportion of damage and thus any subtle differences might be difficult to identify. In contrast to the previous section (5.3), acid treatment was used because the demineralization experiments (see Chapter 3) had suggested this treatment could be used to accentuate differences in the extent of thermal alteration and swelling between different collagen samples.

5.4.1 Materials and methods

Fresh mid-shaft bone samples were obtained from five specimens including pig, sheep, cow, horse and human. The pig (tibia) and sheep (femur, mutton) specimens were obtained from a butcher. The cow (femur, seven years-at-death) specimen was obtained from an abattoir. Details pertaining to the post-mortem treatment of these samples can be found in section 3.2.2.1. Details regarding the procurement of the young human (femur, 14 years-at-death) and old human (femur, 62 years-at-death) can be found in section 3.2.2.2. The horse (radius, two years-at-death) sample came from a filly. It had been stored intact in a freezer for eight years. Prior to any further treatment a section was removed from the mid-shaft of the radius and this was left to defrost at 4°C.

Sub-samples of bone were removed with a water cooled saw. These samples were broken into medium sized shards (typical dimension 5 mm). 180g of the shards were placed in a glass vial and then heated in a pre-heated heating block at 80°C for one hour. After the heat treatment the glass vials containing the samples were quenched in an ice bath to rapidly reduce the temperature. The samples were then split into small shards (largest dimension ≤ 3 mm) with a hammer and then demineralized using the HCl method (section 3.3.2.1). A further set of unheated shards were also demineralized in the same way. Once demineralized the samples were washed several times with ultra pure water and placed in PBS at 4°C until they were prepared for TEM analysis. The TEM sample preparation method is described in section 3.3.4.2.

5.4.2 Results and discussion

It was anticipated from the demineralization experiments (Chapter 3) that the HCl treatment would produce more damaged fibrils than EDTA. A comparison of the TEM results from the EDTA (see Figure 5.3) and HCl (Figure 5.7) demineralized bovine samples confirms that this is the case. The acid treatment was used however to allow differences in the stability of the collagen fibrils (based on the extent of damage and swelling) to be accentuated. The results of the TEM analysis (Figure 5.7) however suggest that collagen from all of the samples heated at 80°C for one hour display a similar amount of damage regardless of species; the majority of the fibrils were in a *Beaded* state with a smaller proportion of *Dumbbell* and *Unaltered* fibrils also present.

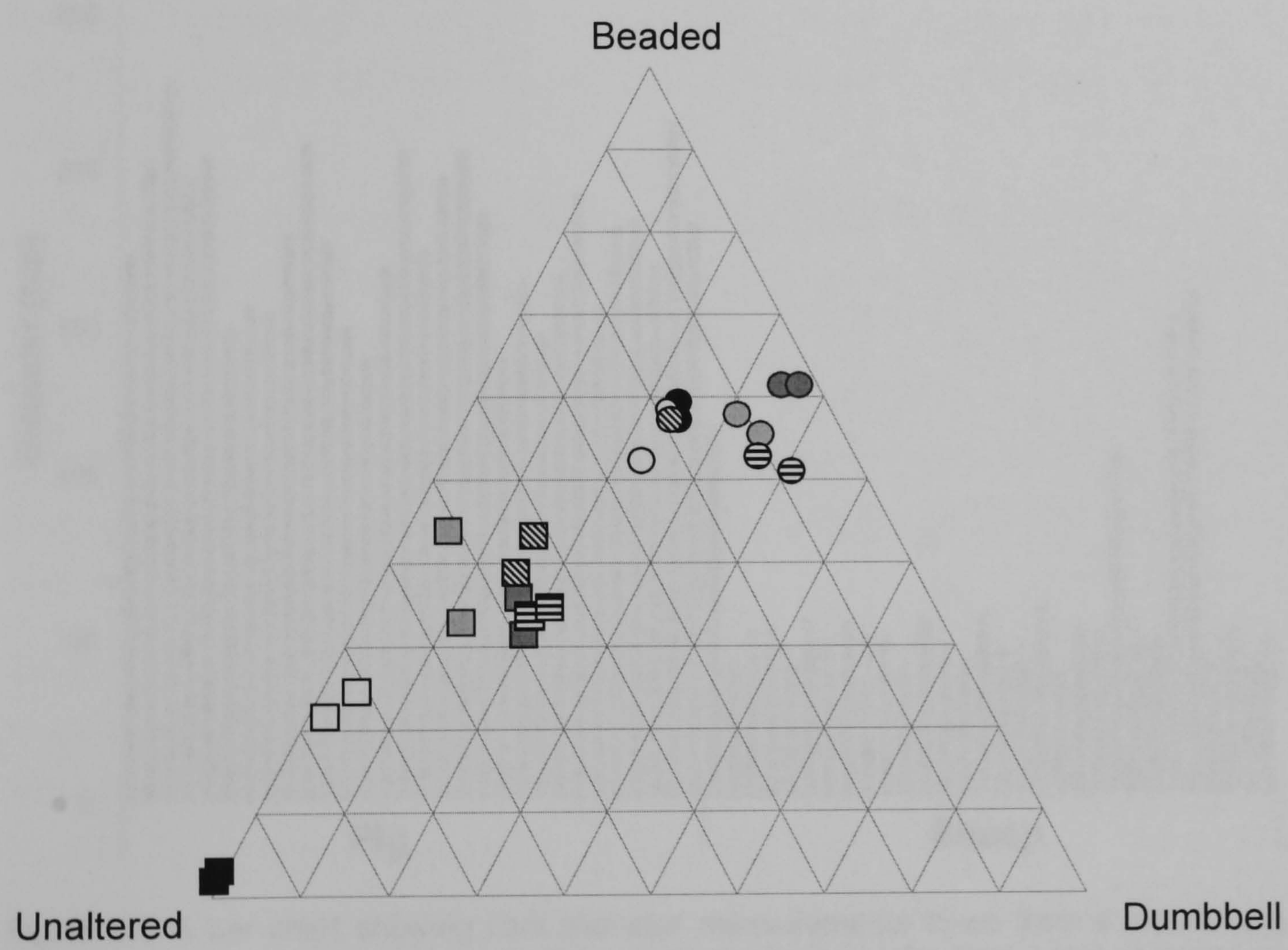


Figure 5.7 A ternplot showing species variability in the stability of collagen fibrils from heated (circles) and unheated (squares) modern samples. TEM analysis was conducted on acid demineralized mid-shaft compact bone shards from pig (light grey), sheep (black), horse (diagonal lines), cow (horizontal lines) and human specimens (14 years; dark grey, 62 years; white). Each point represents the proportion of *Unaltered*, *Beaded* and *Dumbbell* type fibrils recorded for each analysis (two analyses per sample).

There were however marked differences in the extent of swelling observed between samples; the pig and 14 year old human specimens displayed extensive swelling; the cow and horse displayed a mixture of fully swollen and non-swollen fibrils; yet the sheep and 62 year old human specimens did not show any evidence of swelling. Apart from a small proportion of very large fibrils the majority of the fibrils from the sheep sample were between 40-60 nm (see Figure 5.8), which is consistent with values obtained from bone collagen (Tzaphlidou, 2005; Tzaphlidou and Berillis, 2005). By comparison the average diameter of fibrils from the unheated pig sample was approximately 3-4 times wider than that of the unheated sheep sample (see Figure 5.8).

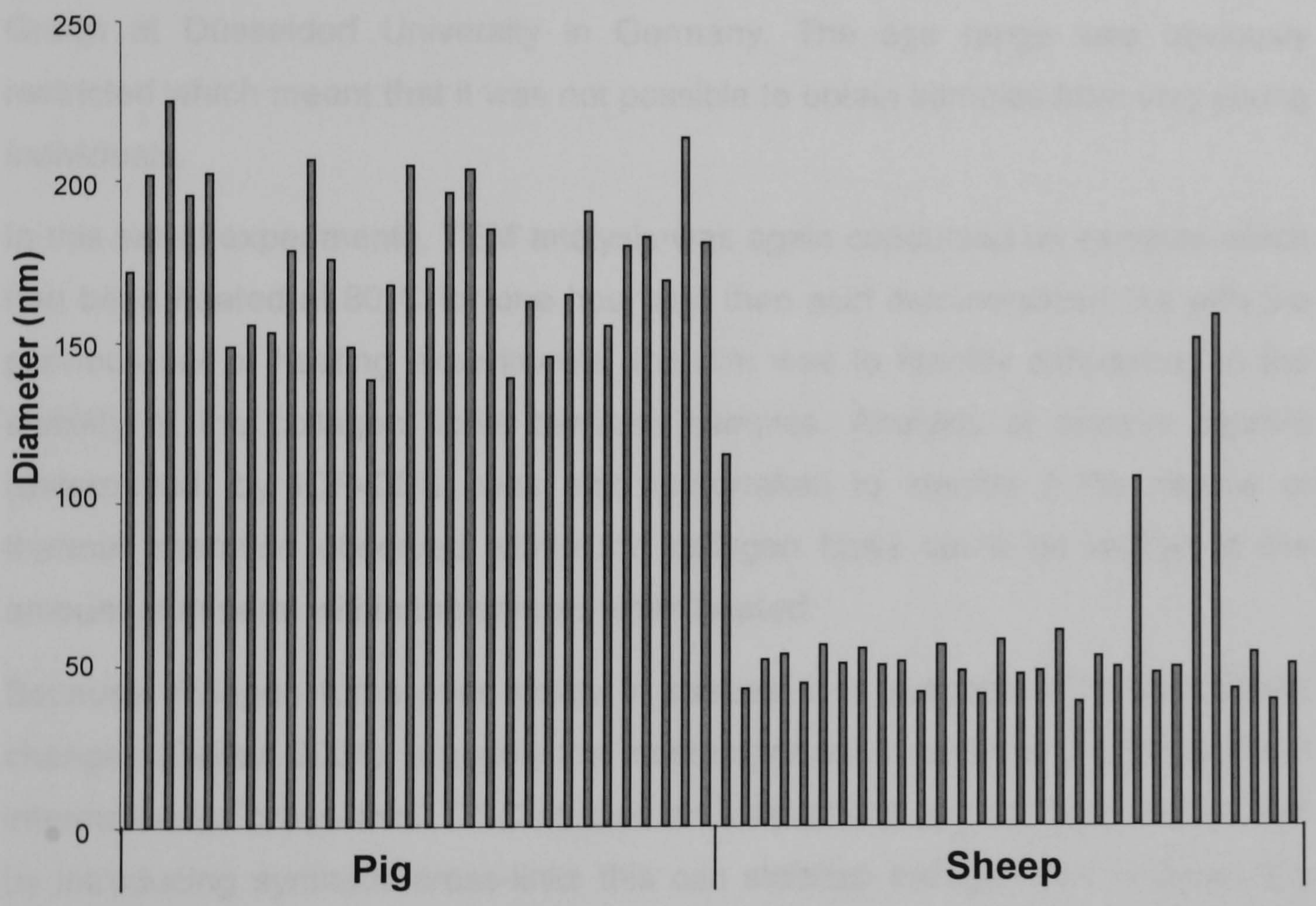


Figure 5.8 A bar chart showing fibril diameter measurements taken from a modern pig and sheep bone samples which had been demineralized in HCl. Each value is based on the average of five measurements taken from undamaged regions along a fibril and 30 fibrils were measured per sample.

The unheated samples from the sheep and 62 year old human specimens also exhibited less damage than the rest. The greater resistance to damage and swelling exhibited by the 62 year old, compared to the 14 year old human and the younger animal specimens may reflect an age related stability and this possibility is explored further in the following section. The stability of the collagen from the

sheep specimen is not easily explained however *Unaltered* fibrils from other sheep bones were also found to be resistant to swelling in HCl (Koon et al., 2003). Acid swelling experiments would have to be conducted on a larger set of sheep and other animal bones of known ages at death to determine whether these differences are consistent and significant.

5.5 Effect of age-at-death on the thermal stability of the bone collagen

To investigate the effect of age on bone collagen stability, a set of modern human bone samples of known age at death were analysed. Human bone covers a wider age range than the animal bone used in the previous section, and it has direct relevance to forensic cases and archaeological inhumations. A small number of forensic samples were used for this study provided by the Ageing Research Group at Düsseldorf University in Germany. The age range was obviously restricted which meant that it was not possible to obtain samples from very young individuals.

In this set of experiments, TEM analysis was again conducted on samples which had been heated at 80°C for one hour and then acid demineralized. As with the previous set of heating experiments, the aim was to identify differences in the stability of the collagen fibrils between samples. Analysis of calcium content (determined, by ICP-OES) was also undertaken to identify if the degree of thermal alteration observed within the collagen fibrils could be related to the amount of mineral within the sample when heated.

Because collagen turns over slowly in tissues, it is susceptible to age-related changes (Bailey, 2001); arguably the most important of which is the formation of intermolecular cross-links. DSC studies on leather and tendon have shown that by introducing synthetic cross-links this can stabilize collagen and increase the temperature at which denaturation takes place (Covington, 2001; Kronick and Buechler, 1986; Miles et al., 2005; Usha and Ramasami, 2000). The same technique will be applied to mineralized and demineralized bone collagen to investigate the stabilizing effect of *in vivo* cross-linking.

5.5.1 Methods and materials

Thirteen modern specimens both male and female and ranging in age from 14 to 73 years were analysed. Cross-sections of bone were provided from non-infectious mid-shaft femur bone (see section 3.2.2.2 for details pertaining to these samples).

Sub-samples of bone were removed from the anterior and lateral aspects with a water cooled saw. These samples were broken into medium sized shards (typical dimension 5 mm). 60 mg of the shards were placed in a glass vial and then heated in a pre-heated heating block at 80°C for one hour. After the heat treatment samples were quenched in an ice bath to rapidly reduce the temperature of the bone, and then split into small shards (largest dimension ≤ 3 mm) with a hammer. A further set of unheated shards were also prepared from the same specimens. 60 mg from each sample was then demineralized using the HCl method (see section 3.3.2.1). The heated collagen samples were prepared for TEM analysis (see section 3.3.4.2 for the preparation method). The unheated demineralized collagen samples were prepared for DSC analysis along with two unheated mineralized samples; one old (62 years) and one young (14 years) (see section 3.3.5.2 for the preparation method). Samples were run in the DSC in medium pressure steel pans using the temperature range 15-195°C and a heating rate of 5°C min^{-1}. A further set of mineralized shards were prepared for Ca analysis using ICP-OES (see section 3.3.3.2 for the preparation method). A summary of the analysis that was undertaken on each sample is provided in Table 5.3.

Table 5.3 A summary of the analysis that was conducted on human samples in order to identify evidence of an age-related thermal stability within bone collagen.

Age at death	**Sex**	**Analysis**		
		TEM	**DSC**	**Ca content**
14	female	yes	yes	no
16	male	yes	yes	yes
17	male	yes	no	no
22	male	yes	no	yes
29	female	yes	no	no
32	male	yes	yes	no
33	male	yes	no	yes
47	male	yes	yes	no
51	female	yes	no	yes
52	female	yes	no	no
52	male	yes	no	yes
62	male	yes	yes	yes
71	male	yes	no	yes

5.5.2 Results

5.5.2.1 TEM analysis

Figure 5.9 shows the proportion of *Unaltered* fibrils observed from TEM analysis of each of the human bone samples. For the male samples (shown in black) there was an increase with age in the proportion of fibrils which were unaffected by heat treatment. There appears to be a sharp increase in the proportion of these more heat stable fibrils in the final stages of skeletal maturation (14-23 years; Buikstra and Ubelaker (1994) and a further increase between 30 and 50 years of age. A much smaller group of female samples (shown in white) did not display an obvious pattern.

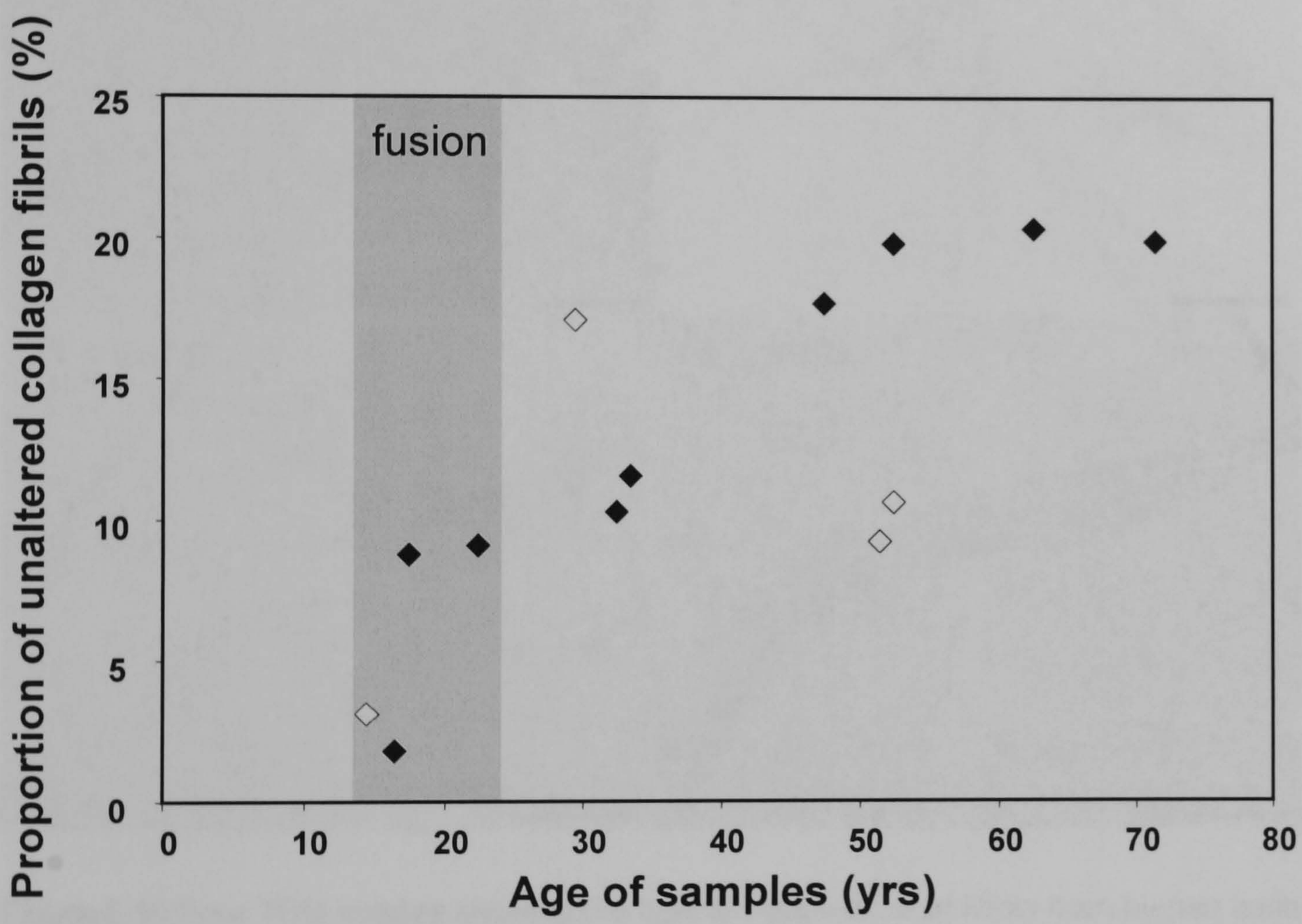


Figure 5.9 A scatter chart showing the relationship between age and susceptibility to thermal alteration. Human bone samples of different ages-at-death were heated (80°C for one hour) and subsequently demineralized in acid. The proportion of *Unaltered* collagen fibrils observed by TEM are plotted against age; the black points indicate male samples and white for female. The grey zone denotes the period within which the final stages of skeletal maturation take place.

As was the case with the animal bones, the human collagen samples differed in the extent to which the fibrils were affected by acid demineralization. There was a marked difference in the appearance of the heat-damaged fibrils between young and old specimens (see Figure 5.10). The fibrils from the two youngest samples

(14 and 16 years) were all swollen and the banding pattern was often completely lost (e.g. Figure 5.10; 14 years). The collagen fibrils from the 17 and 22 year old samples retained their banding pattern but were again completely swollen and faint (e.g. Figure 5.10; 17 years). By comparison, in the older samples the majority of the fibrillar structure remained intact. The fibrils were more resistant to swelling and only discrete regions of alteration were observable along the fibrils and at the ends (e.g. Figure 5.10; 51 and 62 years).

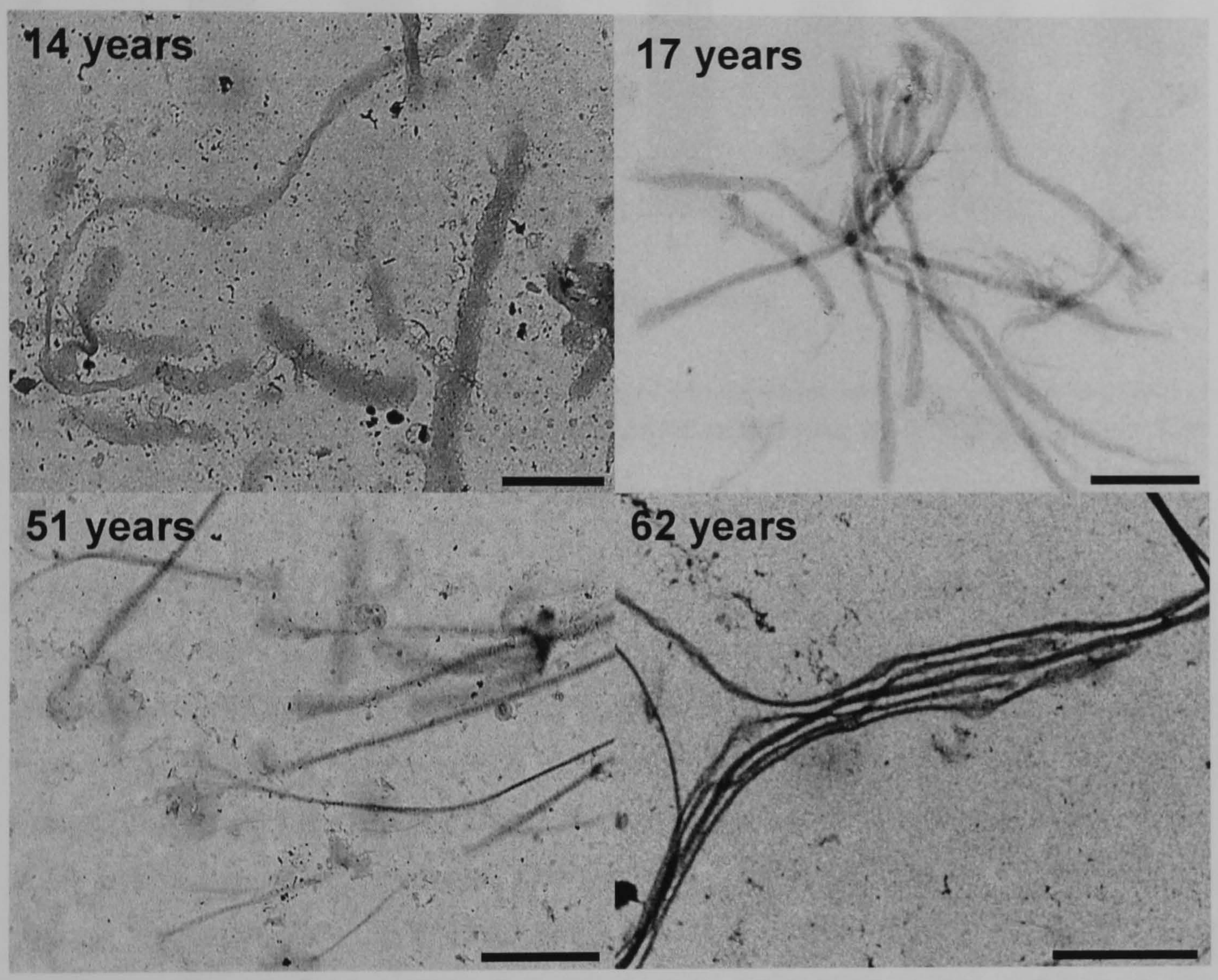


Figure 5.10 Four TEM images showing the typical appearance of fibrils from human bone specimens with different ages at death. The samples had been heated at 80°C for one hour and then demineralized in HCl. The scales bars are 2µm.

5.5.2.2 Calcium content and DSC analysis

The Ca content, determined by elemental analysis, ranged from 25-33% (Figure 5.11). The three adult males (22-52 years) produced almost identical Ca values and these were the highest of the samples analysed. Assuming a general formula of $Ca_{10}(PO_4)_6OH$ for bone apatite this would give values for the mineral content of between 60-78%.

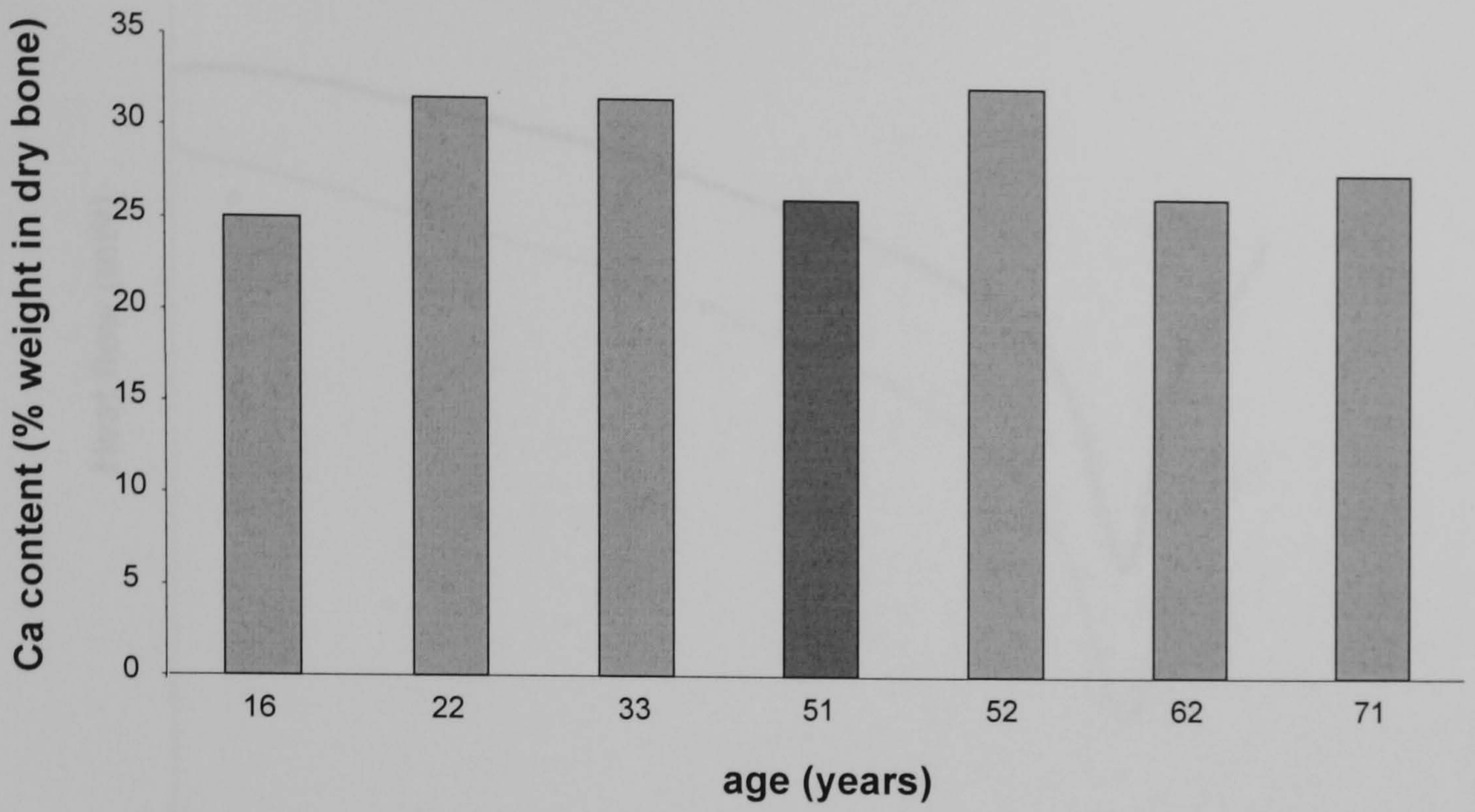


Figure 5.11 A bar chart showing the Ca content of mid-shaft femur bone from humans of different-ages-at death. The one female specimen which was analysed is shown in dark grey.

Figure 5.12 shows a combined DSC trace of mineralized bone from a young (14 years) and old (62 years) human sample. The endotherm was in the same position for both samples; suggesting that this transition is not affected by age. In Figures 5.13 and 5.14 the onset, peak max and peak end values, from DSC traces of five of the demineralized human samples have been plotted. Figure 5.14 shows the values obtained from the first transition. The peak max values ranged between 59°C and 63°C but were not correlated with the age of the specimen. Figure 5.14 shows that there was a change in the position of the second high temperature peak which appeared to be related to the age of the specimen; the peak was broader and shifted to a slightly higher temperature in older samples.

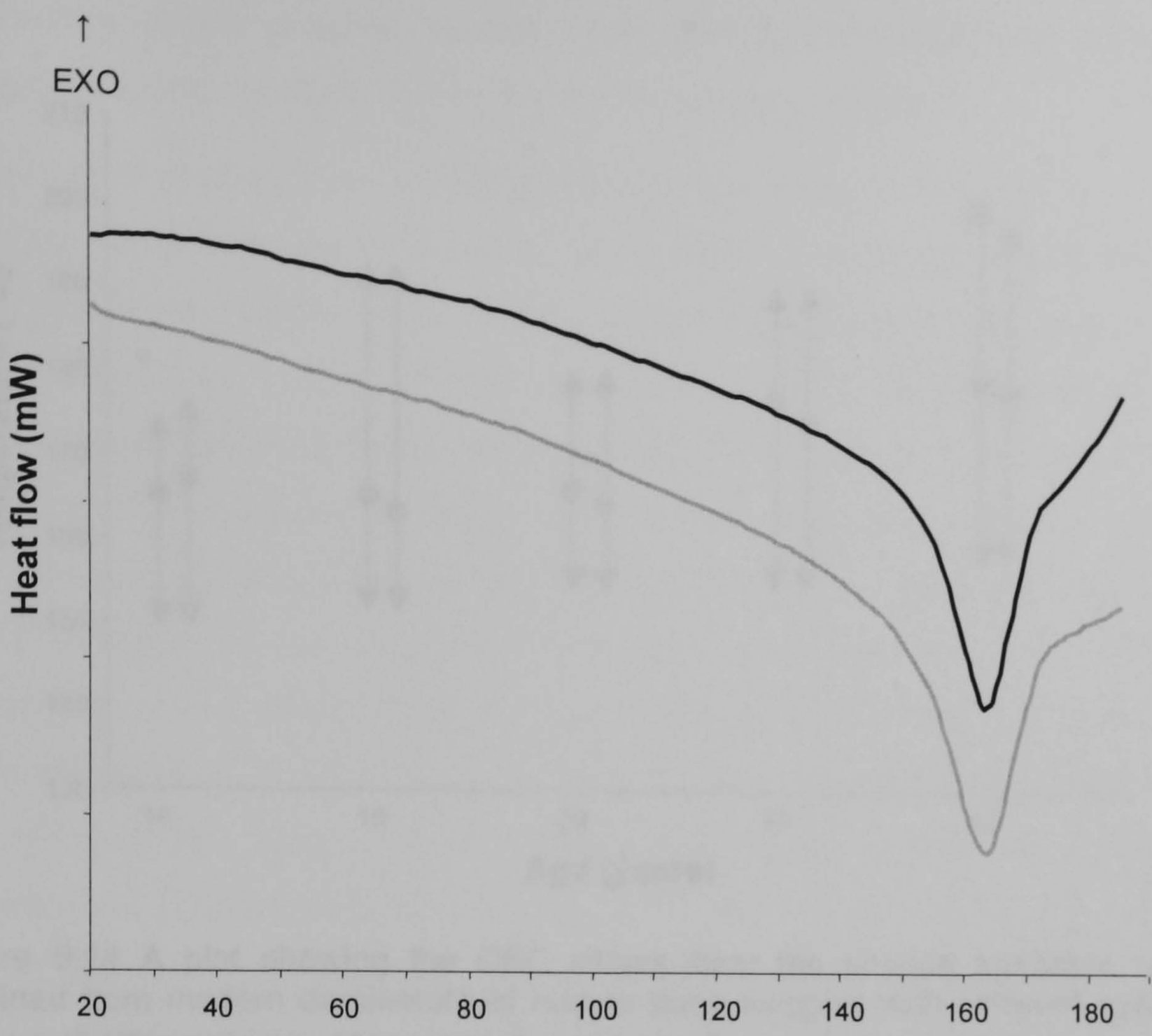


Figure 5.12 A combined DSC trace showing a typical trace obtained from 14 year old (shown in black) and 62 year old (shown in grey) mineralized human bone. The DSC traces are to the same scale but have been offset along the y-axis so that they can be viewed more easily.

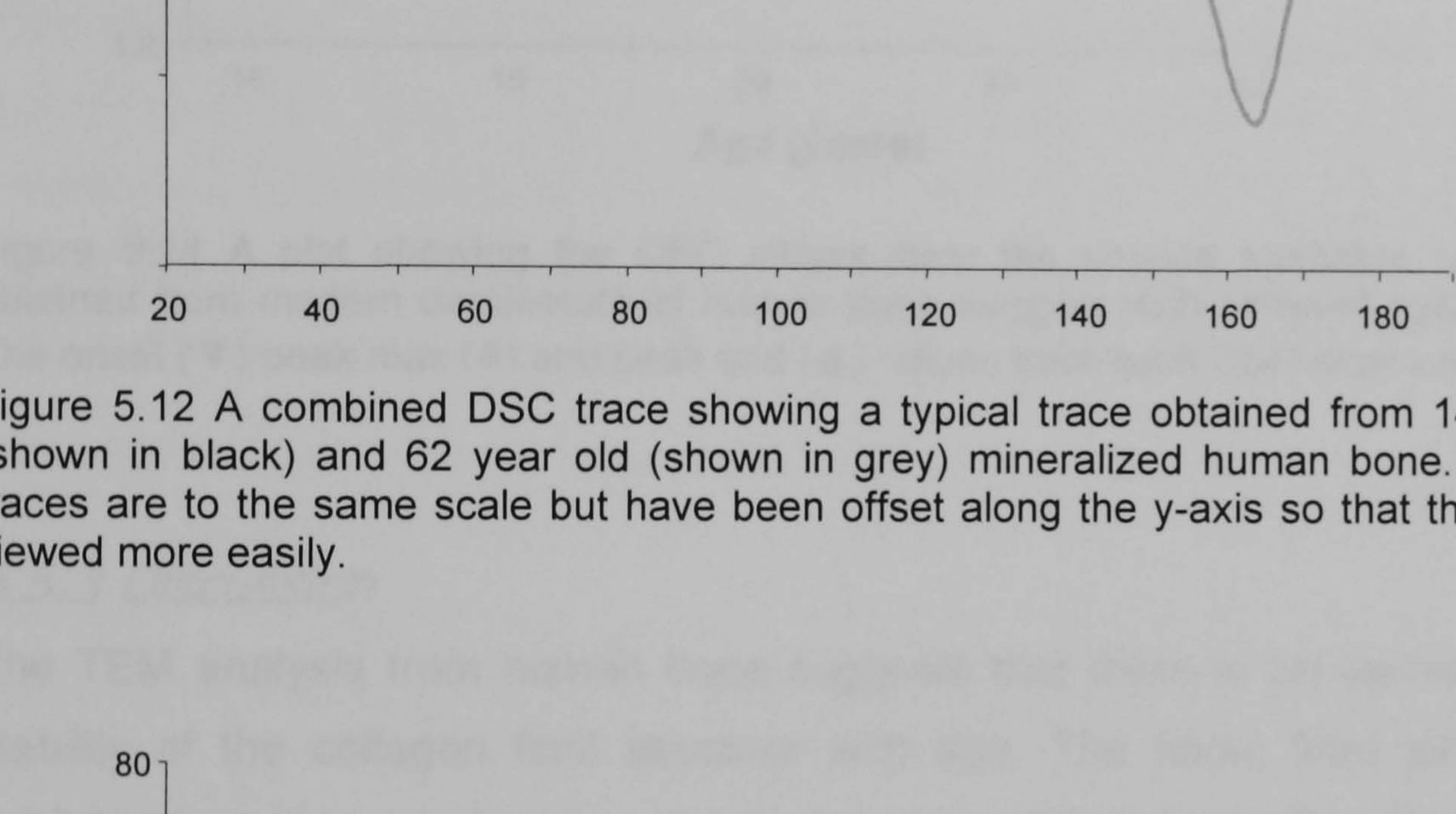


Figure 5.13 A plot showing the DSC values from the first transition which were obtained from modern demineralized human bone samples with different ages-at-death. The onset (▼) peak max (●) and peak end (▲) values from each DSC scan are shown.

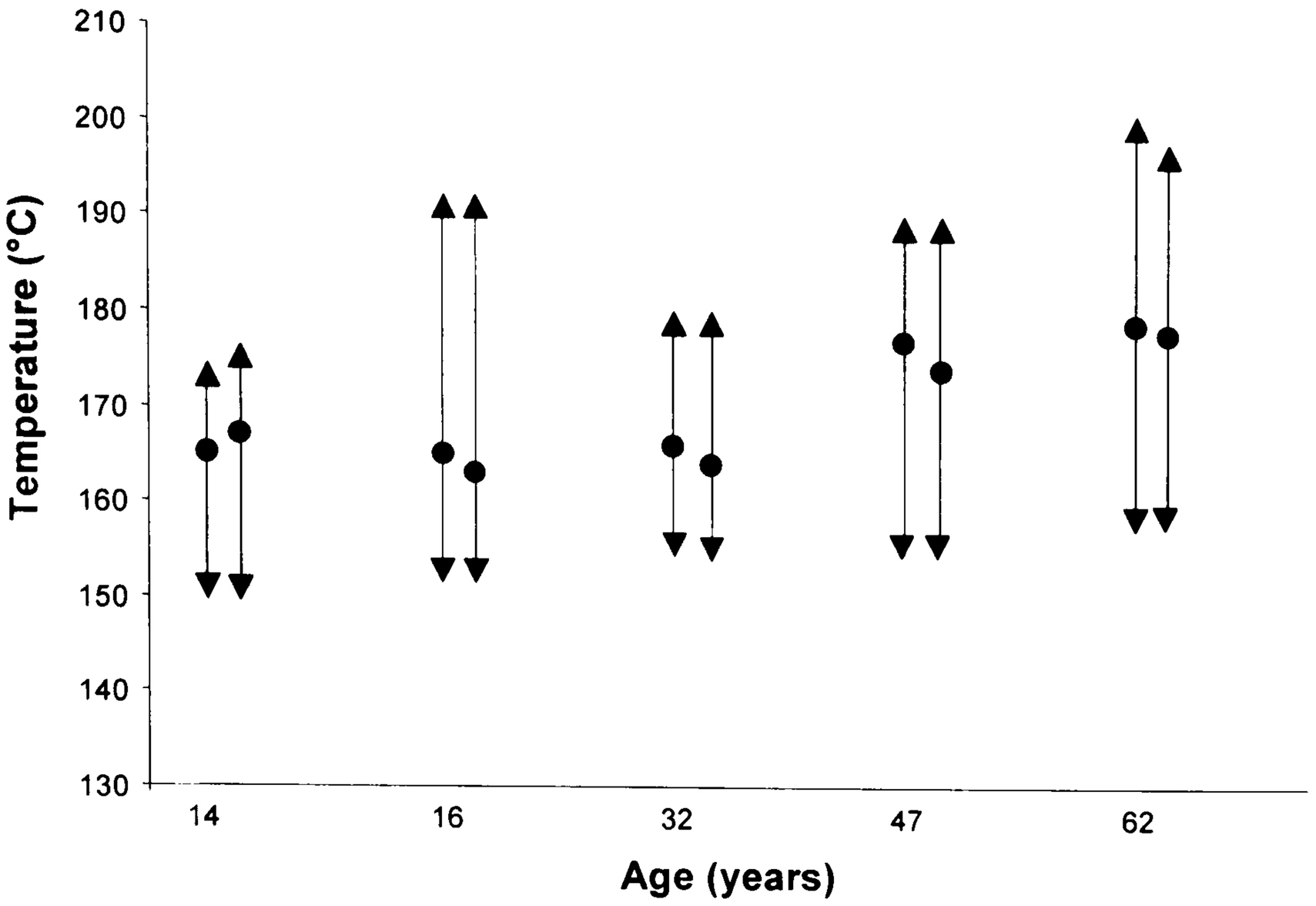


Figure 5.14 A plot showing the DSC values from the second transition which were obtained from modern demineralized human bone samples with different ages-at-death. The onset (▼) peak max (●) and peak end (▲) values from each DSC scan are shown.

5.5.3 Discussion

The TEM analysis from human bone suggests that there is an increase in the stability of the collagen fibril structure with age. The fibrils from older males exhibited less thermal alteration and were more resistant to swelling than those of younger males. The pattern was less clear for the smaller group of females; perhaps reflecting fluctuations in bone turnover due to exposure to different hormonal and physiological events, such as childbirth. The Ca content values suggested that the amount of mineral within the samples was not related to age, except perhaps in the case of the adolescent male (16 years). The bone mineral content of the mid-shaft femur has been observed to increase up to skeletal maturation (Moro et al., 1996); the adolescent sample may not yet have reached skeletal maturation, thus explaining the slightly lower Ca content, compared to the young and middle aged adult males. The lower values obtained for the female and two older males are not so easy to explain; although the mineral content in adult human femur bone has previously been found to be variable and not related to age (Zioupos, 2001). It is clear that the mineral content can not explain the greater integrity observed in the fibrils with age. An increase in the concentration of cross-links could however explain the TEM observations; a large network of

cross-links could prevent swelling and inhibit microstructural damage from spreading along the fibril, by holding the structure together.

A number of studies have investigated changes in the cross-link concentration of collagen as a function of age. In newly formed collagen, the cross-links are divalent and heat labile, however they spontaneously combine to form multivalent stable cross-links in mature tissue (see section 1.3.6). There is a steady increase in the concentration of these mature cross-links up to skeletal maturation. After this point the collagen is still being turned over as bone continues to be remodelled throughout life. Some researchers have suggested that there is a slight increase in the quantity of mature cross-links in bone collagen after maturation, whilst others have recorded no significant change (Bailey and Paul, 1999; Wang et al., 2002b; Zioupos et al., 1999) and even a slight decrease in old age (Eyre et al., 1988). However, there is a steady increase in the stiffness of collagenous tissues with age. This is thought to be due to a secondary cross-linking reaction with glucose or its metabolites that occurs with age as the turnover of collagen is reduced (Bailey and Paul, 1999; Li et al., 2003).

One might expect that an increased quantity of cross-links would elevate the temperature at which bone collagen is denatured. Despite the small number of samples in the current study (n= 5, age range 14-62 years), the denaturation temperature (based on the peak max value) ranged from 59 to 63°C and was not affected by age. This is consistent with the results from Danielsen et al. (1994) who looked at the effect of age on the shrinkage temperature (T_s) of human bone. The large endothermic transition which is observed by DSC is accompanied by a macroscopic shrinkage of the tissue, referred to as the shrinkage temperature (Haines, 1987; Wright and Humphrey, 2002). Danielsen et al. (1994) looked at cortical bone collagen from iliac crest biopsies and found that in normal males the mean T_s varied between 62 and 64°C in the age range of 21-63 years, and was not related to age. Beyond 63 years there was a decline in the T_s to between 59 and 62°C (Danielsen et al., 1994). Zioupos (2001) also obtained T_s values, in this instance from mid-shaft femur, using isothermal tension; mean values ranged from approximately 52 to 58°C and displayed a negative correlation with age. The differences between the values obtained may in part be due to the three different methods which were used to measure the transition; DSC (this study), area shrinkage using spectrophotometry (Danielsen et al., 1994) and isometric tension (Zioupos, 2001). Also in the present study samples were taken from the anterior and lateral regions of the mid-shaft sections. The study by Zioupos

(2001) showed up to a 6°C difference within the same mid-shaft cross-section, depending on whether the sample was taken from the lateral, anterior or posterior aspect.

The apparent age-related stabilization exhibited by the second high temperature peak in demineralized collagen is interesting but, unfortunately without knowing what this peak actually represents, it is not possible to comment on the significance of this finding.

5.5.4 Summary

The aim of this set of heating experiments was to determine the effect of age-at-death on the thermal stability of the bone collagen. This is more complicated for bone than for non-mineralized tissue because it is a composite material containing both collagen fibrils and mineral crystals. Thus the properties of collagen in bone are dependent not only on its structural integrity but also on the presence of the mineral. The analysis of the human samples showed a progressive increase with age in the resistance of the collagen fibril to collapse under the conditions used for TEM visualization. The DSC analysis showed that the low temperature denaturation transition observed in the demineralized samples and the high temperature transition observed in the mineralized samples were not affected by age. It would appear therefore that an age-related mechanism; presumably covalent cross-links helps to preserve the structural integrity of the collagen fibrils in bone when exposed to heat (and acid swelling). This mechanism however is not sufficient to increase the denaturation temperature of the demineralized collagen, and when the mineral is present it is this which gives bone collagen its superior thermal stability not the structural integrity of the fibrils themselves.

Flat fibrils

Collagen fibrils are considered to have a cylindrical structure. Yet during this research a number of unusual flat fibrils were observed, which do not appear to have been previously documented (Figures 5.15). The flat 'ribbons' were found in immature and young adult bone collagen which had been demineralized in acid for 14 days (see section 3.3.2.1). The ribbons were more common in bone samples which had been heated beforehand and were completely absent in the sheep sample and the older human samples (49-71 years). They were also not observed if samples were demineralized using EDTA. The ribbons were less electron dense than the normal fibrils and had diameters of 200-250 nm. The d-

spacing was measured from TEM images at 64 nm +/- 0.4 (to the nearest nm, based on the average value from 30 fibrils, +/- S.D.), consistent with native type I collagen (Kadler, 1994). A number of the ribbon–like fibrils appeared to be in a state of unwinding (an example of this if shown in the top image in Figure 5.15).

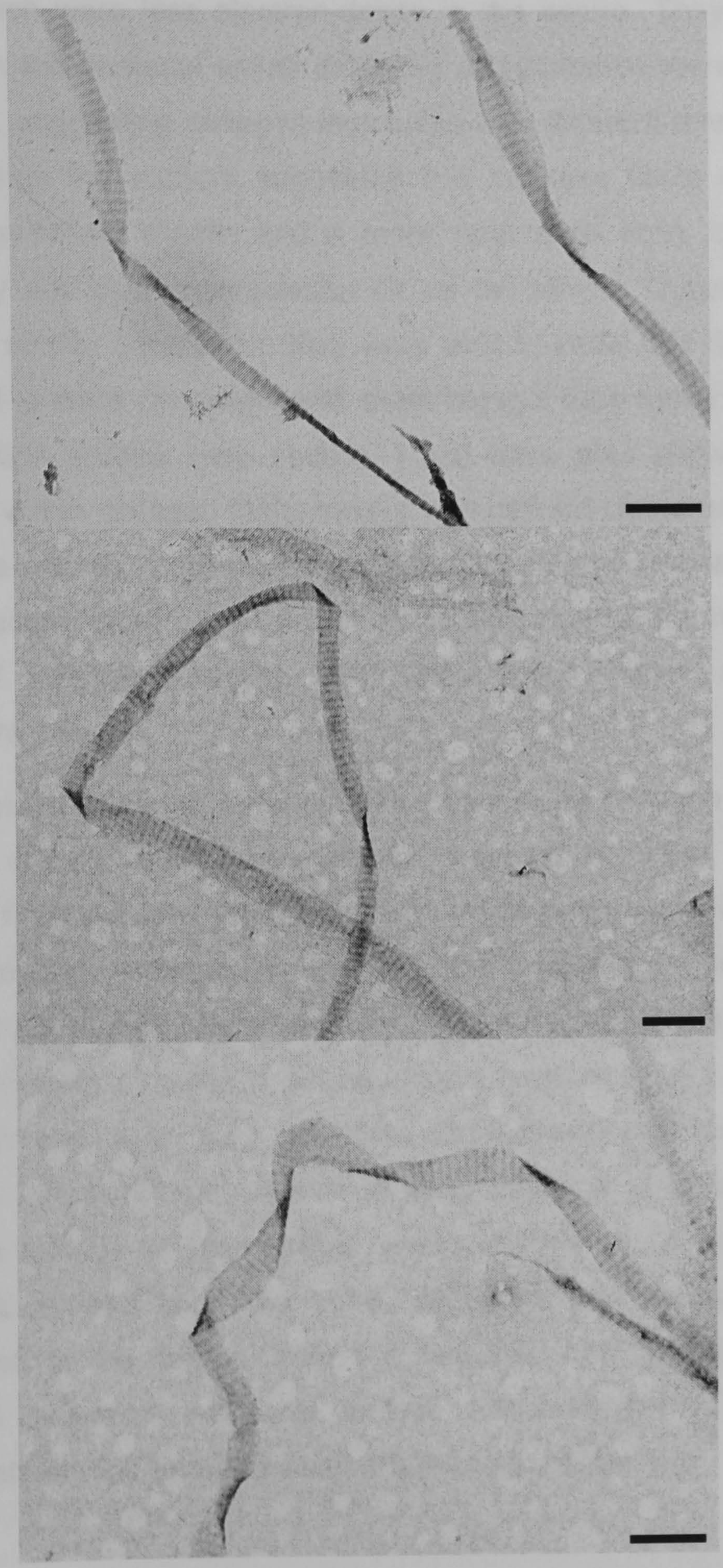


Figure 5.15 TEM images showing examples of flat fibrils from unheated acid-demineralized pig bone collagen. The scale bars are 1μm.

The identification of flat fibrils may relate to recent discoveries which suggest that type I collagen fibrils are in fact tubular in shape with an outer sheath and a soft, inner core (Gutsmann et al., 2003; Kronick et al., 1988; Williams et al., 2005). Kronick et al. (1988) showed images of rat tail tendon collagen fibrils in cross-section which were less electron-dense in the centre. They also showed DSC traces from this material which exhibited a high-temperature shoulder after the main peak, suggesting collagen molecules with different thermal stabilities; from these findings the authors suggested that collagen fibrils are composed of a highly cross-linked sheath and a more heat-labile core. Using Atomic Force Microscopy and micromanipulation on rat tail tendon Gutsmann, et al., (2003) came to a similar conclusion; they were able to show that the way in which the fibrils bend is more consistent with them being a tube rather than a solid rod. X-ray diffraction studies (see section 1.3.5) have also shown that whilst some molecules within collagen fibrils have a well-defined crystal structure many are in a liquid-like disorder (Hulmes et al., 1995; Prockop and Fertala, 1998). Based on x-ray diffraction studies Hulmes et al. (1995) proposed a model for the lateral packing of collagen molecules within fibrils which showed a disordered central core (Figure 1.9).

It is suggested that in the present study by leaving collagen in dilute acid for 14 days the sheath, being the ribbons observed by TEM, somehow became separated from the core (which had presumably been gelatinized and lost into the demineralization mixture). As the flat fibrils were not observed in the older individuals perhaps the heterogeneity within the collagen fibrils is only present in younger collagen. In older individuals where there is a build up of older collagen, the sheath and core may be highly cross-linked and therefore can not be separated. If this is the case it is likely that the collagen fibrils from older individuals would be less flexible which in turn could be detrimental to the mechanical properties of the bone. These images strongly indicate that the organization of the fibril is more complex than has hitherto been appreciated. Interaction between individual helices (cross-linking) is clearly directional, perhaps relating to the original self-organization of the fibril.

5.6 Implications

5.6.1 Effect of mineral on the stability of bone collagen and the implications of this for identifying cooked bone

TEM analysis can be used to highlight the important stabilization which is afforded to bone collagen by the presence of mineral. If the mineral is removed and the insoluble collagen component is heated above 60-65°C, the fibrillar structure collapses (see Figure 4.12). By contrast when mineral is present, bone collagen can be heated at 80°C for at least one hour without sustaining significant damage; when demineralized and viewed by TEM, the majority of the fibrillar structure appears intact.

The collagen within bone is not completely resistant to mild heating, however. Small regions of alteration can be observed within the demineralized collagen fibrils (see Figure 5.4, 5.5); becoming more frequent as the temperature or length of heating is increased. Within the damaged areas there is often a complete loss of banding pattern indicating a disruption to the quarter stagger alignment of the collagen molecules within this region of the fibril. In non-mineralized tissues, during a heating event, the tight packing of the collagen fibrils prevents the molecules from melting at the solution denaturation temperature (of 37°C; Leikina et al., 2002). Once the fibril organization is itself disrupted (when collagen is heated above 65°C) the triple helices would however be free to collapse. It is proposed that when bone is heated above 60-65°C, damage will also accumulate in the fibrils; the presence of mineral however will inhibit the unpacking of damaged regions and the spread of damage along the fibril. It is only when the mineral cage is removed that it is possible to visualize these damaged areas (see Figure 5.16).

This explains the discrepancy between the temperature at which mineralized collagen is thought to be susceptible to thermal alteration, as discussed at the beginning of the chapter. Much of the research pertaining to thermally induced denaturation of collagen has dealt with non-mineralized collagen in soft tissue, demineralized bone collagen or mineralized collagen. In studies utilizing demineralized bone (or non-mineralized collagen) the collagen does not have the stability of the mineral, and therefore is rapidly transformed from an organized fibril structure to a gelatinized material. In studies utilizing bone, no change is observed within the collagen fraction at mild temperatures because it is still restrained within the mineral matrix. With the TEM method, samples are heated in a mineralized state and then demineralized prior to analyses of the collagen

fraction. Using this approach it is possible to visualize subtle, low temperature, morphological changes within collagen fibrils, not commonly noted within the existing literature.

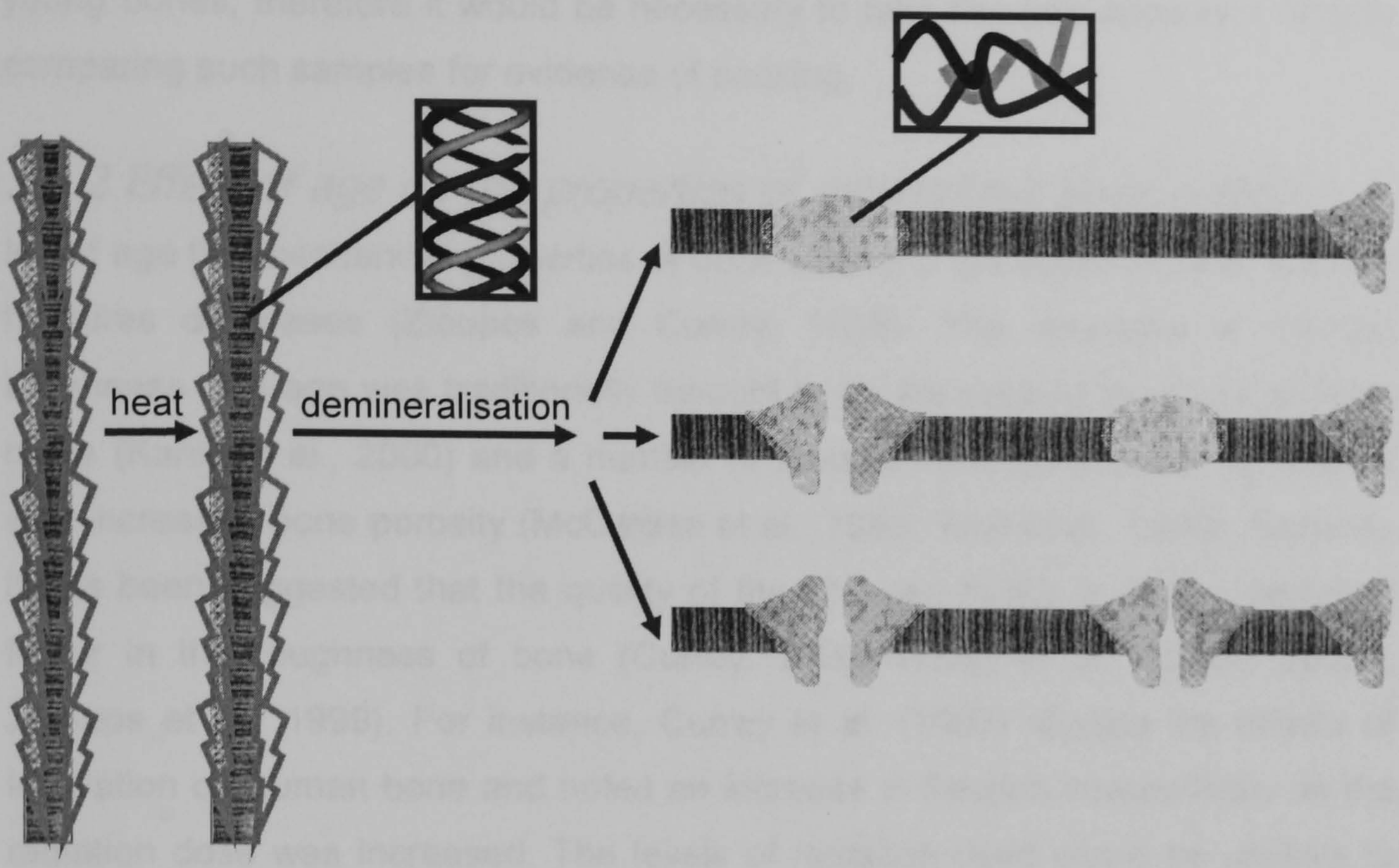


Figure 5.16 A schematic diagram proposing how bone collagen fibrils become degraded by mild heat treatment. When collagen is heated in mineral (i.e. bone) the collagen molecules become damaged (most likely through hydrolysis) but are held in position by a mineral 'cage'. The damaged molecules will disrupt the packing of the collagen fibrils and this can be observed, once the mineral is removed, as *Beaded* regions. At higher temperatures or after prolonged exposure the *Beaded* regions break apart to form shorter *Dumbbells*.

The DSC and TEM results have shown that there are limits to the stabilization afforded by the mineral. When bones are heated for extensive periods, such as nine hours at 100°C, the mineral can no longer protect the collagen from catastrophic failure (complete denaturation). This gross alteration to the collagen component is detectable by DSC as a downward shift in the transition temperature and by TEM as a loss of fibril structure. The TEM results also show a loss of fibrillar structure between 140°C and 170°C; thus dictating the upper limit of this technique for detecting cooked bone. This is probably related to the endothermic transition which occurs in mineralized tissues between these temperatures (Kronick and Cooke, 1996; also see section 5.2 and Chapter 4).

The heating experiments have shown that whilst DSC is able to detect catastrophic damage to the collagen component as a result of heating; it is unable to detect the mild heat alteration that would be caused by a domestic

heating event. TEM on the other hand is able to identify very mild heating, subtle differences in temperature, and also produce repeatable results. However the TEM analysis has also shown slight differences in the thermal stability of old and young bones; therefore it would be necessary to take this into account if directly comparing such samples for evidence of cooking.

5.6.2 Effect of age on the properties of mineralized bone collagen

In old age the mechanical properties of bone alter and the ability of bone to resist fractures decreases (Zioupos and Currey, 1998). The decrease in fracture toughness with age was traditionally thought to be the caused by a loss of bone mass (Kanis et al., 2000) and a number of studies have observed a correlation with increased bone porosity (McCalden et al., 1993; Yeni et al., 1998). Recently it has been suggested that the quality of the collagen matrix is also a dominant factor in the toughness of bone (Currey, 2003; Wang et al., 2002a, 2002b; Zioupos et al., 1999). For instance, Currey et al. (1997) studied the effects of irradiation on human bone and noted an increase in fraction susceptibility as the radiation dose was increased. The levels of radiation used would be unlikely to affect the mineral but would be detrimental to the collagen; thus suggesting that the toughness of bone is determined by the structural integrity of the collagen not the mineral. Only a few studies on the age related changes in collagen and their correlation with the toughness of bone have been reported (Wang et al., 2000, 2002b; Zioupos et al., 1999). Although these studies have demonstrated the involvement of collagen in age related changes to bone quality, the underlying mechanisms are still unclear.

Wang et al. (2000) observed an age-related decrease in the fracture toughness of baboon cortical femur bone which was not related to either bone mineral density (BMD) or porosity but correlated with the amount of denatured collagen. Based on these findings the authors suggested that the collagen molecules become damaged with age, weakening the collagen network and increasing the susceptibility of bone to fracture. This could explain the slight decline in T_s which has been found to occur in old age >60-65 years (Danielsen et al., 1994); as the T_s is indicative of the overall stability of the collagen molecules. However, when Wang et al. (2002b) performed mechanical tests and collagen analysis on human material (aged 19-89 years) they found no relationship between the amount of denatured collagen and age. Li et al. (2003) also observed no relationship between the amount of *in situ* denatured collagen and age. They did however find that collagen thermostability in bone is age-dependent; based on a decreased

susceptibility to heat induced denaturation that was observed with age. This led the authors to suggest that collagen becomes more structurally stable *in situ* as people age.

The results from the present study have suggested that under the conditions used for the TEM visualization, with increasing age the collagen fibrils are more resistant to thermal alteration and swelling in acid. This suggests that the collagen fibrils in older individuals are more structurally stable; consistent with Li et al. (2003). It has been proposed that glycation-induced cross-links may adversely affect the toughness of bone (Viguet-Carrin et al., 2006). Indeed a negative correlation between pentosidine, a non-enzymatic cross-link, and fracture toughness in human femur bone has been identified (Li et al., 2003; Wang et al., 2002b). It is suggested that the apparent age-related stability of the collagen fibrils, observed by TEM, is caused by non-enzymatic cross-links. This would explain the increased resistance to swelling observed in the fibrils with age. If the fibrils are restricted however they are also likely to be less flexible and more brittle; which could also explain the decrease in the fracture toughness of bone collagen with age. A negative relationship between glycation and the ductility of collagen fibrils has been observed; both in naturally cross-linked human cortical femur collagen (aged 49-85 years), and cross-link enhanced (in vitro with ribose) bone collagen (Catanese et al., 1999). Exploring the relationship between the 'stiffening' of the collagen fibrils in bone with its toughness with age may help to elucidate the underlying mechanisms behind the greater fracture prevalence observed in old age. Possibilities for further work in this area are discussed in Chapter 9.

Chapter 6 Analysis of bones from experimental burials

6.1 Introduction

Archaeological faunal remains can be recovered from different types of burial environments; some of which are conducive to good preservation whilst others can have detrimental effects, causing severe alteration to the chemical composition, appearance and even loss of skeletal elements.

In Chapter 2 of this thesis I considered the difficulty of separating the effects of cooking from those of long term burial; because cooking can mimic the alteration that occurs in bone through diagenesis. If one is trying to find a means to identify a cooked bone within the archaeological record it is necessary to investigate not only the effects of thermal alteration on bone but also (i) the effects of the burial environment within which the bone was deposited and (ii) perhaps more importantly the relationship between thermally altered bone and the burial environment because these factors are irrevocably linked in archaeological cooked bone. One way to approach this would be to take suspected cooked and uncooked samples from different time periods and from different burial contexts. In this way one could attempt to build up a picture of the types of environments where this technique could successfully be applied.

It is not feasible, however, to gain a complete set of data from all of the sites and time periods in which cooked bone may be found as this would involve analysing a large number of samples and the researcher would need to be certain that bones had been cooked, and know how and for how long. It would also be necessary to obtain detailed knowledge of the burial history and the specifics of the burial environment of each sample in order to get an idea of how the burial matrix may have influenced the state of preservation of the bone collagen. Even with such a high level of understanding it still may not be possible to separate factors of the burial environment into those which are (a) conducive to good preservation, (b) those which have no effect and (c) those which may damage the collagen fibrils. A further problem is that it may only be possible to obtain one 'cooked' and one 'uncooked' sample from a given deposit. In this case whilst they may have significantly different states of collagen preservation it could not be said that cooking was the only factor, unless the samples were from the same

element of the same animal in exactly the same burial environment. This of course is highly unlikely to occur in archaeological deposits.

A way to bypass some of these problems is to look at samples from experimental burials. The primary advantage of this is that there is a documented taphonomic history of the sample. In addition there is also documentation of the species, the skeletal element that the sample came from, and in some cases age-at-death of the specimen. Consequently a number of factors can be accounted for when determining the state of preservation of the collagen fibrils. Within the burial environment itself different factors can be controlled and monitored over the course of the burial, which is not possible with an archaeological deposit. In an ideal situation, bones would be heated over different time/temperature regimes and buried together with uncooked bone in a whole series of different deposition environments. The relative behaviour of the heated bones to the unheated controls would then be compared after excavation. However this was not feasible given the time limitations of a PhD thesis. An alternative was to use bones from pre-existing burial experiments. This had the advantage that the bones had a known burial history and pre-burial treatment. Also it is possible to analyse bones which have been buried for more than the two or three years allowable had I conducted my own burial experiments.

There were some immediate problems with this approach however, such as the scarcity of burial experiments where paired cooked and uncooked bones are buried together. Another problem with using samples from pre-existing burial experiments is that the samples from different sites are not normally directly comparable. They may have involved different species and skeletal element and been treated differently in terms of cooking temperature, cooking method, depth and duration of burial and post-excavation history. To investigate the impact of diagenesis paired cooked and uncooked bones were sought from burial environments sufficiently corrosive to yield demonstrable alteration to the bone within relatively short time scales.

Outlined below are the main pathways by which bone can be degraded in the burial environment; for reviews of the subject of bone diagenesis see Hedges (2002) and Collins et al. (2002).

6.2 Pathways of diagenesis

If human and animal remains do survive in the archaeological record, they are generally represented by only the hard tissues; the soft tissue is lost and the only organic material to survive is that protected within the bones and teeth by mineral. The diagenetic processes which act upon bone are intimately linked to the burial environment. Bone is essentially composed of two components an organic matrix (principally of type I collagen) which is embedded with small (predominately) plate like minerals of poorly crystalline bioapatite. The diagenesis of bone can therefore be analysed in terms of conditions which lead to the preferential alteration of bone mineral, conditions under which the collagen is preferentially lost or conditions under which these two components are destroyed as a composite (Collins et al., 2002).

6.2.1 Dissolution of mineral

The loss of bone mineral can occur relatively rapidly as the bioapatite is both poorly crystalline and has a high surface area (Weiner and Price, 1986). Apatite mineralogy suggests that apatite is stable at around neutral pH (Hedges and Millard, 1995), but the mineral is increasingly susceptible to dissolution at pH's lower than this (at higher pH, the phosphate dissolves but alternative calcium minerals such as $Ca(OH)_2$ are precipitated). Therefore in environments in which the pH is sufficiently low to promote dissolution and the burial environment favours removal of the dissolved phosphate (i.e. in free draining soils), it is predicted that the bone will rapidly lose its mineral component. In addition to mineral loss, in geochemically less extreme environments reorganization can occur through Ostwald ripening which leads to an increase in crystallinity (Weiner and Bar-Yosef, 1990).

6.2.2 Loss of collagen

Collagen is believed to be protected from biodegradation by the close association with the mineral phase (i.e. Nielsen-Marsh et al. (2000a)), and will therefore decay by chemical hydrolysis if the mineral is present (Collins et al., 1995). Collins et al. (1995) put forward a conceptual model to explain this form of collagen diagenesis based on the assumptions that two underlying processes are involved: (i) the depolymerization of collagen by chemical hydrolysis of peptide bonds and (ii) dissolution of the polypeptide fragments retaining fewer than a critical number of hydrogen bonds.

Collagen deterioration is further promoted by both low pH causing hydrolysis and presence of protons which may cause swelling of the collagen fibrils and high temperature promoting hydrolysis and gelatinization. Both acid burial and cooking are therefore likely to damage the collagen fibril and accelerate the rate of loss of protein from bone.

If the mineral is removed however (see above) collagen will be exposed to rapid hydrolysis by enzymes known collectively as collagenases which are secreted by microbes (Child, 1995).

6.2.3 Degradation of the composite (Microbial attack)

Much bone is subjected to microbial attack by both fungi and bacteria (for an overview of the types of microbial alteration see Hackett (1981)). This attack can occur within a few years (Yoshino et al., 1991) or even months post-mortem (Bell et al., 1996a, although the sample in question was recovered from a predator scat). The microbes demineralize the bone, which leads to the destruction of histological features (Garland, 1987; Jackes et al., 2001) and an increase in porosity (Jans et al., 2004), which in turn can accelerate degradation of the collagen (Nielsen-Marsh and Hedges, 1999). Fungi appear to be mining the bone for phosphate (perhaps associated with a sub-aerial mycorrhizal network). They tend to burrow in from the external surface and cut across bone ultrastructure, presumably using oxalic acid to dissolve the apatite. Fungal mining appears to be different from changes believed to be of bacterial origin which respect bone ultrastructure and do not cross-cement lines between Haversian systems. They follow the anatomy of the bone, and in this way apparently choose to follow the collagen fibrils along lines of least-resistance. Using the analogy of dental caries, it has been speculated that this form of attack is principally associated with conditions under which biodegradation of excess organic matter (e.g. for soft-tissues and body fluids) leads to local anoxia, fermentation and the production of organic acids (e.g. putrefaction) (Collins et al., 2002). Adventitious removal of the mineral exposes the collagen which then is itself biodegraded. The bacterial and fungal alteration collectively termed 'focal destruction' can be observed histologically from thin sections of bone. A histological index has been developed to assess the extent of microbial alteration to bone (Hedges et al., 1995). Using this index it has been shown that bones tend to have either mild or extensive alteration (Hedges et al., 1995; Millard, 2001).

This suggests that unless microbial attack is inhibited in some way (e.g. anoxic, acidic conditions) complete destruction will eventually occur.

6.3 Experimental burial sites

Bones for this study were obtained from three experimental burials. In order to observe accelerated microbial attack bones from a compost heap were analysed. The effects of low pH and hydrology were investigated by looking at bone in saturated (peat bogs) and unsaturated (upland soils) acidic environments (pH 4.6 – 5.6). To assess the impact of any initial destabilization of the collagen through heating, boiled and uncooked bones were examined.

6.3.1 Preserving factors of Peat bogs

In order for preservation to take place the organisms that promote decomposition must be inhibited by environmental factors (Janaway, 1996). In the case of human remains there are several interrelated agents acting on a body; such as internal and external bacteria, fungi, insects and carnivores, which cause decomposition (Bass, 1997). However under exceptional circumstances (anoxia, presence of bactericides, cross-linking agents) biological and chemical deterioration may be retarded or even arrested and collagen may be preserved even in the absence of mineral.

There are a few environments that can cause this exceptional preservation. One such situation can be found in the peat bogs of northwest Europe. Deposition in very specific physical and biochemical environments, namely mildly acidic raised peat bogs in lowland areas with the right climatic conditions, can produce a bog body (Fischer, 1998; Painter, 1991b).

The infrequency with which leather artefacts are recovered from archaeological deposits, attests to the normal low preservation potential of unprotected collagen. The formation of a bog body is the result of several inherently interrelated factors, which deter putrefaction caused by micro-organisms, whilst simultaneously impregnating the soft tissue with a natural tanning agent (Fischer, 1998). The external surface of a body deposited in a bog is inaccessible to insects and carnivore activity, known to be major factors in soft tissue decomposition (Bass, 1997; Turner, 1999). It is also protected from invasion by aerobic bacteria and fungi, due to the lack of oxygen in the bog, and the lack of nutrients which have been sequestered by sphagnan (Mitsch and Gosselink, 1986; Painter, 1991b).

The acidic nature of the bog water may deter some anaerobic micro-organisms (Brothwell, 1986). However this also has a detrimental effect on the bones which become rubbery or in some cases can be completely lost, due to chelation of calcium ions from the bone apatite by acidic bog water (Pyatt et al., 1995). It is also important that the deposition environment is sufficiently cold (<4°C) for two reasons: to inhibit bacterial activity until the bog water has time to infiltrate the body (Andersen and Geertinger, 1984; Fischer, 1998), and to suppress hydrolytic damage from the acidic conditions found within the bog (Collins and Galley, 1998).

Finally, the penetration of bog water enriched with sphagnan and humic acid into the soft tissues halts bacterial growth (Painter, 1991a). When the Sphagnum moss present in raised peat bogs dies and breaks down, the sphagnan contained within is converted into humic acid. These latter two substances form a lattice of cross-links within and between the collagen fibres in the soft tissue, thereby protecting them from hydrolysis by putrefactive bacterial enzymes (Painter, 1991b).

6.4 Materials and Methods

6.4.1 Details of Burial Experiments

As mentioned above, samples were obtained from three different burial environments: acidic upland soil, peat bog and compost heap. This section describes the burial experiments that the samples came from.

6.4.1.1 Acid upland soil

Bone assemblages were buried in Palace Leas Meadow Hay Plots at Cockle Park Farm, Northumberland. The meadow is the world's oldest cutting and grazed grassland experiment and is controlled by the University of Newcastle. The bones were buried in selected plots in April of 1999 as part of a PhD project. Three sets of bovine rib bones were initially buried; these included uncooked bone and bones which had been boiled for between three and 81 hours, only the uncooked bones are discussed here. Due to unforeseen circumstances only the first set of bones, buried for one year, had been excavated. I and a colleague excavated the rest of the bones after four and six years of burial. However the six year set was only removed at the very end of my PhD and are yet to be analysed. The grassland experiment has been the subject of intensive study for the past

100 years; as a result, the soil composition, pH and microbial populations of each plot are closely regulated and well characterized (Hopkins et al., 1997; Shiel and Rimmer, 1984). Plots 7 and 9 lie close to each other in the field and therefore are subject to the same hydrological, topological and climatic regime. The main difference between Plots 7 and 9 is soil pH due to a varied programme of organic (manuring) and inorganic treatments (ammonium sulphate and NPK fertiliser) on the different plots over the years. Plot 9 also has low levels of phosphate. The pH data for this site is shown in Table 6.1.

6.4.1.2 Peat bogs

In 1998 a conservation project was initiated by the Vitenskapsmuseum at the Norwegian University of Science and Technology. The aim of the project was to investigate interactions between the burial environment and selected organic materials that are found preserved in bog environments. The materials included in this study were comprised of those commonly represented in the archaeological record in Northwest Europe: vegetable-tanned leather, unbleached linen textile, dyed and undyed wool textile, raw and boiled bovine metapodials, antler and raw pig skin. The materials were buried in perforated modules at two different bog sites; Rørmyra Nature Preserve, Trondheim, Norway and The Historical-Archaeological Research Centre at Lejre, Denmark. The installation at Rørmyra is located in a raised bog with a subarctic climate. The site at Lejre lies 900km south of Rørmyra and is a lowland bog with a humid oceanic climate shared by Northwest Europe and the British Isles (Peacock *pers. comm.*). At each site four modules were installed, the contents of which are being retrieved over a period of eight years.

6.4.1.3 Compost heap

The compost heap experimental burial forms part of a wider comparative project which was set up by Dr Nicholson (Nicholson, 1992, 1996 and 1998). The aim of the project was to generate a set of data from which to investigate bone diagenesis within different burial contexts. The assemblages, which included cooked and uncooked mammal, bird and fish bone, were buried for seven years in five different environments. I have previously analysed cooked and uncooked sheep metapodials from three of the other sites (acidic moorland, neutral garden soil, deciduous woodland) by TEM (Koon et al., 2003). Comparisons will be made between TEM results from the compost heap and previous analysis from the other sites.

The bones at this site were buried in an urban compost heap approximately 0.2-0.3m above the base of the heap and within a layer of well-rotted compost. The layer consisted mainly of plant residues with a further 0.6 m of semi-rotted vegetable matter on top. At the time of excavation the pH of the burial environment was slightly alkaline and the organic content consisted of about 10% of the weight of the dried soil (Nicholson, 1998).

6.4.2 Samples

Paired boiled and uncooked bones were examined from each site except at Palace Leas where only uncooked bones were included.

The bone samples from both plots at Palace Leas came from intact bovine ribs. After excavation the bones were left to air dry at room temperature under acid-proof paper for one week. After this time the soil adhering to the bones was dry and crumbly and could be easily removed. The bones from the four year excavation were then bagged and stored at 4°C. The bones from the one year excavation had been stored in a freezer.

The bone samples from both bog sites came from bovine metapodials. Prior to burial a 15 cm section of the mid-shaft of the metapodials had been cut out with a hacksaw. These were mechanically cleaned of soft tissue and marrow then rinsed under running water. For the cooked bone the mid-shaft was cut into three cross-sections and boiled for approximately six hours (Turner-walker, *pers. comm.*). The samples were provided for analysis as one centimetre cross-sections which had been stored in a freezer. The burial experiments at Rørmyra and Lejre provide a unique opportunity to look at collagen preservation in an acidic environment which is known to degrade bone collagen but at the same time one in which bog-chemicals promote the preservation of collagenous materials. As there was the opportunity to examine differences in preservation of mineralized and non-mineralized collagenous materials buried in this environment the pig skin and vegetable tanned leather, which had been buried with the bones at Rørmyra, was also analysed.

The samples from the compost heap included three uncooked sheep metapodials and three of the same elements which had been boiled for one hour (including 20 minutes for the water to boil) prior to burial. Three uncooked cow metapodials were also analysed. The only post-excavation treatments were the manual removal of soil adhering to the bones and drying at room temperature. The bones

were subsequently stored at room temperature and were generally whole except where cross-sections had been removed for histological analysis.

6.4.3 Analysis

In order to determine the state of preservation of the collagen component Transmission Electron Microscopy (TEM) was used to visualize the collagen fibrils and detect alteration to the collagen fibril structure. Differential Scanning Calorimetry (DSC) was used to measure the degree of deterioration or stabilization of the collagen. Details of samples that were analysed from each site and the methods that were used are displayed in Table 6.1.

6.4.4 Bone sample preparation

Only compact bone was used in these experiments. As the bovine rib samples from the Palace Leas burials also contained trabecular bone this was first removed with a scalpel. Five millimetre cross-sections were taken from the midpoint of each bone using a water-cooled electric saw. In the case of the bog samples these had already been cut into sections with an electric saw so the outer two millimetres from each side of the cross-sections was removed with the water-cooled saw to try to remove any heat-damage produced during the previous sectioning. The sections were then crushed into small shards (largest dimension ≤ three millimetres) using a hammer. In each case 60 mg of bone shards were demineralized using the EDTA method (section 3.3.2.2)

6.4.5 TEM sample preparation

Details of the TEM sample preparation can be found in section 3.3.4.2. Analysis was conducted using an FEI Tecnai G2 Transmission Electron Microscope fitted with a CCD camera. The analysis method is described in full in section 3.3.4.4. In brief, in order for comparisons to be made between samples, for each grid (two grids per sample) the first 100 fibrils observed are counted and classified as *Unaltered*, *Beaded* (where swollen areas appear at discrete positions along the fibril and at the ends) or *Dumbbell* (a short fragment of a fibril with swollen ends).

Table 6.1 Details of the samples that were analysed from the different burial experiments. * Roberts (2003) (unpublished PhD thesis), measurements taken at time of burial. †Peacock *pers. comm.*, measurements taken at time of burial. ‡ Nicholson (1998), measurement taken at time of excavation.

Site	Soil water pH	Sample	Burial period (years)	Analysis
Palace Leas Plot 7	4.69 ±0.30*	Bovine rib bone uncooked	1	TEM
Palace Leas Plot 7	4.69 ±0.30	Bovine rib bone uncooked	4	TEM
Palace Leas Plot 9	5.58 ±0.28*	Bovine rib bone uncooked	1	TEM
Palace Leas Plot 9	5.58 ±0.28	Bovine rib bone uncooked	4	TEM
Lejre	5.6†	Bovine metapodial bone uncooked	1	TEM, DSC
Lejre	5.6	Bovine metapodial bone boiled 6hrs	1	TEM, DSC
Lejre	5.6	Bovine metapodial bone uncooked	4	TEM, DSC
Lejre	5.6	Bovine metapodial bone boiled 6hrs	4	TEM, DSC
Rørmyra	5.0†	Bovine metapodial bone uncooked	1	TEM, DSC
Rørmyra	5.0	Bovine metapodial bone boiled 6hrs	1	TEM, DSC
Rørmyra	5.0	Bovine metapodial bone uncooked	4	TEM, DSC
Rørmyra	5.0	Bovine metapodial bone boiled 6hrs	4	TEM, DSC
Rørmyra	5.0	Vegetable tanned leather	1	TEM, DSC
Rørmyra	5.0	Pig skin	1	TEM, DSC
Compost heap	7-7.5‡	Bovine metapodial bone uncooked	7	TEM
Compost heap	7-7.5	Bovine metapodial bone uncooked	7	TEM
Compost heap	7-7.5	Sheep metapodial bone uncooked	7	TEM
Compost heap	7-7.5	Sheep metapodial bone uncooked	7	TEM
Compost heap	7-7.5	Sheep metapodial bone boiled 1hr	7	TEM
Compost heap	7-7.5	Sheep metapodial bone boiled 1hr	7	TEM

6.4.6 DSC sample preparation (leather, skin and demineralized bone)

The first two millimetres was removed from the skin samples (which included the outer (epidermal) surface and the underlying collagen-rich dermal layer). The leather was cut into short strips and no discrimination was made between the outer and inner surface. The leather, skin and demineralized bone samples were then soaked in a pH 7.0 PBS for 24 hours prior to analysis to ensure a neutral pH and that the samples were fully hydrated. 10-20 mg of each sample were then blotted dry and sealed into Al DSC pans. The pans were heated from 15-95°C at a heating rate of 5°C min^{-1} in a DSC822e differential scanning calorimeter, (Mettler Toledo, Leicester, UK) fitted with a nitrogen gas intracooler, using a sealed empty pan as a reference. The onset, peak max and peak end temperatures were recorded for each endotherm.

6.5 Results and discussion

6.5.1 Palace Leas, Lejre and Rørmyra (saturated and unsaturated acidic soils)

6.5.1.1 Physical Changes

Palace Leas

Clear differences in the state of preservation of the bones and the burial environment were observed when we excavated the bones from Palace Leas after four years. The soil in both plots was moist and cloggy but not waterlogged. In Plot 7, the highly acid plot, there was no insect activity. There was however a considerable amount of earthworm activity in Plot 9. The bones recovered from Plot 7 were very fragmented and exhibited severe cortical exfoliation (flaking and erosion of the bone surface). The bones were dark yellow/orange in colour (7.5YR 7/8), had a wet wood-like appearance and could be easily broken. The bones from Plot 9 by contrast appeared macroscopically to be considerably better preserved. These bones were cream (10YR 8/3) with mottled brown staining (7.5YR 4/2) and showed some channelling and pitting across the surface. There were also some patches of cortical exfoliation revealing the underlying cancellous bone but the ribs were otherwise intact.

Lejre and Rørmyra

The cooked and uncooked bones from Lejre and Rørmyra were all intact at the time of excavation. The bones displayed some cortical exfoliation and some mottled dark brown (10YR 4/1) staining which was more extensive after four years. The staining was most prominent at the exposed ends of the boiled bone samples from Rørmyra. However in cross-section it could be seen that this staining did not penetrate further than the very outer rim. The stained region was friable and could be easily removed exposing the underlying bone which was yellowish white (2.5YR 8/4).

The leather samples from both Lejre and Rørmyra appeared to be unaffected by their time in the bog. Compared to the control the only apparent difference was a mottled dark brown staining (7.5YR 4/2). The main difference in preservation at the two sites was seen in the pig skin samples. After one year the Lejre sample was very friable and was only held together by soil which had adhered to it. By contrast at Rørmyra the skin samples were largely intact but again had dark brown mottled staining (7.5YR 3/1).

6.5.1.2 TEM analysis

Results from Palace Leas and the two bog sites are displayed together on a ternplot (Figure 6.1).

Palace Leas

Between one and four years in the acidic soil of Plot 7 there was a large shift in the proportion of *Unaltered*, *Beaded* and *Dumbbell* alteration observed by TEM. The number of *Unaltered* fibrils dropped from 60% to 40%. This was accompanied by a 5% increase in *Beaded* but a 15 % in *Dumbbells*. In both the bone collagen samples from one and four years approximately half of the fibrils appeared swollen.

Although also an acidic plot, the collagen fibrils from the bones buried at Plot 9 did not show the rapid alteration that was noted in Plot 7 and the fibrils were long and showed no evidence of swelling. Between one and four years of burial there was a 10% decrease in the proportion of *Unaltered* fibrils observed and a small increase in both *Beaded* and *Dumbbell* alteration.

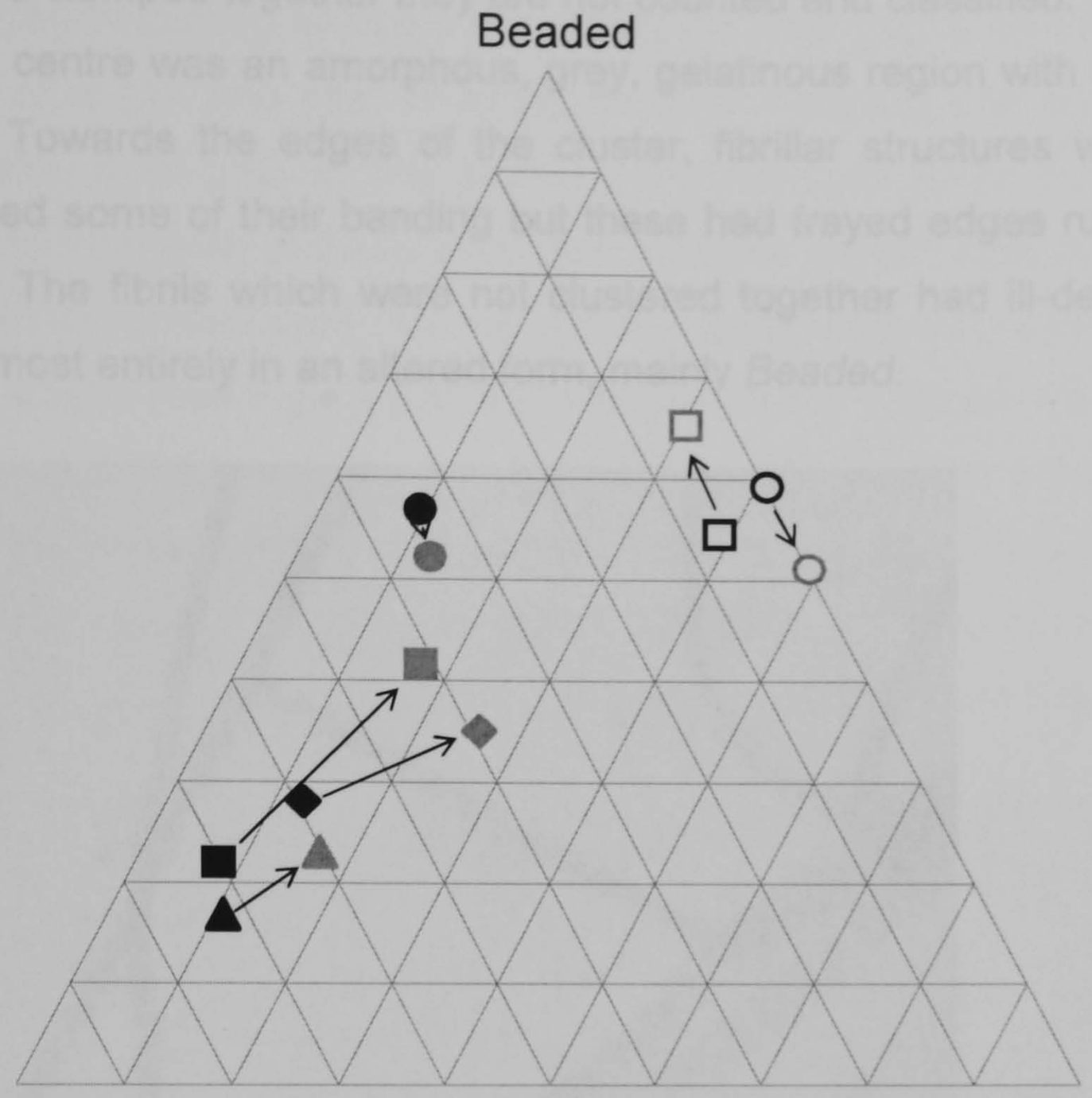


Figure 6.1 Ternplot showing the results of the TEM analysis from the unheated Palace Leas bone samples from Plot 7 (♦) and Plot 9 (▲) and the unheated and boiled samples from Rørmyra (●) and Lejre (■). The boiled samples are shown with a white centre. The arrows on the ternplot show the change in the proportion of *Unaltered*, *Beaded* and *Dumbbell* type fibrils observed over time. They point from samples buried for one year (black) to those buried for four years (grey).

Lejre

After one year of burial the uncooked sample from Lejre contained collagen fibrils which were well stained and the majority of which were long with an equal diameter along their length (see Figure 6.2). Although most fibrils appeared in this *Unaltered* state a small proportion of damaged fibrils in both *Beaded* and *Dumbbell* forms were also observed. By four years at Lejre the proportion of *Unaltered* fibrils had decreased and more *Beaded* alteration was observed.

The collagen fibrils from the boiled samples at Lejre by comparison were quite faint and mainly swollen. They varied little in their appearance between one and four years though surprisingly there was a slight decrease in the number of *Dumbbells*, but an overall increase in the number of altered fibrils. The collagen fibrils were also often found clumped together (see Figure 6.3). This may explain

the apparent decrease in the proportion of *Dumbbells*; if a number of *Dumbbell* fragments are clumped together they are not counted and classified. Within these clusters, the centre was an amorphous, grey, gelatinous region with no evidence of banding. Towards the edges of the cluster, fibrillar structures were evident which retained some of their banding but these had frayed edges running along their length. The fibrils which were not clustered together had ill-defined edges and were almost entirely in an altered form, mainly *Beaded*.

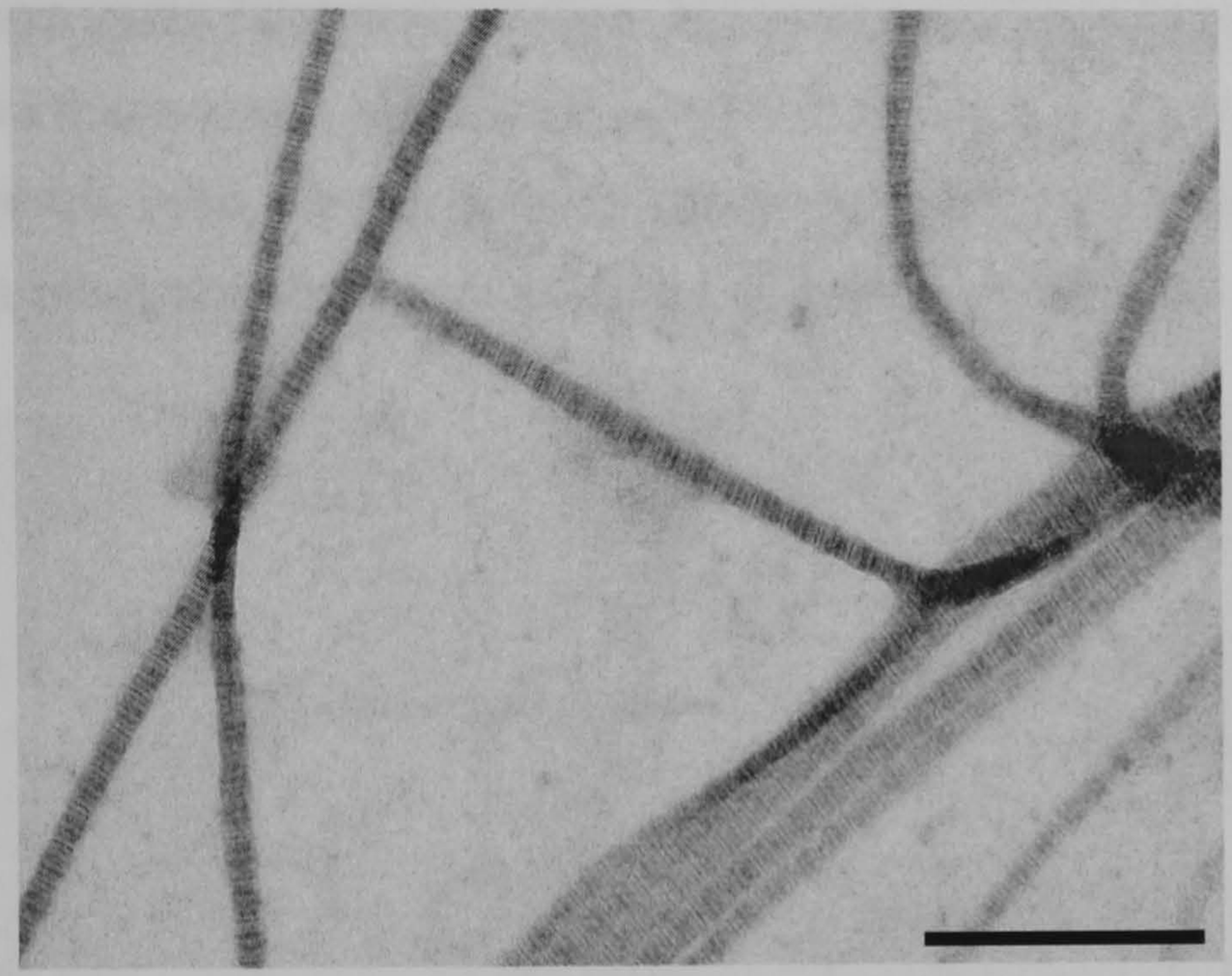


Figure 6.2 TEM image from the Lejre one year uncooked bone sample. The scale bar is 500 nm. The majority of the collagen was in an *Unaltered* state as shown below.

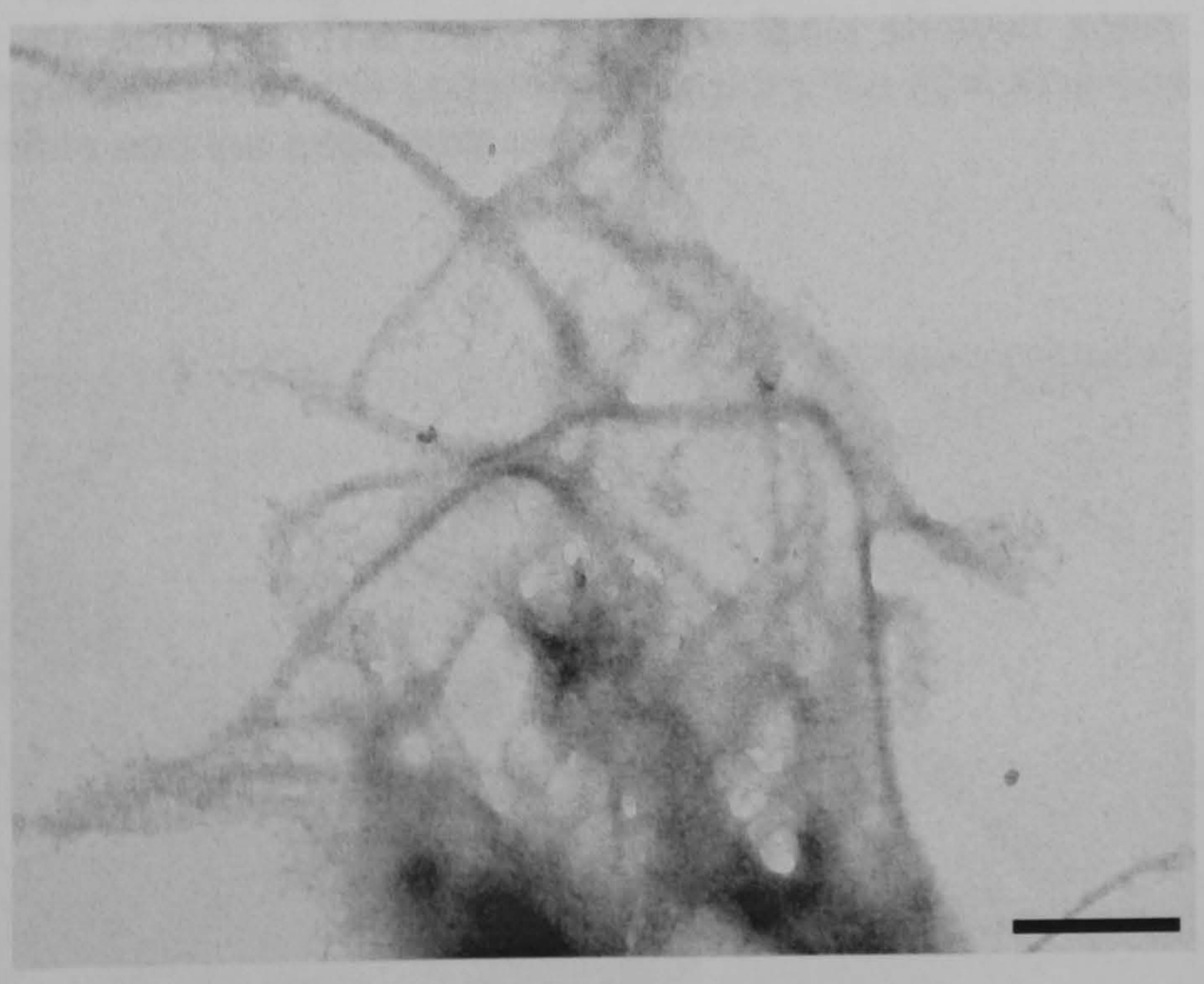


Figure 6.3 TEM image from the Lejre one year boiled bone sample. The scale bar is 500 nm. The fibrils appear faint with ill-defined edges and are often clumped together as shown below.

Rørmyra

Unlike Lejre the uncooked samples from Rørmyra displayed a significant degree of alteration after just one year of burial, however between one and four years there was little difference in the state of the collagen fibrils observed. The collagen fibrils from both of these uncooked samples were mainly in the *Beaded* form although the undamaged regions of these fibrils appeared well stained with well defined edges (see Figure 6.4). By comparison the collagen from the boiled samples was quite faint and swollen. No *Unaltered* fibrils were present in either sample and there was a slight increase in the number of *Dumbbells* between one and four years. Also like the boiled samples from Lejre the fragments of collagen fibrils were often observed within a large gelatinous mass (Figure 6.5).

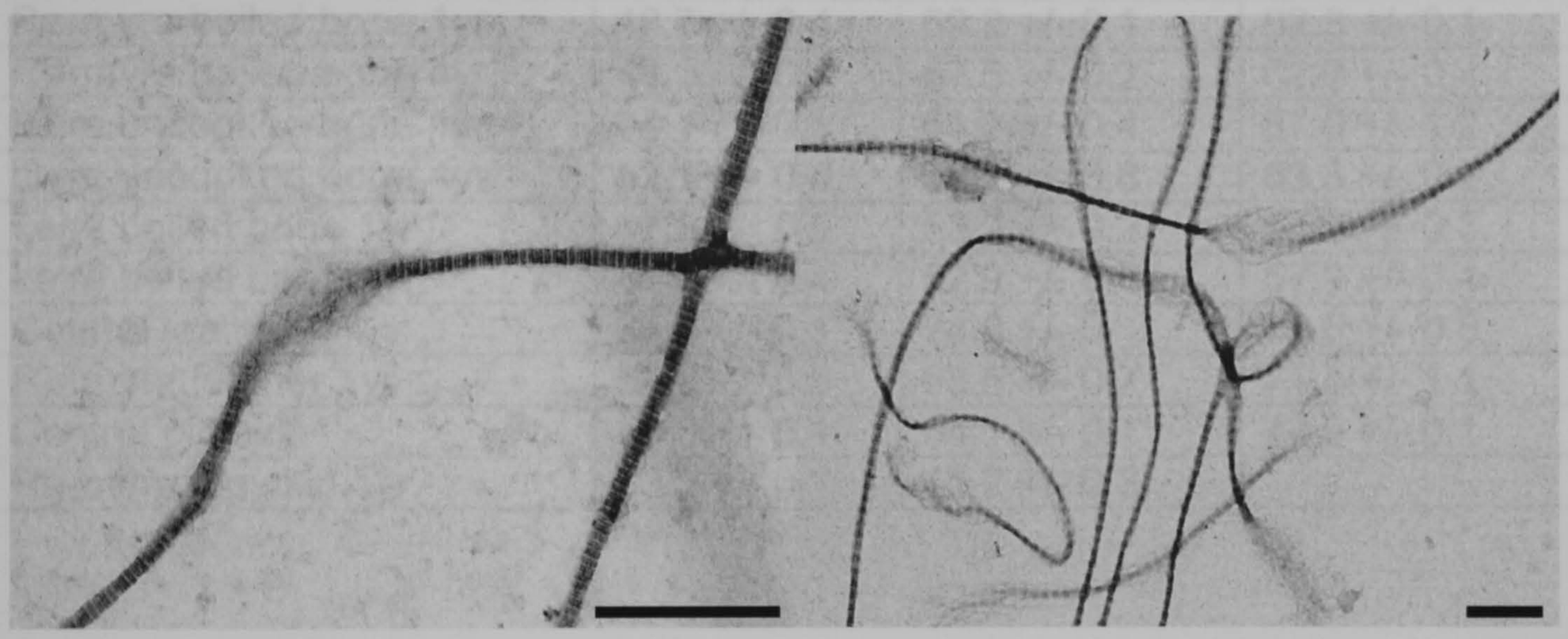


Figure 6.4 Two TEM images from the Rørmyra one year uncooked bone sample. The scale bars are 500 nm. The majority of the fibrils showed some evidence of *Beaded* alteration, however within the undamaged regions the fibril structure is intact, the banding is clearly visible and the edges are well defined.

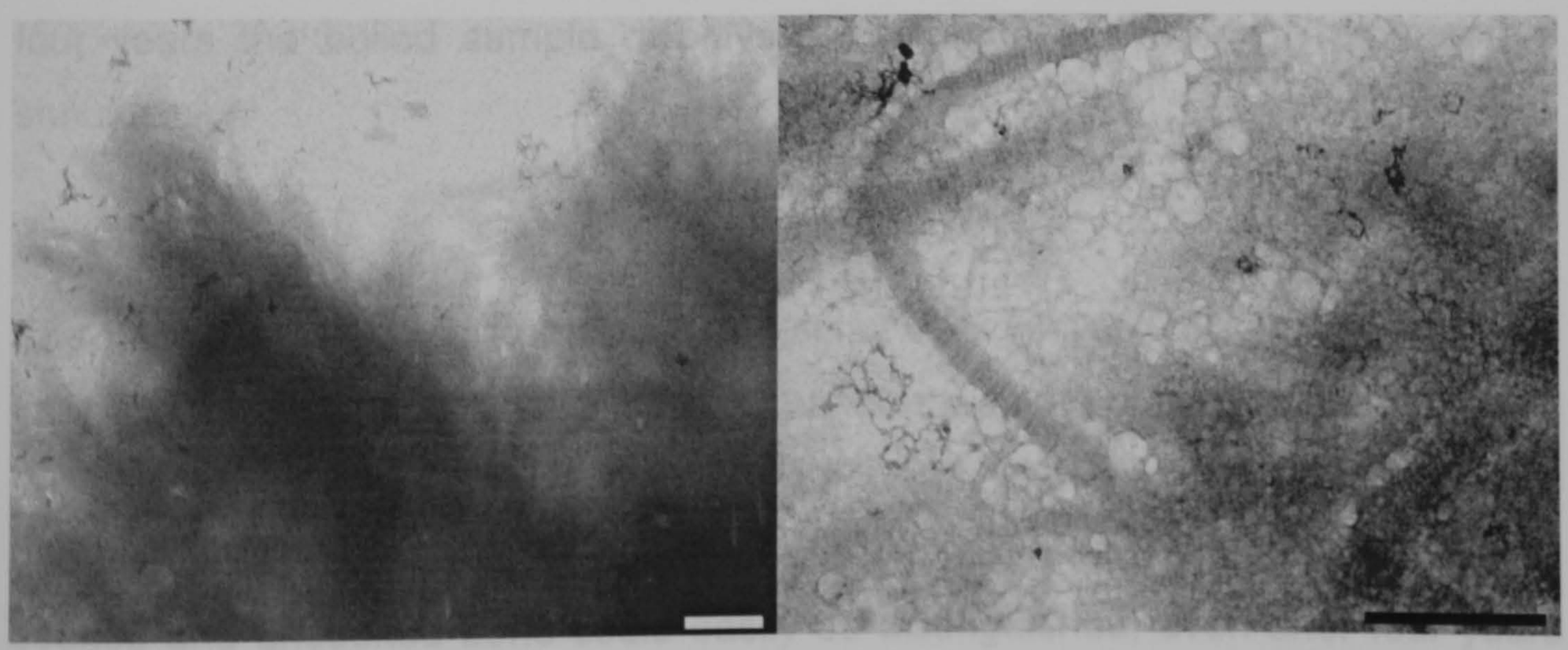


Figure 6.5 Two TEM images from the Rørmyra one year boiled bone sample. The scales bars are 500 nm. Many short fragments of fibrils were found held within a large mass of grey amorphous material which is presumably gelatin.

6.5.1.3 DSC analysis (Lejre and Rørmyra only)

The results of the DSC analysis are shown in Table 6.2. There are also three summary DSC charts showing a typical trace for each of the demineralized bone samples and the leather samples (Figures 6.6, 6.7 and 6.8). The DSC traces are all to the same scale but have been offset along the y-axis so that they can be viewed more easily.

Table 6.2 Results of the DSC analysis conducted on bone, leather and skin peat bog samples (*n* =3 per sample, +/- S.D.).

Sample	Onset (°C)	Peak max (°C)	Peak end (°C)
Rørmyra uncooked bone 1yr	53.3 +/- 0.8	59.1 +/- 0.8	66.2 +/- 0.4
Rørmyra uncooked bone 4yr	54.5 +/- 0.4	59.1 +/- 0.8	66.6 +/- 0.4
Rørmyra boiled bone 1yr	48.7 +/- 0.4	59.2 +/- 0.1	62.3 +/- 0.1
Rørmyra boiled bone 4yr	48.3 +/- 0	57.5 +/- 0.2	62.9 +/- 0.4
Lejre uncooked bone 1yr	54.1 +/- 0.1	61.2 +/- 0.4	67.0 +/- 1.2
Lejre uncooked bone 4yr	52.1 +/- 0.4	59.1 +/- 0.8	63.3 +/- 0.9
Lejre boiled bone 1yr	47.8 +/- 0.5	53.2 +/- 0.2	54.9 +/- 0.3
Lejre boiled bone 4yr	47.9 +/- 0.4	52.2 +/- 0.6	57.9 +/- 0.4
Control leather	65.6 +/- 0.1	74.8 +/- 0	83.5 +/- 0.5
Rørmyra leather 1yr	59.5 +/- 0.6	63.5 +/- 0.7	71.1 +/- 1.1
Control pig skin	58.3 +/- 0.1	64.1 +/- 0.1	74.9 +/- 0.1
Rørmyra pig skin 1yr	33.3 +/- 0.1	45.2 +/- 0.3	-

Lejre

The DSC traces show that boiled and uncooked samples could be distinguished by a downward shift in the temperature of the denaturation peak in the cooked samples (see Table 6.2). There was little change in the DSC traces between one year and four years of burial at Lejre (Figure 6.6). One slight difference is that after four years the peak for the uncooked sample is slightly broader. Also after four years the boiled sample displays a peak with a small high temperature shoulder.

Rørmyra

The uncooked samples from this site display very similar DSC traces after one year and four years of burial (Figure 6.7) and as was the case with the uncooked Lejre samples these peaks are consistent with those produced by modern demineralized bone collagen. Also in common with the samples from Lejre, cooked and uncooked bone could easily be distinguished, even after four years of burial. Both of the boiled samples show endothermic transitions which have a lower temperature shoulder before the main peak. The four year sample also

shows a downward shift in this peak by approximately 2°C (see Peak max from Table 6.2), compared to the one year sample suggesting that preheating had left the collagen more susceptible to damage within this burial environment. Overall the peaks for the Rørmyra samples are sharper and better defined than for Lejre, suggesting greater uniformity within the collagen samples from this site.

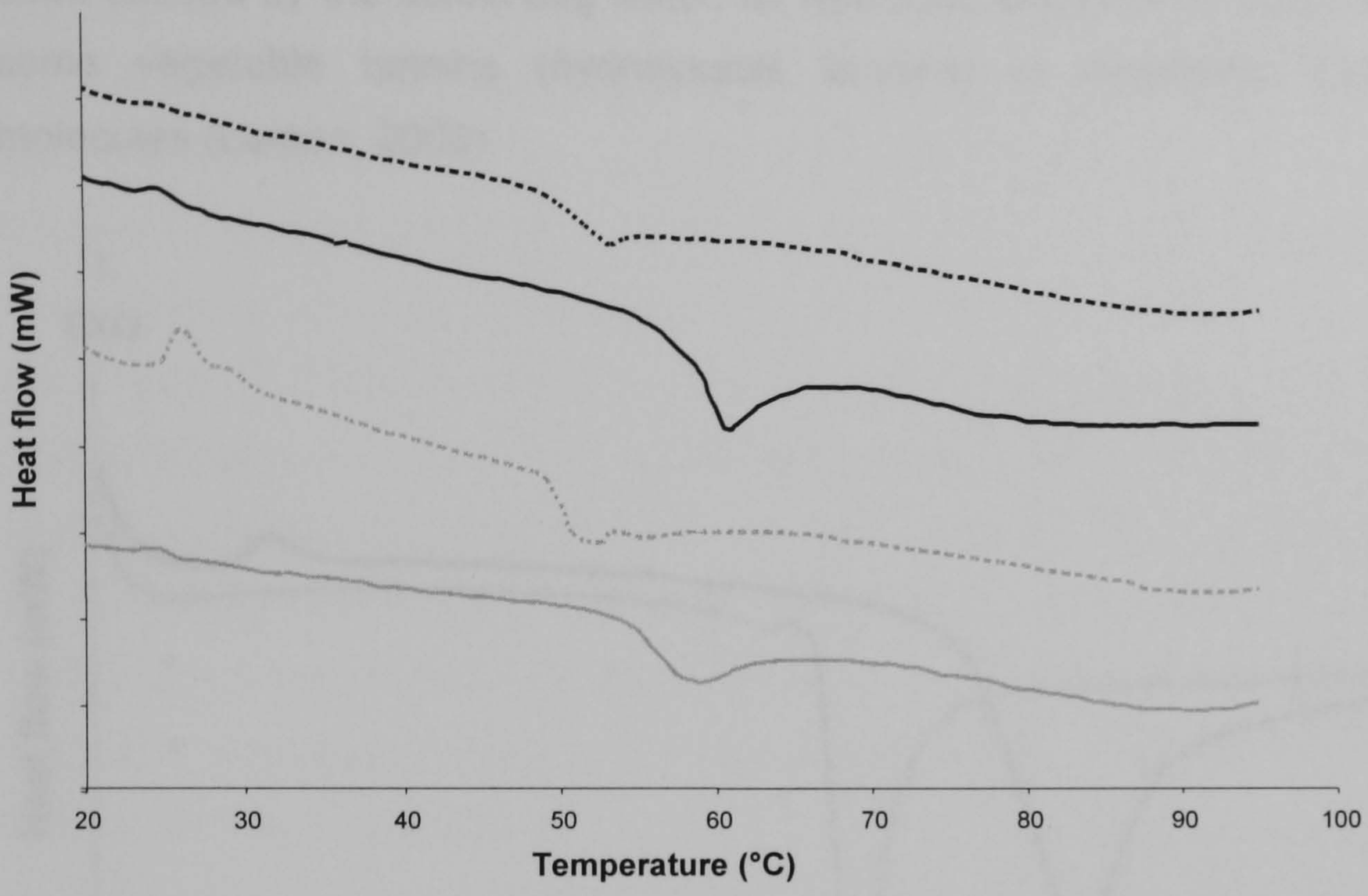


Figure 6.6 DSC traces from the Lejre bone samples. The samples which were buried for one year are shown in black and those buried for four years are shown in grey. The dotted lines denote the boiled samples.

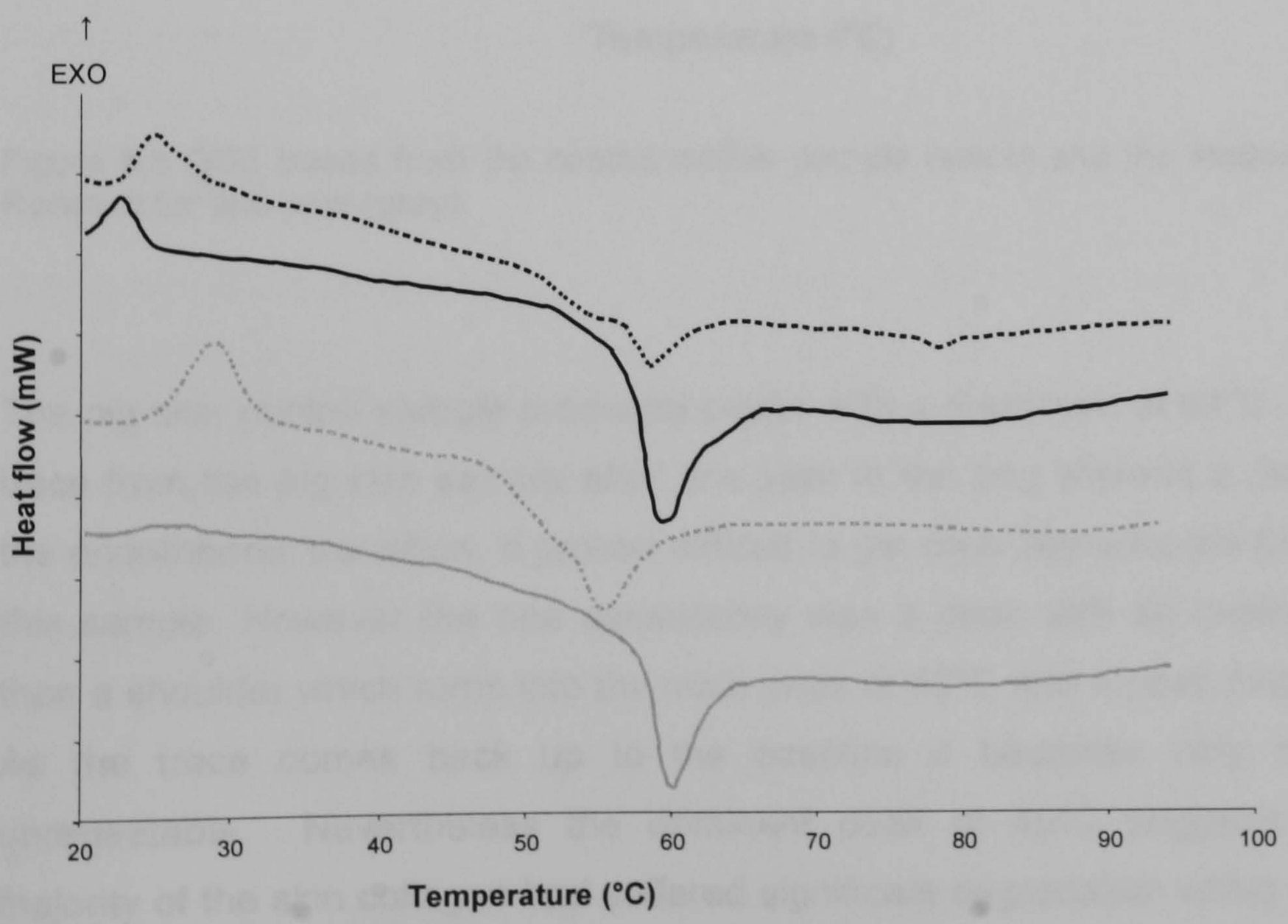


Figure 6.7 DSC traces from the Rørmyra bone samples. The samples which were buried for one year are shown in black and those buried for four years are shown in grey. The dotted lines denote the boiled samples.

The leather control sample produced a sharp peak at 78°C. This value falls within the range for a vegetable tanned leather of 75°C to 85°C (Haines, 1987). The DSC results for sample buried at Rørmyra for the one year however produced a peak at 65°C (see Figure 6.8). This suggests that the tanning process was reversed within a year of the samples being in the bog. This reversal could have been caused by the acidic bog water; as hydrolytic enzymes or acids can cause some vegetable tannins (hydrolysable tannins) to dissociate into smaller molecules (Larsen, 2000).

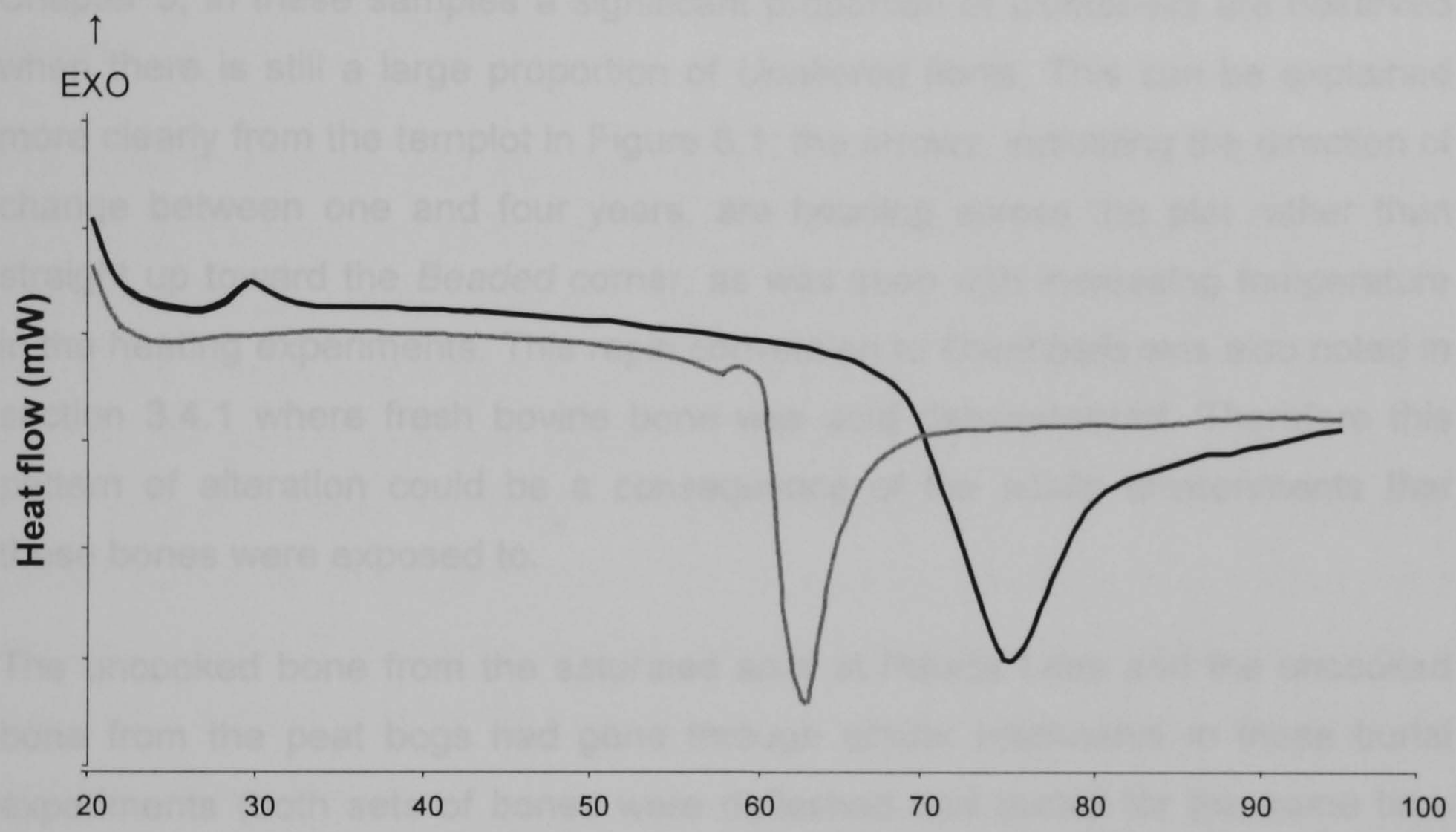


Figure 6.8 DSC traces from the control leather sample (black) and the leather buried at Rørmyra for one year (grey).

The pig skin control sample produced peaks with a maximum at 64°C. The DSC trace from the pig skin sample after one year in the bog showed a decrease in the endothermic transition. It proved difficult to get clear reproducible traces from this sample. However the one consistency was a peak with an onset at 33°C, then a shoulder which turns into the main peak at 43°C and a peak max at 45°C. As the trace comes back up to the baseline it becomes very noisy and unrepeatable. Nevertheless the dominant peak at 45°C suggests that the majority of the skin collagen had suffered significant degradation whilst within the bog.

6.5.1.4 Comparison of results between the saturated and unsaturated acidic soil sites

Overall the TEM analysis suggested that there was an increase in the extent (proportion of altered fibrils) and degree of damage (proportion of *Dumbbells*) between one year and four years of burial, except in the cases of the uncooked Rørmyra samples which were indistinguishable. Between one and four years the samples showed a significant increase in the proportion of *Dumbbells* (again with the exception of Rørmyra). In contrast to the heating experiments, discussed in Chapter 5, in these samples a significant proportion of *Dumbbells* are observed when there is still a large proportion of *Unaltered* fibrils. This can be explained more clearly from the ternplot in Figure 6.1; the arrows, indicating the direction of change between one and four years, are heading across the plot rather than straight up toward the *Beaded* corner, as was seen with increasing temperature in the heating experiments. This rapid conversion to *Dumbbells* was also noted in section 3.4.1 where fresh bovine bone was acid demineralized. Therefore this pattern of alteration could be a consequence of the acidic environments that these bones were exposed to.

The uncooked bone from the saturated soils at Palace Leas and the uncooked bone from the peat bogs had gone through similar treatments in these burial experiments (both sets of bones were defleshed and buried for the same time periods (one and four years)). However after this relatively short period of burial the bones displayed differences in their gross appearance and at a nanoscopic level differed in the extent of damage to the collagen fibrils as detected by TEM.

Taking into account the pathways to diagenesis discussed above the unsaturated soils from Palace Leas should have produced the most damaged bone (and bone collagen). The free draining acidic soils at this site should have caused the rapid dissolution of bone hydroxyapatite into the surrounding soil (Pike et al., 2001). This in turn would have exposed the collagen component to biodegradation by bacteria and both swelling and hydrolytic damage from the acidic environment. This is consistent with the results from the bones in Plot 7, the high acidity soil. The appearance of the bone from this plot was macroscopically the most degraded. Also the TEM analysis showed that the collagen from this plot was significantly degraded and after four years of burial the collagen fibrils exhibited the most *Dumbbell* alteration. However Plot 9 also contains a free draining acidic soil and has low phosphate levels which should have helped to facilitate the

dissolution of the mineral. Also the soil from Plot 9 has been shown by biomass and bacterial counts to have much more microbial activity than the more acidic environment of Plot 7 (total number of bacteria per gram soil, Plot 7 =$1.05x10^9$ g^{-1}soil; Plot 9 = $5.68x10^9$ g^{-1} soil, Roberts, 2003), (microbial biomasses, calculated from CO2 evolution from glucose induced respiration, Plot 7 = 327 µg C g^{-1}soil; Plot 9 = 514 µg C g^{-1}soil, Hopkins et al., 1997). However the material from Plot 9 showed considerably better macroscopic preservation than the bones from Plot 7. It also showed the least damage of all of the Lejre, Rørmyra and Palace Leas samples, after both one and four years of burial, in terms of the TEM analysis.

The collagen from the bog sites was more damaged than predicted. By four years TEM analysis from the uncooked samples at both sites showed less than 40% *Unaltered* fibrils. Both Lejre and Rørmyra are waterlogged raised peat bogs which are known to produce exceptional preservation of collagenous materials. Further the leather and skin samples from Rørmyra displayed markedly better macroscopic preservation than the samples from Lejre, and were stained dark brown (which is often attributed to the presence of humics (van Klinken and Hedges, 1995)). It was postulated that the differences in preservation of leather and skin samples may have been due to the action of stabilizing cross-linking agents at Rørmyra.

By comparing the DSC and TEM results from the skin, leather and bone collagen buried at Rørmyra it would appear that hydrolysis, possibly due to the continuous exposure of the bones to a low pH aqueous environment, played an important part in the early degradation of collagen within this bog. This caused the reversal of the tanning process in the leather, significant degradation of the skin and damage to the bone collagen.

It is unclear from the results of this study if any cross-linking had actually occurred through the action of sphagnan within the bog. The brown staining need not necessarily be attributed to the presence of cross-linking compounds; it could equally be due to the uptake of metals such as iron from the burial environment. Studies on the trace element composition of bones from waterlogged sites have shown a u-shaped distribution in the uptake of metal ions; with higher concentrations at the periosteal (outer) and endosteal (inner) surfaces of the bone and low concentrations in the middle (Hedges and Millard, 1995; Reiche et al., 1999). This is consistent with the pattern of staining observed on the bones

from the peat bogs. However the considerably better macroscopic preservation of the pig skin at Rørmyra does suggest that cross-linking may be involved. And one could speculate that the lack of *Dumbbell* formation at the Rørmyra site may suggest cross-links are holding the fibril together and that otherwise the pattern of alteration would be more similar to that seen at the other acidic burial environments.

6.5.2 Compost heap (microbial attack)

6.5.2.1 Physical Changes

The immediate burial environment at the time of excavation was a humose sandy loam, with clay pockets as well as ash, eggshell, stone and pottery fragments (Nicholson, 1998). Earthworms, woodlice, centipedes and millipedes were noted by Nicholson to be present within the heap at the time of excavation, though it is not clear whether they were found within the layer of the compost heap that contained the bones.

At the time of excavation the state of preservation of the bones was described in detail by Nicholson (1998). Briefly, both the cow and sheep bones were described as being macroscopically well preserved and intact, although no soft tissue remained. All of surfaces of the bones were covered with a mottled yellowish-red to reddish-brown staining. Under an optical microscope Nicholson observed channelling and pitting on the bone surface of the cow and sheep metapodials and this was attributed to fungal hyphae. The metaphyseal surface (surface at the end of the shaft in the unfused bone) was also covered with fungal hyphae. The histological analysis of the boiled sheep bone showed areas of bone loss, mainly below the external surface. This was attributed to focal destruction (see Garland, 1987) of the forms linear longitudinal and lamellate (see Hackett, 1981). These types of alteration are thought be caused by bacteria (Jans et al., 2004). In general the cooked bone was described as being slightly less well preserved than the uncooked material although "the difference was insufficient to separate the bones into 'cooked' and 'uncooked'" (Nicholson, 1998).

6.5.2.2 TEM analysis

Figure 6.9 shows the results from the TEM analysis of the compost heap samples. The ternplot shows the proportion of *Unaltered*, *Beaded* and *Dumbbell* type collagen observed for each sample and samples have been coloured white for the cow bones, grey for the uncooked sheep and black for the boiled sheep bones. The uncooked sheep bones display the least alteration and the cooked and uncooked sheep bones can easily be distinguished based on the relative proportions of unaltered and altered collagen fibrils which were observed by TEM. The TEM analysis shows that the majority (70-80%) of the collagen observed from the uncooked bones was *Unaltered* with almost no *Dumbbells* evident. By comparison only 10% of the observed collagen was *Unaltered* in the cooked bone, with the majority in a *Beaded* state. The two cooked sheep bones and the two uncooked sheep bones show very similar values indicating that the TEM values give a good representation of the general state of the collagen from the bones at this site.

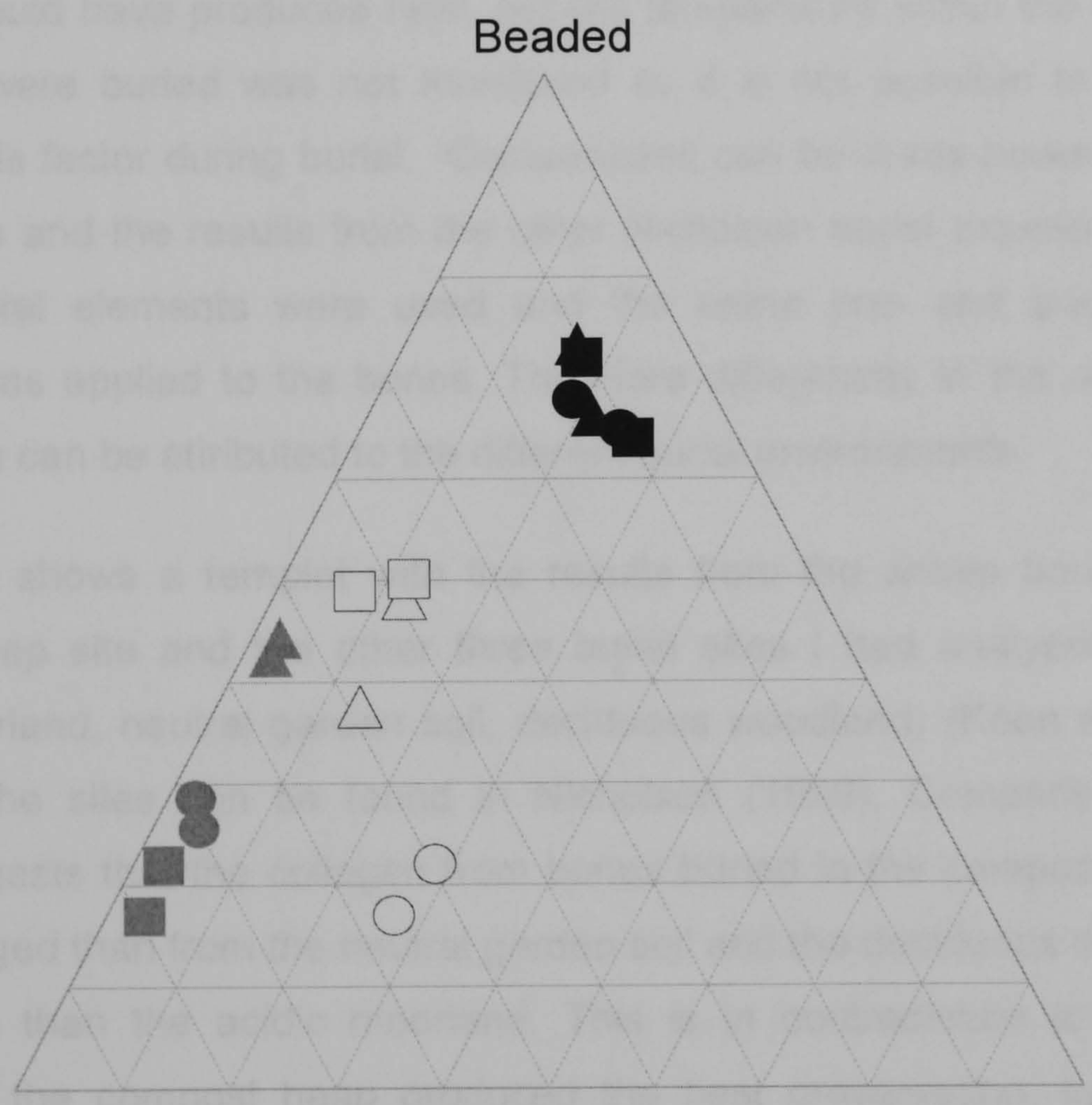


Figure 6.9 Shows the results from the TEM analysis of the compost heap samples. The ternplot shows values obtained from each bone (two analysis per bone) the different symbols denote different bones. The results from the unheated sheep bones are shown in grey, the unheated cow bones are shown in white and the boiled sheep bones in black.

Uncooked cow bone was also included within this study to determine if there was any species variation in the preservation of the collagen fibrils from this site. This appears to be the case; cow bones show considerably more alteration than the uncooked sheep bones. The preliminary experiments that were conducted on fresh cow and sheep bone; which are described in Chapter 5, also suggested that the cow bone collagen fibrils were less stable than those of the sheep bone.

6.5.2.3 Comparison of the compost heap results with other sites in the Nicholson burial experiment

The compost heap is a complex burial environment making it difficult to attribute the state of the collagen fibril degradation to particular factors within this environment. Further the immediate environment around the bones may have changed during the period of burial as the organic matter degraded and new material was applied. For instance it is possible that localized areas of acidity could have formed during this period, either through microbial activity or percolation of rain water down through the heap. Also the microbial activity within the heap would have produced heat, but the temperature within the heap where the bones were buried was not monitored so it is not possible to assess the impact of this factor during burial. Comparisons can be made however between these bones and the results from the other Nicholson burial experiments as the same skeletal elements were used and the same pre- and post-deposition treatment was applied to the bones. Therefore differences in the collagen fibril preservation can be attributed to the different burial environments.

Figure 6.10 shows a ternplot with the results from the sheep bones from the compost heap site and the other three burial sites I had analysed previously (acidic moorland, neutral garden soil, deciduous woodland) (Koon et al., 2003). Details of the sites can be found in Nicholson (1996). Comparison of these results suggests that the collagen from bones buried in the compost heap were more damaged than from the neutral garden soil and the deciduous woodland but slightly less than the acidic moorland. This is in contradiction to Nicholson's finding that the compost heap produced the best preservation; based on the number of bones recovered and macroscopic condition (Nicholson, 1998).

The Nicholson study suggests that the reason for the good preservation in the compost heap could be due to cross-linking compounds known as humics, which inhibited microbial attack. Humic substances consist of a heterogeneous mixture of compounds producing complex aromatic macromolecules; they can be formed

during decomposition of plant and animal matter and have been identified in archaeological bone (Tuross, 2002). The humics form non-specific random cross-links within and between collagen molecules (van Klinken and Hedges, 1995). It has been shown that the presence of bulky humic substances hinders enzymatic digestion of collagen by collagenase (van Klinken and Hedges, 1995). The TEM analysis of the collagen however does not suggest that humic substances have preserved the collagen beyond that which was found in the garden soil or the woodland. It is possible that humics have provided some protection and that without them the considerable fungal and bacterial activity which would have taken place within the compost heap would have caused significantly more damage to the bones. It should be noted however that no soft tissue was preserved. If humic substances were in fact protecting the bones by inhibiting microbial attack to the collagen, one might expect that the same would have happened to some degree in the soft tissue, especially in this case where reactions would not be hindered by the presence of mineral. van Klinken and Hedges (1995), observed a rapid uptake of humic acid to collagen in experimental studies using bovine Achilles' tendon.

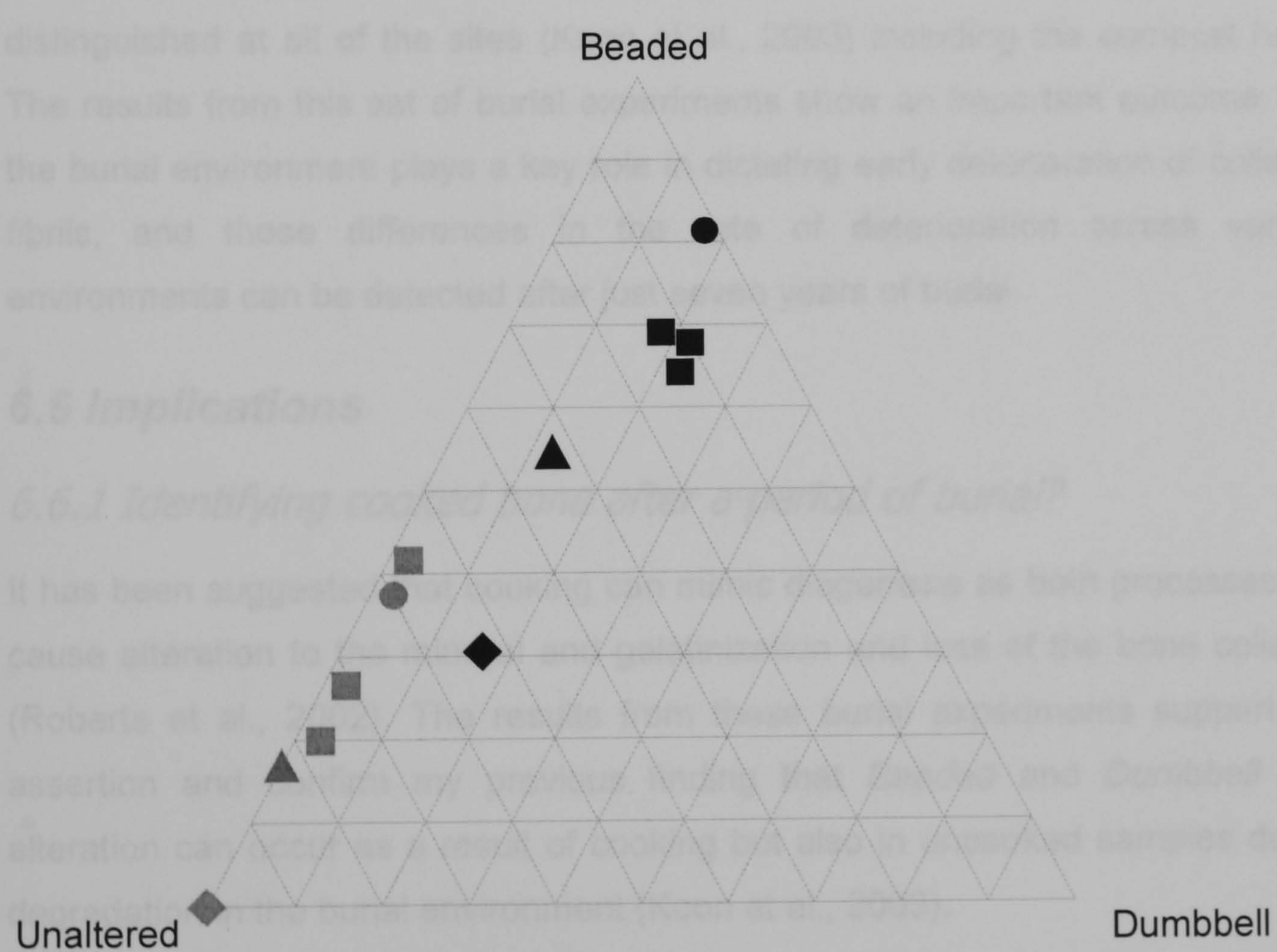


Figure 6.10 Ternplot showing the TEM results from cooked (black) and uncooked (grey) sheep bones which formed part of a seven year experiment to compare bone preservation in different burial environments. The sites include a neutral garden soil (♦), woodland (▲), acidic moorland (●) and a compost heap (■). All points are the average values of two analyses per bone.

The acidic moorland produced the most damage and the least alteration was found from the neutral garden soil. In my 2003 paper I suggested that soil pH is an important factor in dictating the early degradation of collagen fibrils within buried bone. The neutral pH of the soil in the compost heap might in part explain why the collagen fibrils from this site appeared less damaged than at the more acidic moorland site. However soil pH can not explain why the deciduous woodland environment, which is also acidic, yielded collagen (especially from the cooked bone) which was less altered than the compost heap samples. Nicholson also noted considerably better preservation at the woodland site when compared to the moorland samples (based on the amount of fungal hyphae present on the bones and extent of cortical exfoliation and bone loss) despite similar soil pH and drainage (Nicholson, 1996). There is no obvious explanation for the better preservation at the woodland site.

Lastly, Nicholson concluded that cooking did not appear to have greatly altered either the appearance of the sheep bone or its ability to survive burial in the compost heap and at any of her other sites (Nicholson, 1998). From TEM analysis of the sheep bones cooked and uncooked bone could easily be distinguished at all of the sites (Koon et al., 2003) including the compost heap. The results from this set of burial experiments show an important outcome: that the burial environment plays a key role in dictating early deterioration of collagen fibrils, and those differences in the rate of deterioration across various environments can be detected after just seven years of burial.

6.6 Implications

6.6.1 Identifying cooked bone after a period of burial?

It has been suggested that cooking can mimic diagenesis as both processes can cause alteration to the mineral and gelatinization and loss of the bone collagen (Roberts et al., 2002). The results from these burial experiments support this assertion and confirm my previous finding that *Beaded* and *Dumbbell* type alteration can occur as a result of cooking but also in uncooked samples due to degradation in the burial environment (Koon et al., 2003).

However, whilst in all cases cooking had little effect on the macroscopic appearance of the bones after burial, at each site the boiled samples exhibited significantly more microscopic alteration than the uncooked bones, allowing these samples to be identified as cooked using TEM. It remains to be seen whether in

archaeological timescales the effects of diagenesis will overwhelm any damage which has been caused by cooking. The application of this TEM technique to identify cooking in an archaeological context is investigated in Chapter 8.

The DSC traces also allowed boiled and uncooked samples to be distinguished from both bog sites. In all cases the uncooked samples displayed a peak at around 60°C which is consistent with unaltered demineralized bone collagen. The boiled samples displayed a downward shift in the temperature of the peak to varying degrees. Also in most cases these peaks have shoulders, indicating that there is collagen with different thermal stabilities within the same sample. However these samples had been boiled for six hours and judging from the heating experiments discussed in Chapter 5 it is doubtful that the DSC would pick up the less extreme heat treatment that would be associated with cooking.

Part 3 Case Studies

Chapter 7 Application to forensic science

7.1 Introduction to the case studies

7.1.1 The need for detecting mild heating of bone in forensic contexts

One area where this research may have particular application is in the field of forensic science. The burial experiments, discussed in the previous chapter, suggest that the effects of the burial environment can mirror alteration caused by cooking. However in all of the short term burials cooked and uncooked bones from the same contexts could be distinguished. The technique should therefore be particularly applicable to forensic contexts in which it is necessary to identify low temperature heating events. In these instances the remains would have been buried for a relatively short period or the body may not have been interred at all.

There are a number of types of criminal investigation in which it would be helpful to be able to identify short term heating. One such type of investigation would be instances of suspected cannibalism or ritual cooking. A brief search through the BBC news web pages produced over 20 cases of such acts in just the last 15 years. For instance in 2002, a group from Zhytomyr, Ukraine were said to have scalped a young woman, boiled her decapitated head and to have eaten it as part of a satanic ritual (http://news.bbc.co.uk/2/hi/europe/2129910.stm 15 July 2002).

7.1.2 The case of Roger Frisby – failure identifies the need for a new approach

An alterative type of enquiry is one in which the bones have been heated in an attempt to destroy evidence. An example is the case of Roger Frisby, a postman who had come home from work one day and decided to kill and dismember his wife. To avoid suspicion he roasted her head, hands and feet in the oven in an attempt to prevent identification. He then buried the remains on a golf course, but they were discovered a year later.

The case was brought to the attention of forensic archaeologists at Bradford University. The witness had given a statement, later withdrawn, in which he described heating a human head in a domestic oven, and the investigating authorities requested help to ascertain if this was indeed the case. Bradford University in turn contacted the Ancient Biomolecules Group at Newcastle University. The main emphasis of the research at Newcastle had focused on

bringing together a suite of experimental techniques that could characterize boiled bone (see Roberts et al. (2002); the results of this work are discussed in Chapter 2). A blind test was set up in an attempt to identify bone heated to different temperatures in a domestic oven (Nicholson et al., 2000). Pig skulls (as analogues for human material) were heated at Bradford, at temperatures between 200-250°C and for time durations of 2-5 hours, before samples were sent blind to Newcastle for analysis. The bones, along with an unheated control sample and unheated weathered samples, were assessed in terms of gross changes to both their organic and mineral component. Using the methods then available it was not possible to determine if the observed changes (either to the pig or human material) were either the result of mild heating or weathering (Nicholson et al., 2000). Subsequent studies with cattle bone also failed to discriminate between cooking and diagenesis (Roberts et al., 2002).

7.1.3 TEM as a means to detect low temperature cooking in forensic samples

In Chapter 5 it was demonstrated that even very mild heating events lead to changes which are detectable using the TEM approach. Furthermore, the TEM results from Chapter 5 and 6 show that the amount of alteration to the collagen fibrils that would result from the heating regime claimed in the Frisby case (200°C for several hours) would be much greater than that caused by one year of burial even in an aggressive environment such as an acidic peat bog or acidic moorland soil (Koon et al., 2003).

During the course of this thesis, I was approached on two occasions to help resolve questions of post-mortem treatment of human bone. Curiously, unlike the Frisby case, the treatments were conducted in order to preserve rather than destroy the remains. The first occasion concerned defleshing, a key step in the preparation of specimens for anatomical and museum collections. The second occasion involved the stabilization of decomposing remains awaiting burial. In the wider context of modern forensic analysis, although both of these processes do preserve specimens at a macroscopic level, it is increasingly important to understand their impact at the molecular level.

The following section is arranged as two separate forensic investigations. The first study was submitted as a report to Dr Ian Barnes (UCL) and the curators at the Cleveland, Ohio museum. It is our intention that the second forensic case will

be published in the journal Forensic Science International in collaboration with the Armed Forces DNA Identification Laboratory (AFDIL).

7.2 Case study 1: Defleshing

The Hamann-Todd osteoarchaeological collection, Cleveland; using TEM to understand the 'Leonhart's live-steam method'

7.2.1 Introduction

The Hamann-Todd skeletal collection is an extremely important resource for osteologists and anthropologists. The human bone collection alone consists of 3100 skeletons for which age, sex, ethnicity and cause of death have been recorded. The collection has been used extensively as a reference for studies of biological variation and paleopathological analysis. It has also been used for the development of osteological techniques; which in turn provide sex, age and stature estimations for use on archaeological populations and in forensic cases (Lovejoy et al., 1985; Meindl et al., 1990). Measurements taken from the Hamann-Todd human skeletal remains form part of FORDISC, an essential database used by forensic anthropologists to aid in the identification of remains.

Collections such as the Hamann-Todd are now being turned to for molecular as well as morphologial investigation. One such study attempted to extract DNA from the Gram positive pathogen *Mycobacterium tuberculosis*, of skeletons diagnosed as having died from TB. The research team had failed to detect *M. tuberculosis* DNA in any of the skeletons studied and became concerned that this may have been due to destruction of the DNA by the defleshing process. If this was the case then it would imply that this important osteological collection would be of limited value for molecular studies. Dr Ian Barnes and colleagues at UCL, who were conducting this work, were unable for find detailed accounts of the method of defleshing. It is unfortunate that at a time when museum collections increasingly are seen as an important resource of preserved molecular data, there is surprisingly little knowledge of the historical preparation methods used.

The majority of the bones from the Hamann-Todd skeletal collection were amassed during the 1920's and 30's from the county morgue and city hospitals. The remains went through a period of sitting in boxes, but for the last 20 years the collection has been restored by the Cleveland Museum of Natural History. Records of how these skeletons had been defleshed are incomplete but there

was mention of a 'Leonhart's live-steam method'. No details of the method could be found however. I was approached by Dr Ian Barnes and the collection manager at Cleveland to establish if the skeletal specimens had indeed been subjected to a heating event.

7.2.2 Materials and methodology

Four samples from the Hamann-Todd collection were analysed, consisting of small quantities of compact bone shards with no visible signs of pathological alteration (Table 7.1). Modern compact bone shards taken from the right mid-shaft femur of a 29 year old individual and compact bone from the anterior mid-section of a modern bovine rib were used as controls. Samples were demineralized in dilute acid using the method described in section 3.3.2.1 and then prepared for TEM analysis. A full description of the preparation and analysis method can be found in section 3.3.4. For comparison the TEM results from a further set of heated (one hour at 80°C; Chapter 5) compact bone shards were also included in this study. All were modern bone from male individuals with a similar age-at-death (29-52 years) as the Hamann-Todd samples.

Table 7.1 Sample details for the Hamann-Todd and unheated modern control

Sample	Skeletal element	Sex	Age (yrs)	Cause of Death
HTH0643	undetermined	Male	26	TB
HTH0475	rib	Male	35	Pneumonia
HTH0038	rib	Male	38	TB
HTH0646	undetermined	Male	40	TB
Modern Human	femur	Male	29	-
Modern Bovine	rib	-	-	-

7.2.3 Results and discussion

The results of the TEM analysis are shown in Figure 7.1. Three of the collagen samples (HTH0643, HTH0475, HTH0646) displayed a similar amount of alteration and appeared to be extensively damaged; consisting solely of *Beaded* or *Dumbbell* type fibrils. Sample HTH0038 was in a less damaged state than the other three samples and included 14% *Unaltered* fibrils and only 25% *Dumbbell* alteration as opposed to the 55 - 60% seen in the other Hamann-Todd samples. However all of the Hamann-Todd material is significantly more damaged than the

modern unheated controls. Sample HTH0038 shows a similar degree of alteration to the modern human bone samples heated at 80°C, but the other three samples are more damaged, suggesting that they have been subjected to a more aggressive heated event. The alteration observed in the collagen fibrils can be caused by both heating and diagenesis in the burial environment. However as the Hamann-Todd remains were never buried the damage probably reflects thermal alteration. The reason for the variation in damage between the sample HTH0038 and the other three could be due to differences in the heating regime or due to differences in body weight between the individuals themselves. We have previously conducted research using fleshed and defleshed animal bone and have found that the amount of soft tissue surrounding the bone can affect the temperature that the bone itself reaches; the insulating effect of the flesh means that the bone temperature rarely reaches above 100°C within a domestic cooking environment (Koon et al., 2003).

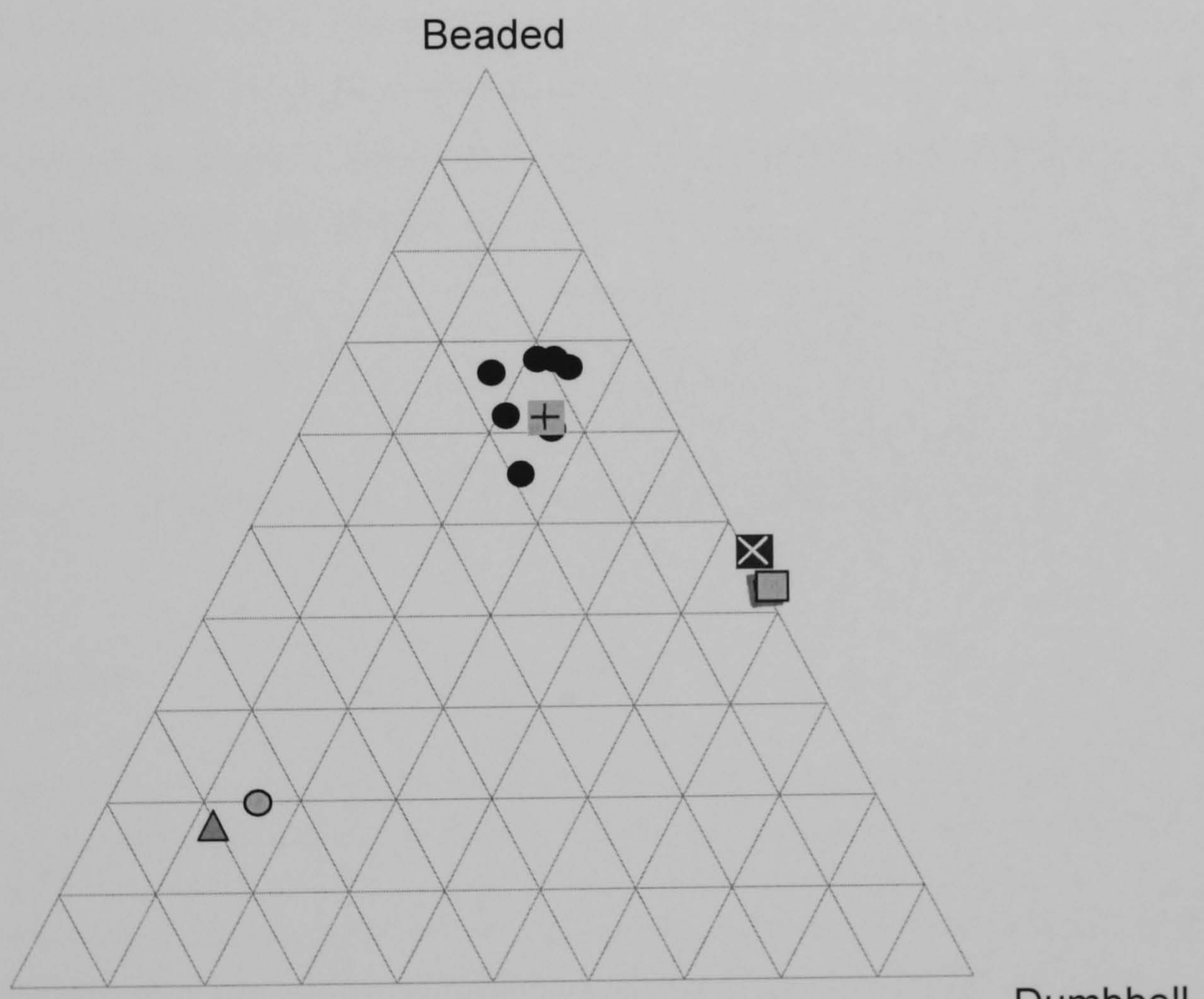


Figure 7.1 Ternplot showing the results of the TEM analysis form the Hamann-Todd samples (⊠ HTH0646), (+ HTH0038), (■ HTH0475 almost hidden) and (□ HTH0643), the heated (●) and unheated (○) age-matched modern human controls and the unheated bovine rib (△).

7.2.4 Conclusions

TEM analysis suggests that the collagen in all of the Hamann-Todd bones is more damaged than modern unheated age-matched equivalents. The most probable explanation for this is that the samples have been exposed to mild heating at some stage during their post-mortem history, presumably as part of the 'live-steam' process. Comparison with unfleshed modern bone fragments heated at 80°C for one hour suggest that most of the Hamann-Todd material have been exposed to a more aggressive heating regime (time and / or temperature). It is notable that whilst in most cases samples have suffered similar degrees of damage, one sample was better preserved. Factors which may have contributed to the reduced deterioration of the collagen include variation in the length and extent of the defleshing treatment and factors which may have reduced the temperatures attained within the cortical bone, notably body mass.

7.3 Case study 2 Embalming

Why was the US Armed Forces DNA Identification Laboratory unable to amplify DNA from the Punchbowl cemetery?

Abstract

The skeletal remains of unknown US soldiers who died during the Korean War were exhumed from Punchbowl, the National Memorial Cemetery in Hawaii. The remains were tested for DNA, but after rigorous extraction procedures, DNA could not be successfully amplified in the majority of cases. TEM and DSC analysis of the collagen component of these bones suggested they had been treated with a cross-linking agent. The DSC results showed collagens with a range of different thermal stabilities and when compared with the DNA amplification results, a clear correlation between successes and failures was evident. This suggests that in some cases cross-linking may play an important role in preventing DNA extraction. Within this unusual context, DSC analysis could be used as a rapid screening technique to assess the likelihood of successful DNA extraction and amplification.

Key words

DNA, cross-linking, collagen, bone, mortuary practices, TEM, DSC

7.3.1 Introduction

Scientists at AFDIL do not yet know why they have not been able to extract DNA from remains that were disinterred from the Punchbowl Cemetery in Hawaii over the last couple of years. The problem appears to come from the preservative powder that was sprinkled on both WWII and Korean War remains years ago. The powder either degraded the DNA so that none is left to be extracted, or it somehow bound the DNA to the bone, making extraction by traditional means impossible.

Jim Canik, director at AFDIL, says his researchers have not given up and they are not yet out of ideas. Unless the DNA has been destroyed, there should be a way to separate it from the bone. AFDIL researchers are now networking with scientists from the Smithsonian Institute and other facilities who have experience that might be helpful."

/www.coalitionoffamilies.org 14 February 2004

Punchbowl, the National Memorial Cemetery of the Pacific in Hawaii holds the remains of US servicemen who died in conflicts around the Pacific. Many of these remains are buried in unnamed graves. Concern has been raised about the speed of identification of these remains (http://www.coalitionoffamilies.org), which has been delayed because all but one of the exhumed remains processed through Camp Kokura had failed to yield reproducible mitochondrial DNA sequences. As time is passing in relation to social issues and the community of affected family members is aging, it has become increasingly important to resolve the Punchbowl problem.

The "Punchbowl' itself is a large crater formed during volcanic activity 75,000 to 100,000 years ago. During the late 1890s it was recommended that the Punchbowl should become the site for a new cemetery to accommodate the growing population of Honolulu. Although initially rejected, in 1947 the issue re-emerged. Congress and veteran organizations had been placing a great deal of pressure on the military to find a permanent burial site in Hawaii for the remains of thousands of World War II servicemen on the island of Guam awaiting permanent burial. The Army began planning the Punchbowl cemetery and in February 1948, Congress approved funding; construction began and the first interment was made Jan. 4, 1949. The cemetery was also used to house the remains of individuals who died in action during the 1950-53 Korean War. Following the end of fighting in 1953, the North Korean government returned remains believed to be those of U.S. servicemen. A total of 866 sets of remains marked as "unknown" from the Korean War are buried at Punchbowl.

From 1999, the remains of some of the "unknowns" have been exhumed for possible identification. To date bone and tooth samples from eleven individuals buried at Punchbowl have been submitted to the Armed Forces DNA Identification Laboratory (AFDIL) in Rockville, Md. Five individuals from the 7 Dec 1941 loss of the USS Oklahoma during the attack on Pearl Harbour, have all yielded sequence data without unusual problems. However four of these eleven exhumations were from Korean unknowns processed through Camp Kokura and in all of these latter cases, the samples yielded no usable mitochondrial DNA. It was surprising that DNA could not be extracted from these relatively recent remains. The burials themselves were unusual however. The bones had an unusual shiny aspect (Figure 7.2) and they were also covered with a white powder. A further set of remains from the 7 Dec 1941 loss of the USS Curtis were

found in a well sealed casket and again a quantity of white powder covered the remains. Although initial efforts to sequence these remains were unsuccessful, a sequence was eventually obtained. One case was exhumed relating to the 7 Dec 1941 loss of the USS Arizona and DNA testing was successful. When this casket was open it was found to be flooded with water. Finally an additional case pertaining to the white powder issue is a post World War II non-Punchbowl burial. This casket contained remains covered in a similar white powder and also failed to yield any reportable DNA. To summarize, five of the six remains with white powder failed to yield amplifiable DNA and the six samples without white powder provided sequences.

It was suggested that the unknowns which were causing problems for DNA amplification may have been treated with one or more mortuary compounds. The unusual thing about these samples was that DNA was visible (on an extraction gel), but for some reason it was not amplifiable with human primers. One possibility was that the observable DNA might just be fungal or bacterial with none of the original human DNA remaining. This would suggest that the chemical treatment that the bones had been subjected to might have included an agent that would aggressively attack the DNA, such as lime. It was also postulated that the native DNA had been modified due to a stabilizing agent and that the white powder might be the remnants of a hardening compound such as formaldehyde, used as part of a mortuary/conservation practice.

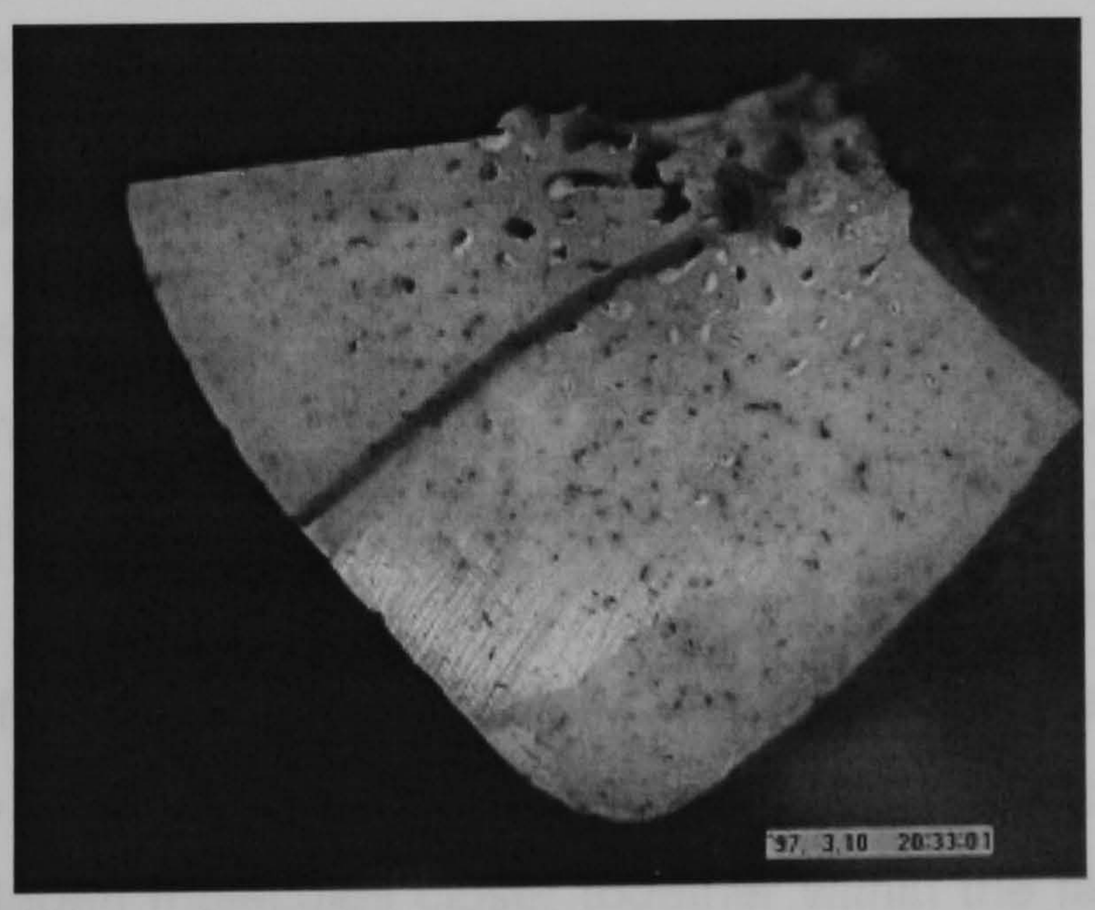


Figure 7.2 Image showing a cross-section of one of the Punchbowl bones from the Korean War. The bones have an unusual shiny aspect.

With more than 800 Korean unknowns buried at Punchbowl and with the four Korean cases sampled all giving negative results for DNA, I was asked to find out what kind of taphonomic process had affected the DNA and to establish a quick and simple way to screen bone samples for those likely to yield DNA.

7.3.2 Approach

In order to understand whether the inability to amplify DNA was the result of excessive damage or cross-linking this investigation targeted bone collagen. Bone collagen is the most abundant organic component of bone and like DNA is a helical polymer. If the treatment of the bones had damaged the DNA the collagen would also be damaged (making the helix more prone to unravel to form gelatin). Conversely, if the treatment led to molecular stabilization via cross-linking, the collagen helix would be more resistant to denaturation.

Three alternative properties of the collagen were investigated (i) acid swelling, (ii) hydrothermal stability and (iii) denaturation temperature.

(i) Acid swelling

Bone collagen is composed of type I collagen which is a fibrillar form. If a collagen fibril is highly cross-linked it will be resistant to acid swelling, determined by comparing the physical dimensions of the collagen fibrils (for an explanation of this phenomenon see section 3.1.1). If the collagen is degraded it will swell more readily and may even collapse to form gelatin.

(ii) Hydrothermal stability

Hydrothermal stability has been commonly investigated in relation to collagen tanning and other forms of cross-linking. Type I collagen denatures if it is heated to approximately 60-65°C, but if it is treated with an aldehyde the denaturation temperature is raised to above 70°C (Covington, 2001; Usha and Ramasami, 2000). In brief, synthetic cross-links such as aldehydes are believed to stabilize collagen in two ways; firstly by physically holding the structure together, but also by drawing collagen molecules closer together within the fibril thereby removing water and dehydrating the whole structure (Covington, 2001; Miles et al., 2005) (this topic is discussed in more detail in section 1.3.7). If the Punchbowl samples were demineralized then heated to 65°C, a resistance to thermal alteration would indicate a cross-linking agent was used whilst untreated or degraded material would denature.

(iii) Denaturation temperature

The denaturation temperature of the collagen can be measured by heating the sample at a constant rate and measuring heat flow associated with denaturation of the fibrils. Chemical treatments which damage the macromolecules will lower and broaden the temperature of this transition. Conversely, stabilization of the fibril will elevate the transition temperature.

7.3.3 Techniques

7.3.3.1 Transmission Electron Microscopy (TEM)

TEM is a method by which the collagen component of bone at the level of the collagen fibril can be visualized. It has been used previously to identify heat damage to fish (Richter, 1986) and mammal bone (Koon et al., 2003) collagen fibrils. Within this thesis, TEM has also been used to assess differences in the thermal stability of collagen from different tissue types and between bones from different species including human. The extent of thermal alteration and acid swelling would be assessed using a TEM approach.

7.3.3.2 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is regularly used within the leather industry to assess the denaturation temperature of the collagen (Bosch et al., 2002; Kronick and Buechler, 1986) and has been used to analyse the state of collagen preservation within archaeological bone collagen (Nielsen-Marsh et al., 2000a). The DSC allows changes in the enthalpy of the sample to be monitored as the temperature is increased at a constant rate. It is a particularly useful tool for studying collagen, because denaturation of type I collagen is associated with a large endothermic transition, which typically occurs at 60-65°C. This transition can be shifted to a higher temperature by introducing synthetic cross-linkers such as aldehydes.

7.3.4 Materials

Small quantities (approx 2-3 grams) of dense compact bone from the Punchbowl were supplied by Central Identification Laboratory, Hawaii (CILHI), with a coded identification number. All other details were withheld so that the analyses would be conducted blind. The samples of compact bone were crushed into smaller shards ≤3 mm using a hammer and these smaller shards were used for the subsequent analyses.

7.3.4.1 Initial Blind study

In the first instance four bone samples some of which had been exposed to white powder were tested to validate the approach.

7.3.4.2 Extended Blind Study

Following success with this pilot study a further 15 samples were analysed. These samples included bone from burials which contained the white powder and for which DNA amplification was not possible or difficult and bones with a similar length of burial but were not associated with white powder and DNA extraction was normal. They also included samples that had been taken from different bones from the same individual. Once again this analysis was conducted as a blind test.

7.3.5 Methodology

Slightly different methodologies were applied to the initial and main blind studies. The first study of four bones was subjected to a wider range of analyses to characterize changes to the bone collagen. In the second blind study only DSC was used in order test for a more routine screening approach that could be used on the potential large number of Punchbowl cases that AFDIL have to process.

7.3.5.1 Acid swelling

For the acid swelling experiment 60 mg of bone shards from each sample were placed in 2 ml 0.6 M HCl and stirred continuously on a rocker at 4°C. The demineralizing solution was changed after 7 days then the samples were left to demineralize for a further 7 days.

7.3.5.2 Thermal stability

For the heating experiment 60 mg of bone shards from each sample were placed in 15 ml 0.1 M EDTA and stirred continuously on a rocker at 4°C. The demineralization solution was changed after 7 days then the samples were demineralized for a further 7 days. Once demineralized, the samples were washed with ultra pure water then placed in a glass tube with 5 ml PBS. Before the samples were added the PBS had been previously heated on a heating stage at 70°C for 10 minutes to ensure it had reached the desired temperature. The samples were then added and heated at 70°C for one hour. After the heating

regime was completed the tubes containing the samples were quenched in a bath of crushed ice.

7.3.5.3 TEM sample preparation

Once demineralized the insoluble 'collagen' residue was washed thoroughly with distilled water, to remove any remaining salt and soluble gelatin. The preparation uses a positive staining technique that was adapted from Richter (1986). Samples were placed in a 25 ml universal tube with approximately 3 ml phosphotungstic acid solution (1% w/v), which had been adjusted to pH 7 with 0.5M NaOH. In order to liberate the collagen fibrils the solution was homogenized for three minutes using a homogenizer. In order to maintain a low temperature the universal tube was placed in a beaker of ice and homogenization was conducted in 30 second bursts with 30 second intervals.

Afterwards the supernatants were discarded and the remaining pellets were re-suspended in 1 ml of phosphotungstic acid and mixed using an Autovortex mixer. A drop from the solution was pipetted onto two formvar grids (300 mesh 3 mm Cu grids carbon coated), and allowed to air dry for five minutes, after which time the excess liquid was removed. Finally the formvar grids were floated on a drop of uranyl acetate (2% solution in 50:50 ethanol/water mix) specimen side down, for 30 minutes. Once the grids had been rinsed with 50:50 ethanol/water mix and distilled water the excess liquid was removed with filter paper and they were left to air-dry. Analysis was conducted using a FEI Technai G^2 Transmission Electron Microscope fitted with a CCD camera. Measurements were taken from the digital images using Technai G^2 software. The measurements quoted in the text are the average of 10 measurements each from 30 fibrils.

7.3.5.4 DSC sample preparation

DSC samples were soaked in pH 7.0 PBS for 24 hours prior to analysis to ensure a neutral pH. 10-20 mg of demineralized bone shards from each sample was then blotted dry and sealed into Al DSC pans. The pans were heated from 15-95°C at a heating rate of 5°C min^{-1} in a DSC822e differential scanning calorimeter (Mettler Toledo, Leicester, UK) fitted with a nitrogen gas intracooler, using a sealed empty pan as a reference. The onset, peak max and peak end temperatures for each endotherm were measured using Mettler STARe integration software where possible or else manually using a printout of the thermal scans.

7.3.5.5 DSC control samples

For comparison two additional DSC traces are also presented. The scans were produced from 18 month old modern bovine bone collagen. One sample was untreated, the other treated with 1% glutaraldehyde. In both cases the samples consisted of 60 mg of small bone shards that had been demineralized in 0.1 M EDTA for 14 days. One of the samples was then prepared for DSC analysis as described above, the other was placed in 20 ml of a 1% glutaraldehyde solution (2 ml Gluteric dialdehyde 50%, 98 ml PBS) and stirred on a rocker at room temperature overnight. This sample was then removed from the solution and left to soak in deionized water for three minutes. The sample was then blotted dry with filter paper, sealed into an Al DSC pan and heated from 15-95°C at a heating rate of 5°C min^{-1}.

7.3.6 Results and discussion

7.3.6.1 Appearance after acid demineralization

After the samples had been demineralized and swollen in dilute HCl they had slightly different characteristics. Sample A was cream / brown in colour (2.5YR 7/3), translucent and was very flexible when pressed with tweezers. Sample C was also translucent and flexible when pressed with tweezers but in this case was cream / white in colour (2.5YR 8/3). Samples B and D by comparison were opaque and white in colour (5YR 8/1) after treatment with HCl. They also had the consistency of hard rubber when pressed with tweezers.

7.3.6.2 Acid swelling visualized by TEM

Two images from each sample are presented in Figure 7.3. The images are representative of the state of the collagen fibrils after they had been swollen by acid. The degree of swelling is markedly different between the four samples. In sample A the majority of the collagen had unaltered regions and areas of swelling within the same fibril, as can be seen in Figure 7.3A. The fibrils from sample B had a very different appearance and resembled those of sample D. These fibrils had clearly defined edges and uniform diameters. They were also mainly observed on the TEM grid as bundles (as seen in Figure 7.3B) and rarely as individual fibrils. Sample C by contrast displayed significantly more swelling than any of the other samples. The collagen fibrils from this sample appeared as flat 'ribbons' which were faint and often had ill-defined frayed edges.

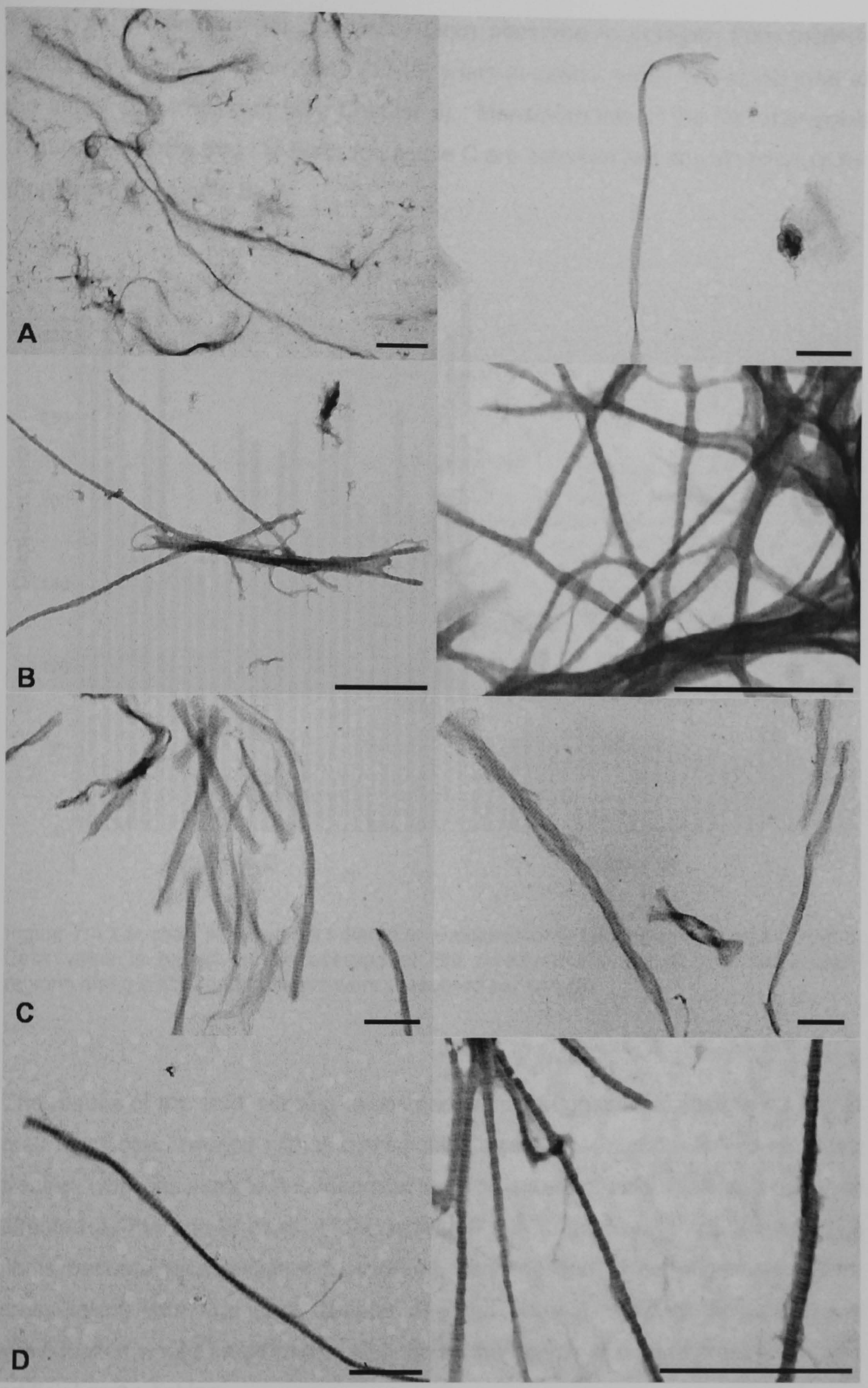


Figure 7.3 TEM images from samples A-D showing the effects of acid swelling to the collagen fibrils. The scale bars are 1 μm.

This type of swelling has previously been observed in collagen from modern young adult human femur bone (17-29 years-at-death) which has undergone to the same acid treatment (see Chapter 5). Measurements of the fibril diameters (Figure 7.4) show that the fibrils in sample C are between two and six times wider than those of sample B.

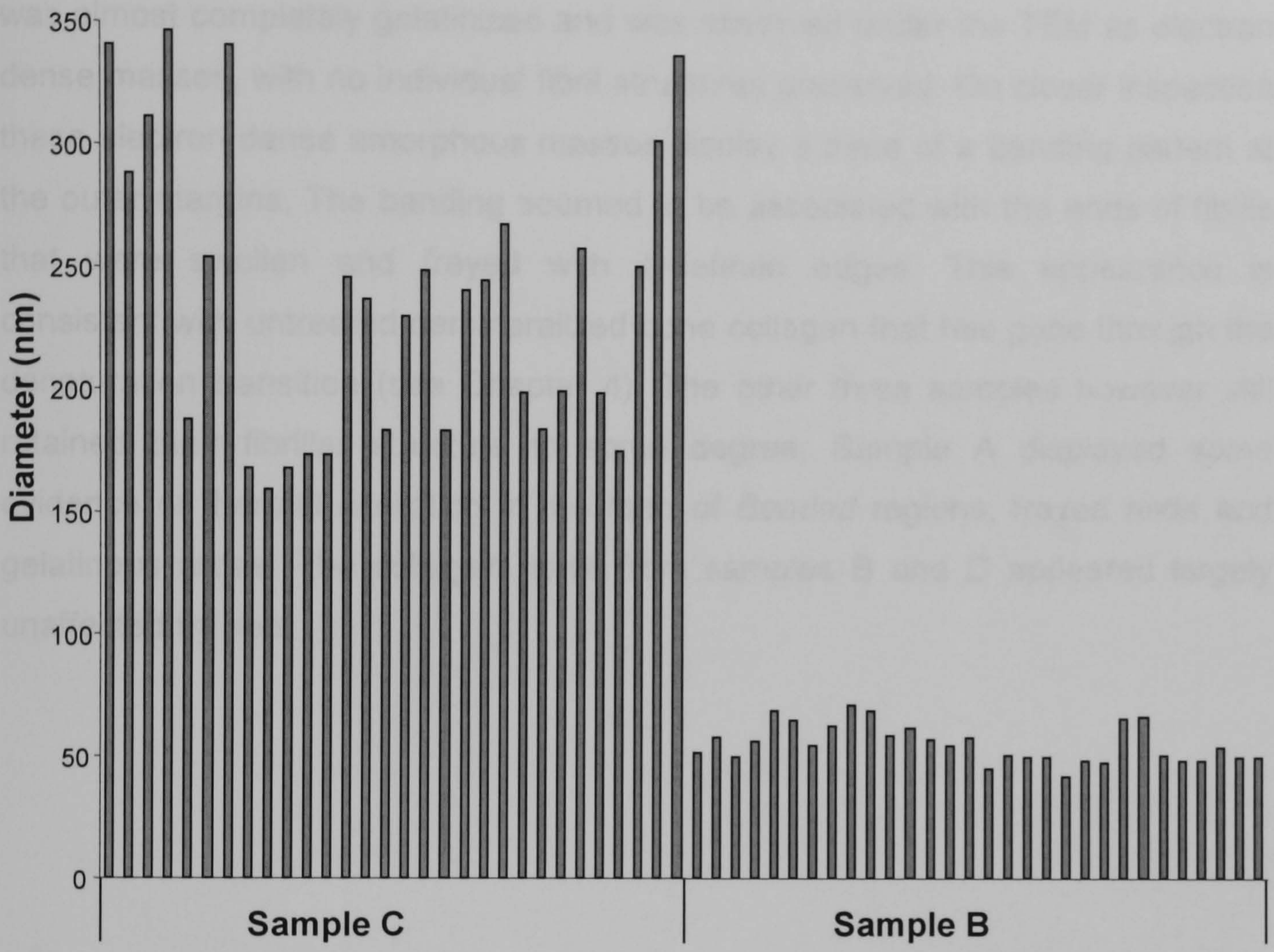


Figure 7.4 bar chart showing fibril diameter measurements taken from samples C and B. Each value is based on the average of five measurements taken from undamaged regions along a fibril and 30 fibrils were measured per sample.

The results of the acid swelling experiment would suggest that samples B and D may have been treated with a cross-linking agent; that sample C had not been treated; whereas sample A is inconclusive. The extent of acid swelling is however affected by the age-at-death of the individual. With increasing age the collagen fibrils become less resistant to swelling, perhaps due to increased secondary cross-linking with age (see Chapter 5). Thus without knowing the age of the individuals it would be difficult to distinguish the effects of natural cross-links from artificial cross-linking.

7.3.6.3 Thermal alteration visualized by TEM

Again for each sample two images are presented that are representative of the state of the collagen fibrils, in this case, after they had been demineralized and then heated in PBS at 70°C for one hour (Figure 7.5). Sample C had undergone significantly more thermal alteration than the other three samples. This sample was almost completely gelatinized and was observed under the TEM as electron dense masses, with no individual fibril structures preserved. On closer inspection these electron dense amorphous masses display a trace of a banding pattern at the outer margins. The banding seemed to be associated with the ends of fibrils that were swollen and frayed with ill-defined edges. This appearance is consistent with untreated demineralized bone collagen that has gone through the denaturation transition (see Chapter 4). The other three samples however still retained their fibrillar structure to some degree. Sample A displayed some evidence of thermal alteration in the form of *Beaded* regions, frayed ends and gelatinous areas. The collagen fibrils from samples B and D appeared largely unaffected by heat.

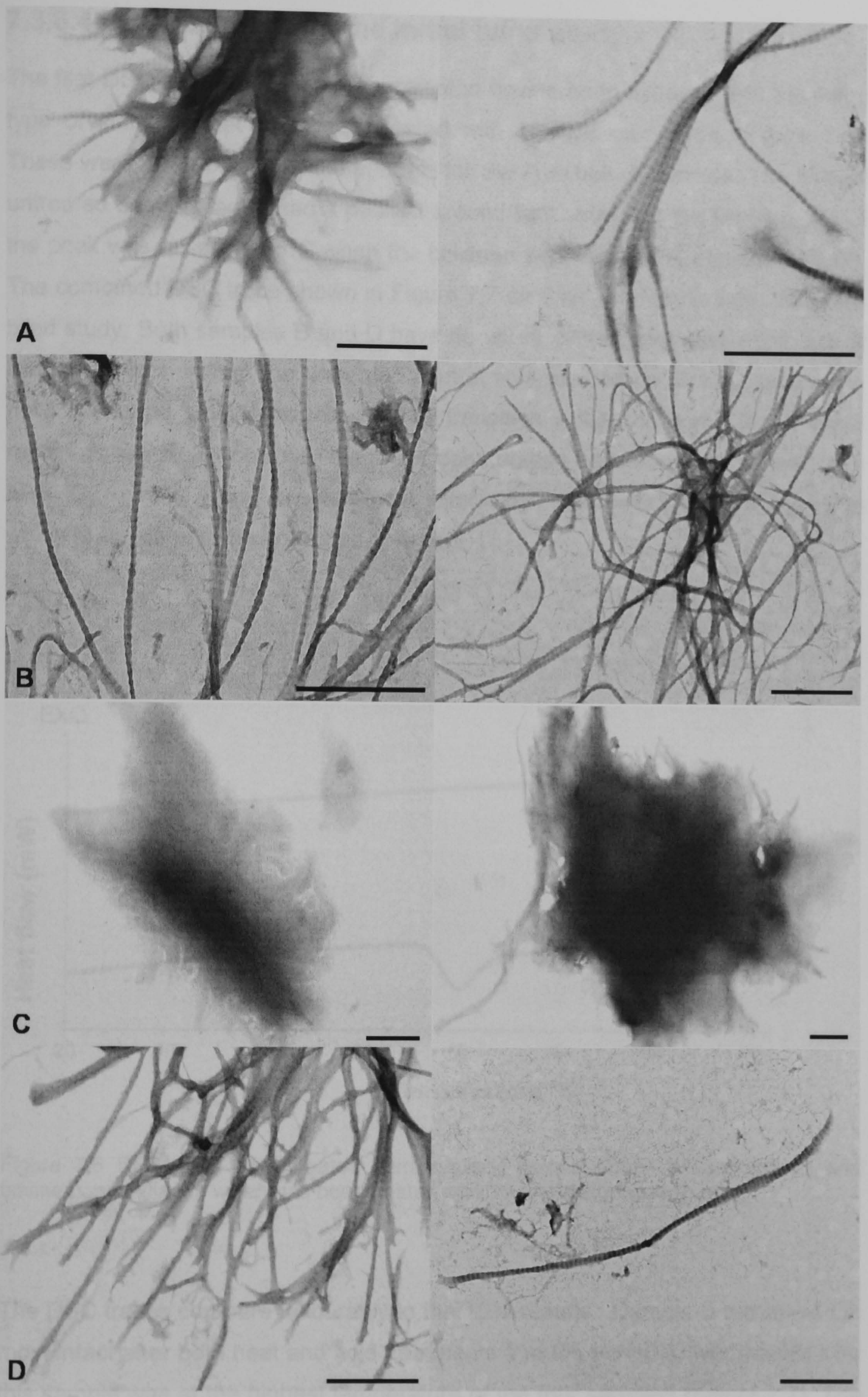


Figure 7.5 TEM images from samples A-D showing the effects of thermal alteration on the collagen fibrils. The scale bars are 1 μm.

7.3.6.4 DSC results from the Initial blind study

The first DSC trace shows normal untreated bovine bone collagen and the same type of collagen that had been treated with 1% glutaraldehyde (Figure 7.6). These were included to provide controls for the Punchbowl samples. The normal untreated bone collagen had a peak at around 58°C however the temperature of the peak was raised to 85°C when the collagen was treated with glutaraldehyde. The combined DSC trace shown in Figure 7.7 displays the results from the initial blind study. Both samples B and D have denaturation temperatures which are at least 10°C higher than the untreated control sample. Sample C also appears to have a second small high temperature transition within the same temperature region as sample D; however the major denaturation transition gives a peak max of 60°C. Sample A also shows a peak max within this lower temperature region which is consistent with untreated collagen.

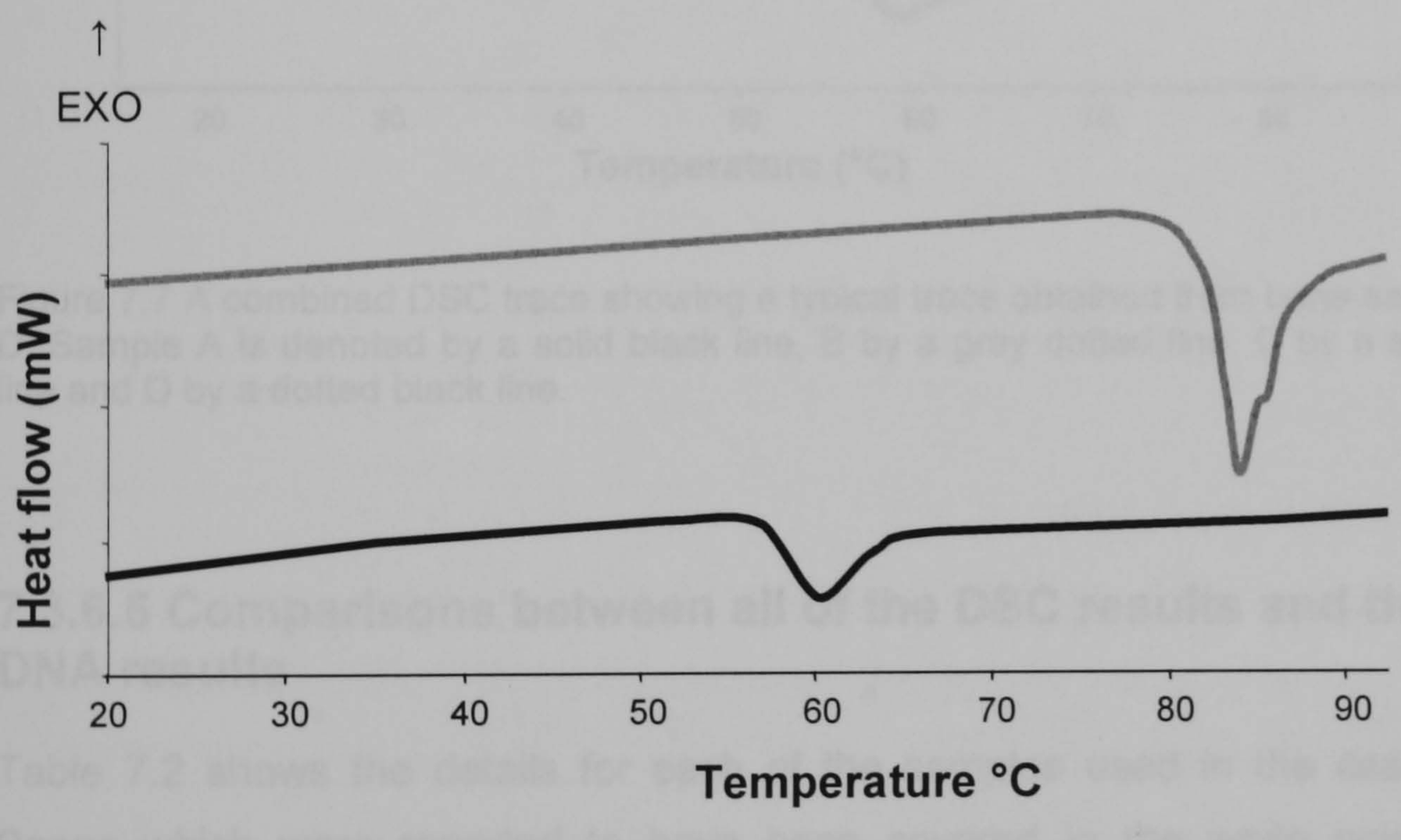


Figure 7.6 DSC trace of untreated demineralized bovine bone collagen (black) and bovine bone collagen which has been treated with 1% glutaraldehyde (grey).

The DSC traces compare favourably to the TEM results. Sample B remained the most intact after both heat and acid treatments and the endothermic transition for this sample was at the highest temperature of the four samples (Figure 7.7). The shape of the peak was also sharp and well-defined, indicating that the collagen from this sample was uniformly stabilized. Samples A and C were observed to be

more damaged after the acid swelling and thermal alteration treatments and they had considerably lower peak max temperatures.

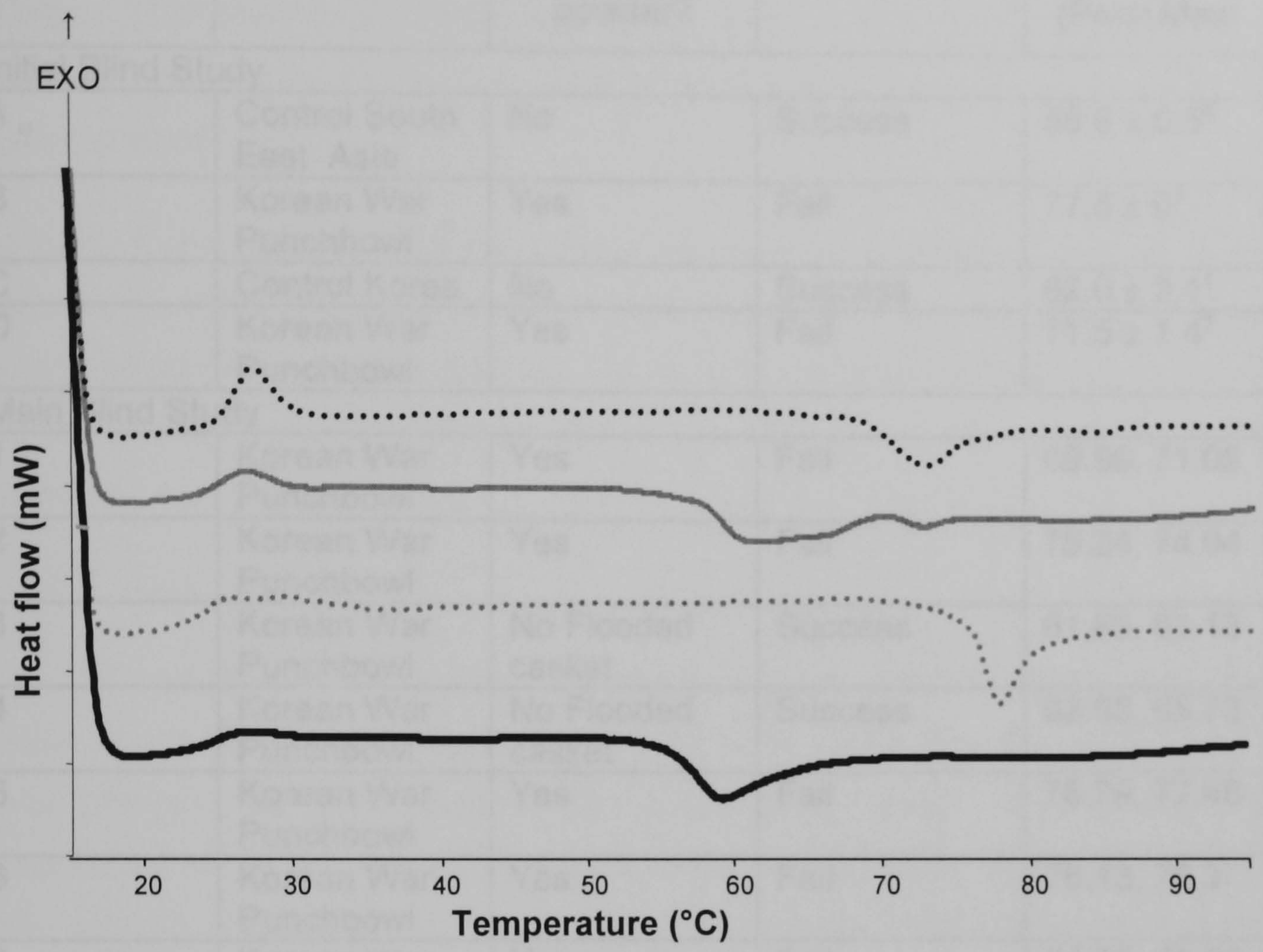


Figure 7.7 A combined DSC trace showing a typical trace obtained from bone samples A-D. Sample A is denoted by a solid black line, B by a grey dotted line, C by a solid grey line and D by a dotted black line.

7.3.6.5 Comparisons between all of the DSC results and the DNA results

Table 7.2 shows the details for each of the samples used in the case study. Bones which were reported to have been covered in the white powder are indicated, as are those that yielded amplifiable DNA. The DSC results showing the average onset, peak max and peak end values for all the samples are also displayed in the table.

Table 7.2 Details of the bone sample analysed in the initial and main blind studies and the DNA and DSC results. † *n* =3 (+/- S.D.)

Label	Details	White powder?	DNA result	DSC result (Peak Max)
Initial Blind Study				
A	Control South East Asia	No	Success	58.6 ± 0.5†
B	Korean War Punchbowl	Yes	Fail	77.6 ± 0†
C	Control Korea	No	Success	62.0 ± 0.1†
D	Korean War Punchbowl	Yes	Fail	71.5 ± 1.4†
Main Blind Study				
1	Korean War Punchbowl	Yes	Fail	69.99, 71.05
2	Korean War Punchbowl	Yes	Fail	75.24, 74.94
3	Korean War Punchbowl	No Flooded casket	Success	61.89, 61.13
4	Korean War Punchbowl	No Flooded casket	Success	63.93, 65.73
5	Korean War Punchbowl	Yes	Fail	78.79, 77.48
6	Korean War Punchbowl	Yes	Fail	76.13, 76.3
7	Korean War Punchbowl	Yes	Fail	74.97, 75.99
8	Korean War Punchbowl	Yes	Fail	69.74, 71.57
9	Korean War Punchbowl	Yes	Fail	69.65, 70.06
10	Control South East Asia	No	Success	58.25, 61.53
11	Control South East Asia	No	Success	59.63, 60.95
12	Control WWII	No	Success	61.86, 61.24
13	Control Bitz	No	Success but difficult	67.28, 68.99
14	Control	No	Fail	60
15	Control Korea	No	Success	61.57, 59.83

7.3.6.5.1 Initial blind study

Both TEM and DSC analysis suggested that samples B and D behaved differently from normal bone collagen. From Table 7.2 it can be seen that both samples B and D did in fact come from the Punchbowl excavations and amplifiable DNA was ***not*** extracted from these remains. The positions of the DSC peaks for these samples are consistent with the suggestion of a treatment which

has stabilized the collagen – e.g. cross-linking. The stabilizing agent would have been added to the corpse and consequently tanning of the mineralized collagen would be anticipated to be less effective than tanning of non-mineralized collagen. This could explain the lower denaturation temperatures and less sharp peaks obtained for the Punchbowl samples when compared to the glutaraldehyde treated control.

Samples A and C were non-Punchbowl bones and amplifiable DNA could be extracted from these bones. TEM and DSC results indicate that these samples behave as normal untreated collagen. However the differences in thermal stability and resistance to swelling between the two controls A and C can not be readily explained.

7.3.6.5.2 Main blind study

The DSC results from samples 1-15 show a good correlation with the DNA results and support the findings of the initial blind study: that the samples with white powder had been treated with a stabilizing agent. The denaturation temperature for untreated, fully hydrated, demineralized bone collagen at neutral pH is approximately 58-63°C. It can be seen from Table 7.2 that all but one of the samples with peak max values within this range came from control specimens where no post-mortem treatment was suspected and DNA extraction had been successful (see samples 10-12 and 15). As mentioned above, stabilization with aldehyde cross-linking agents can raise the transition temperature over 70°C and all of the Punchbowl samples with white powder and which failed to yield amplifiable DNA, had peak max values over 70°C (see Table 7.2, samples 1,2 and 5-9) .

In Figure 7.8 the onset (the point at which the DSC trace begins to deviate from the baseline at the start of the endothermic transition), peak max (maximum of the peak) and peak end (the point at which the DSC trace rejoins the baseline at the end of the endothermic transition) values from the DSC traces of each sample have been plotted together. A dividing line has also been drawn between the peak max values of the samples for which DNA extraction was successful (plotted in white) and those Punchbowl samples which failed to yield amplifiable DNA (plotted in black); there is a clear relationship between the temperature of the endotherm and the DNA results. Samples with values similar to normal unaltered collagen allowed successful extraction of the DNA but those samples

where the collagen had been stabilized (most likely through aldehyde cross-links) failed to yield sufficient DNA for amplification.

However, there is one exception; sample 14 was provided as a control sample which was non-Punchbowl and was not associated with white powder, but had failed to yield DNA. The DSC analysis suggest that the collagen from this sample is unaltered (neither stabilized nor degraded) and therefore the problem with DNA extraction from this sample is unclear but it is unlikely to be due to stabilization through cross-linking.

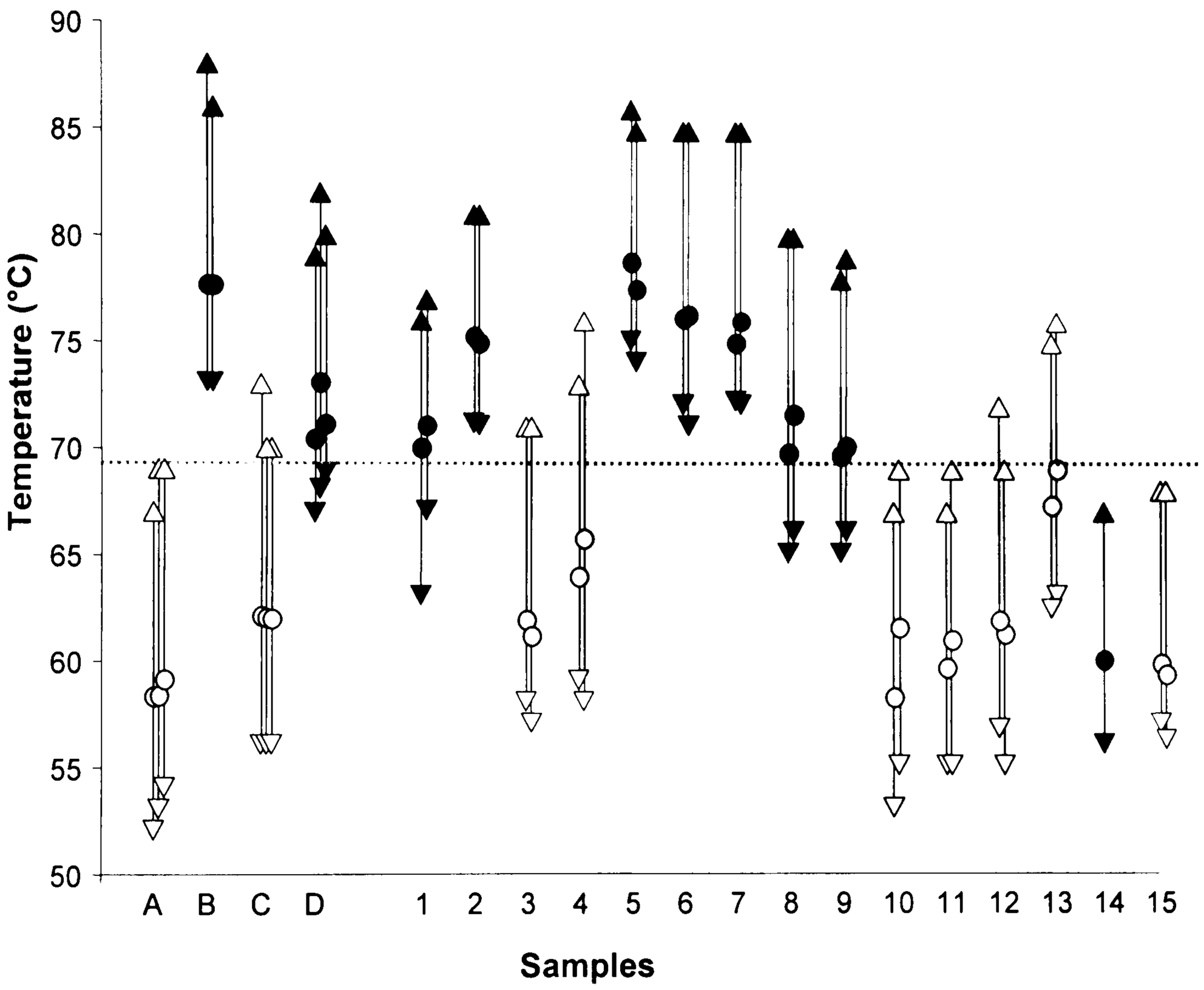


Figure 7.8 A plot showing the DSC values obtained from all of the samples in the embalming case study. The onset (▼) peak max (●) and peak end (▲) values from each DSC scan are shown. Black indicates a DNA failure and white a success. The dotted line represents the division in peak max values between the Punchbowl samples which failed to yield DNA amplifiable and the rest of the samples, the peak max values are consistently elevated in these Punchbowl samples.

There is an opportunity with these samples to look at the reproducibility of the results and hence the reliability of the technique to distinguish between treated and non-treated remains. Multiple runs were undertaken on each sample and the

results of each run are clustered together on the plot in Figure 7.8. For each sample the peaks were in a very similar but not identical position, the onset and peak max proved to give the most consistent values, the peak end values being slightly more variable. Three of the samples were used in both the initial and main blind study and were therefore replicated on two different occasions (A =11, C =15 and D =19). The peak max values for these samples are similar but not identical; also the peaks from the first run tended to be broader (as can be seen from the distance between the onset (▼) and peak end (▲) values). Also some of the bones used in the study were different elements which came from the same individual (1 and 2, 3 and 4, 5 and 6 and 10 and 11). As would be expected the two bones (10 and 11) which came from the untreated control showed the least variability between samples. The elements which came from the remains with white powder and the two samples from the flooded casket (3 and 4) show varying degrees of stabilization but do not cross the dividing line between DNA successes and failures.

Three samples are worth mentioning in greater detail. The first two come from different elements from the same set of remains (3 and 4). As mentioned above these remains came from the Korean War and were buried at Punchbowl however the casket was flooded with water and there was no sign of white powder. DNA extraction from these remains had not caused any problems and it was speculated that the water in the casket had washed away the chemical treatment. The DSC results suggest that if these remains had been treated with white powder it did not cause significant stabilization to the collagen, or the stabilization treatment had been removed by water flushing the casket. The other interesting sample is 13; The DSC results from this sample suggest that the collagen had been stabilized in some way and the peak max values are just under the dividing line indicating DNA successes and failures. This sample did not come from Punchbowl and was not associated with white powder yet the DNA extraction and amplification had proved very difficult (Parsons *pers. comm.*).

One possible reason for this apparent stabilization to the collagen (and DNA) from this sample is natural cross-linking in the burial environment. There are two ways in which these cross-links can occur in buried bone, through the influx of humics from the soil or in situ by the condensation of carbohydrate and predominantly collagen (van Klinken and Hedges, 1995). Humic substances consist of a heterogeneous mixture of compounds including complex aromatic

macromolecules; they can be formed during decomposition of plant and animal matter and have been identified in archaeological bone (Tuross, 2002). The humics could potentially form non-specific random cross-links within and between collagen molecules. In order to investigate this further, more non-Punchbowl bones would have to be tested which had proved difficult or impossible for DNA extraction but yet still had an organic content.

7.3.7 Conclusion

To summarize, the U.S. Army Central Identification Laboratory has been trying to identify the remains of American servicemen buried as an "unknown" from the Korean War. The remains were tested for DNA. However, after rigorous extraction procedures, DNA could not be successfully amplified in the majority of cases. The Punchbowl bones were covered with a white powder and it was suspected that they had been treated with one or more mortuary compounds. It was decided to look at the collagen component of these bones to get an idea of what these compounds could be.

TEM analysis of collagen fibrils from the Punchbowl remains showed that they were more resistant to swelling and heat treatment than those from untreated modern human material. This suggested that the white powder contained a cross-linking agent. DSC was then used to further assess the stabilizing effect of the suspected cross-linker and to provide quantification. Aldehyde cross-linkers such as formaldehyde have a denaturation temperature of above 70°C with an optimum stabilizing effect with a denaturation temperature of 85°C. The peak max temperatures obtained from the Punchbowl samples that had been treated with white power were consistent with them having been treated with an aldehyde based cross-linker. The DSC results show collagens with a range of different thermal stabilities and when compared with the DNA amplification results there was a clear correlation between successes and failures. Thus applying DSC to bone collagen could be used as a rapid screening technique to assess the likelihood of successful DNA extraction and amplification within this unusual burial context. The technique is at present being implemented by AFDIL to identify those Korean War remains which would be most likely to yield amplifiable DNA.

Chapter 8 Application to Archaeology

8.1 Introduction

The main aim of this thesis is determine if it is possible to identify cooked archaeological bone. In Part 2 it was shown that mild heating can lead to subtle but detectable changes in the collagen component of bone. In Part 3 bone samples from experimental burials added a layer of complexity by showing that the same type of damage can be observed in unheated bone which has been subjected to a period of burial. In this chapter the aim was to determine if the TEM approach could be applicable to ancient bone collagen. This raised two questions:

(1) Could intact and partially degraded collagen fibrils be extracted from archaeological bones ?

(2) Could evidence of cooking be distinguished from the effects of long term burial ?

Previous studies of the collagen fibril have resorted to either fixation such as using formaldehyde (Thalhammer et al., 2001) and/or stabilization, such as by resin embedding (Tuross et al., 1980). It may therefore be the case that the TEM approach, developed using modern bone and successfully tested on forensic material, may have no application to archaeological material if the fibrils rapidly lose their integrity post-mortem. The first part of this chapter therefore describes a preliminary experiment to test this first question. The study examined fibril preservation in archaeological samples, using a small selection of archaeological bones from different time periods and with varying macroscopic preservation.

The second question, that of identifying cooked bones which have been buried in the ground for hundreds of years, is problematic for an obvious reason - it is not possible to obtain treated (i.e. cooked) and unheated control samples; there is, as yet, no unambiguous test for cooked archaeological bone. In order to explore this second question, a blind test was developed with a zooarchaeologist, who used evidence from both the type of skeletal element and location of disposal within an archaeological site to identify probable 'cooked' and 'uncooked' bone. This part of the chapter- a case study in which the TEM approach was applied to

a set of archaeological remains from an urban site containing large quantities of butchered bones and domestic waste deposits - is set out in the format of an article intended for publication.

8.2 Preliminary experiments on archaeological material

8.2.1 Materials and methods

The samples that were used for the preliminary experiments are presented in Table 8.1. The samples selected included a range of ages and material which may have been cooked (sheep and cattle bone) and samples which were extremely unlikely to have been cooked (dog and human). On visual inspection, the Clad Hallan dog humerus and the cow metapodial and the sheep ischium (pelvic bone) from Kilham appeared to be the most degraded. The bones had a chalky appearance and were soft and powdery. As an initial test, these three samples were demineralized using both the EDTA and HCl standard methods, all the other samples were subsequently demineralized using only the EDTA method. Details of the demineralization methods can be found in section 3.3.2 and the TEM methods for preparation and analysis can be found in sections 3.3.4.

Table 8.1 Details of the samples used in the preliminary TEM analysis of archaeological material.

Sample	Site	Age
Dairy (?) cow fused metapodial	Dun Vulan, Bornais, Outer Hebrides	Iron Age 500BC-500AD
Young (?) cow unfused metapodial	Dun Vulan, Bornais, Outer Hebrides	Iron Age 500BC-500AD
Dog humerus	Cladh Hallan, South Uist Western Isles	Bronze Age
Cow metapodial	Kilham, East Yorkshire	Anglo Saxon 500AD
Sheep ishium	Kilham, East Yorkshire	Anglo Saxon 500AD
Cow tibia	York, North Yorkshire	Anglo Scandinavian AD 800-1000
Human rib Age 46 years	London, West Kensington	Victorian 1853

8.2.2 Results and discussion

8.2.2.1 Comparative demineralization

There was a marked difference between the HCl and EDTA demineralization of the three poorly preserved samples. When these bones were demineralized in acid after just two days the demineralization solutions had turned a yellowish brown and the shards were very small and almost transparent, they had a mushy texture and separated into smaller fragments when pressed with tweezers. By contrast after 14 days the EDTA demineralized shards were rubbery, translucent and still retained their original shape.

Under the microscope the HCl demineralized fibrils generally appeared very faint and swollen. TEM analysis also revealed that the fibrils were more degraded by HCl than by EDTA (greater proportion of *Beaded* and *Dumbbell*) and when acid demineralized there were less than 100 fibrils actually found on each grid. After EDTA demineralization (except in the case of the dog humerus where only 46 and 32 fibrils could be observed from each analysis) at least 100 fibrils could be counted and classified from each grid and the results of the analysis are shown in Figure 8.1. This suggests that during the HCl demineralization a large amount of the collagen had been dissolved into solution. As a result of these observations only EDTA demineralization was used in subsequent analysis of archaeological samples.

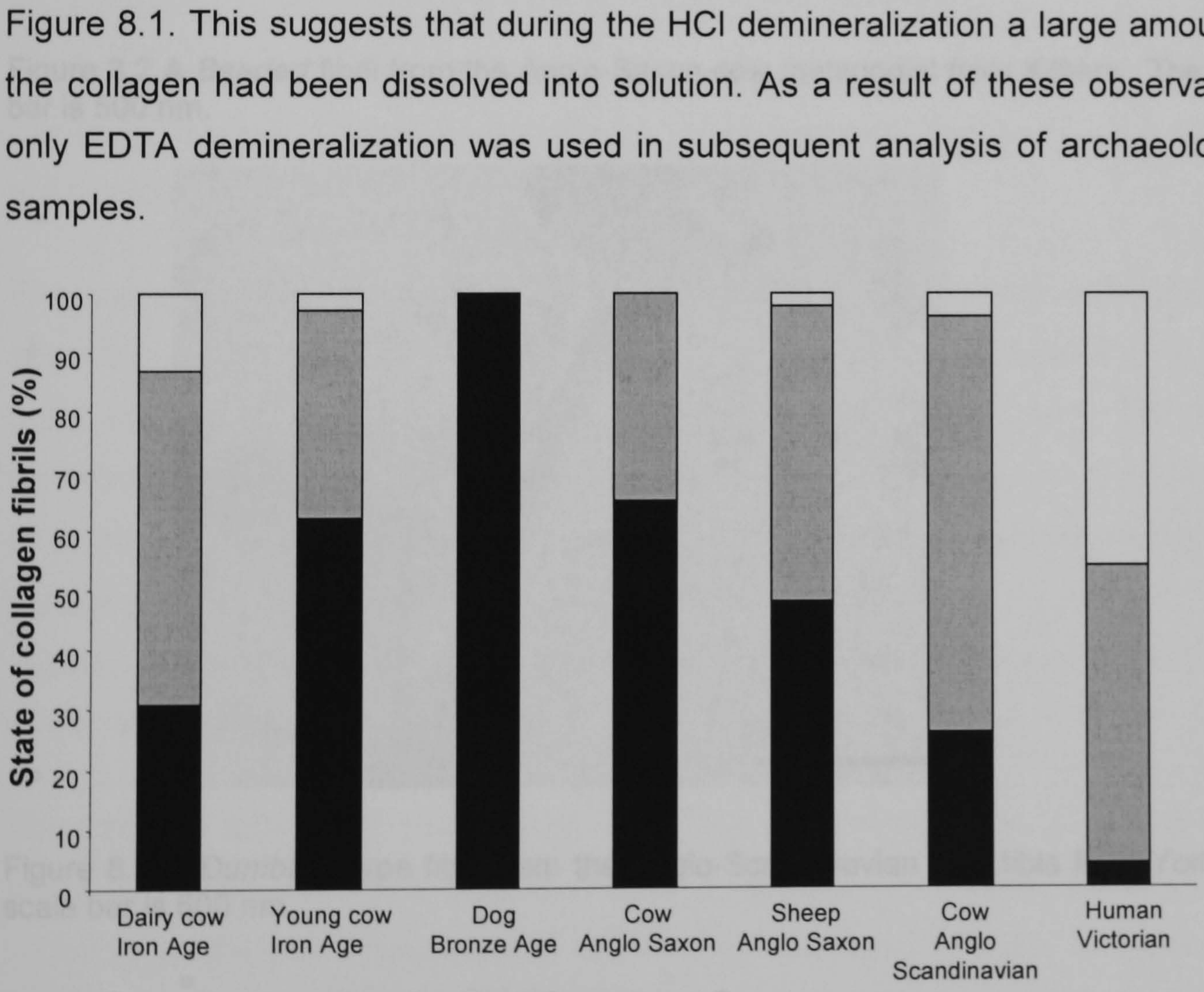


Figure 8.1 A stacked bar chart showing the proportion of *Unaltered* (white), *Beaded* (grey) and *Dumbbell* (black) type fibrils recorded from various EDTA-demineralized archaeological samples (average of two analysis per sample).

8.2.2.2 Visualization

Using the EDTA demineralization technique, fibrillar collagen can be extracted from archaeological bones and viewed under the TEM *without* the need for a fixative or embedding agent. Using this approach partially degraded collagen fibrils, exhibiting *Beaded* and *Dumbbell*-type alteration could be observed (see Figures 8.2 and 8.3).

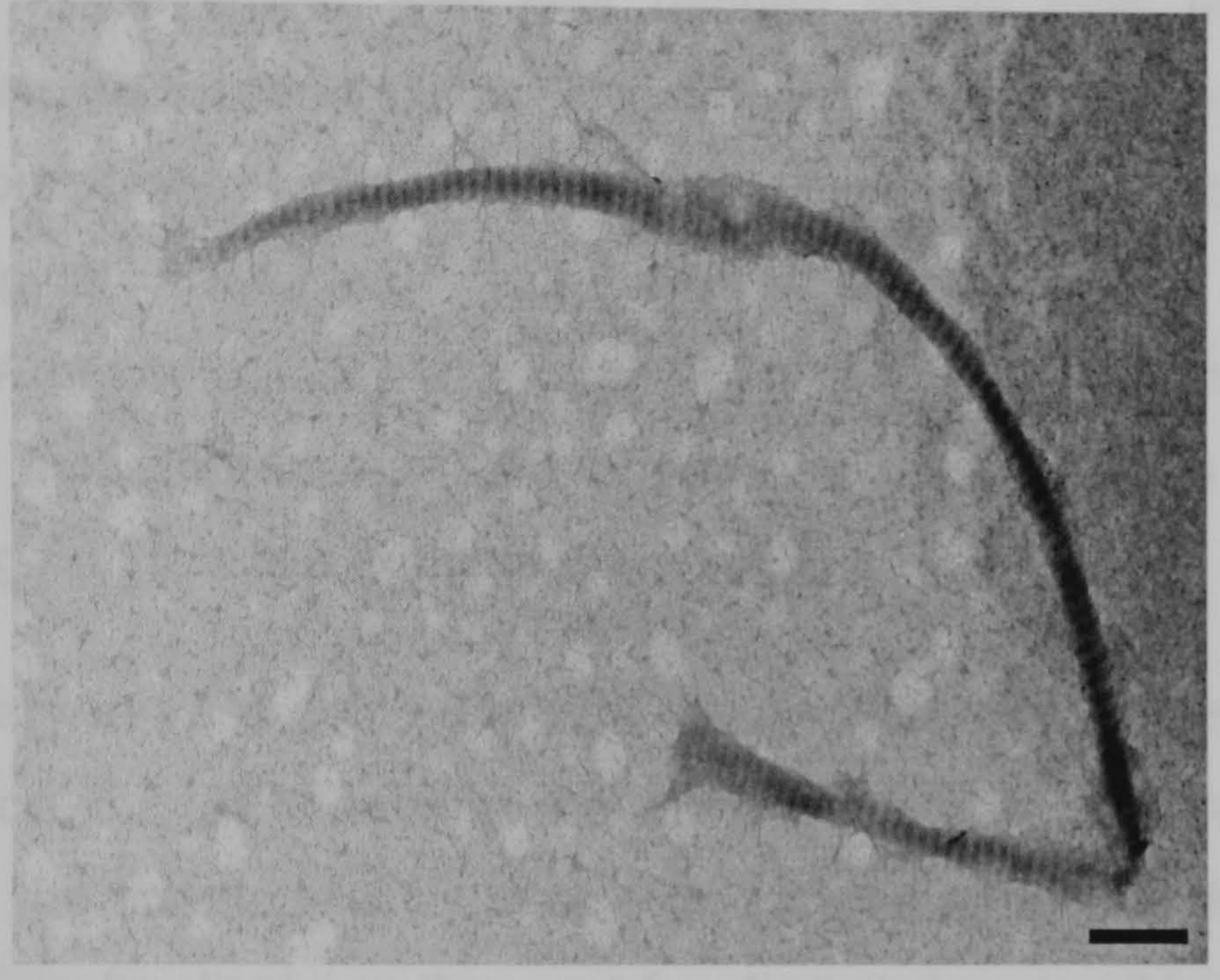


Figure 8.2 A *Beaded* fibril from the Anglo-Saxon cow metapodial from Kilham. The scale bar is 500 nm.

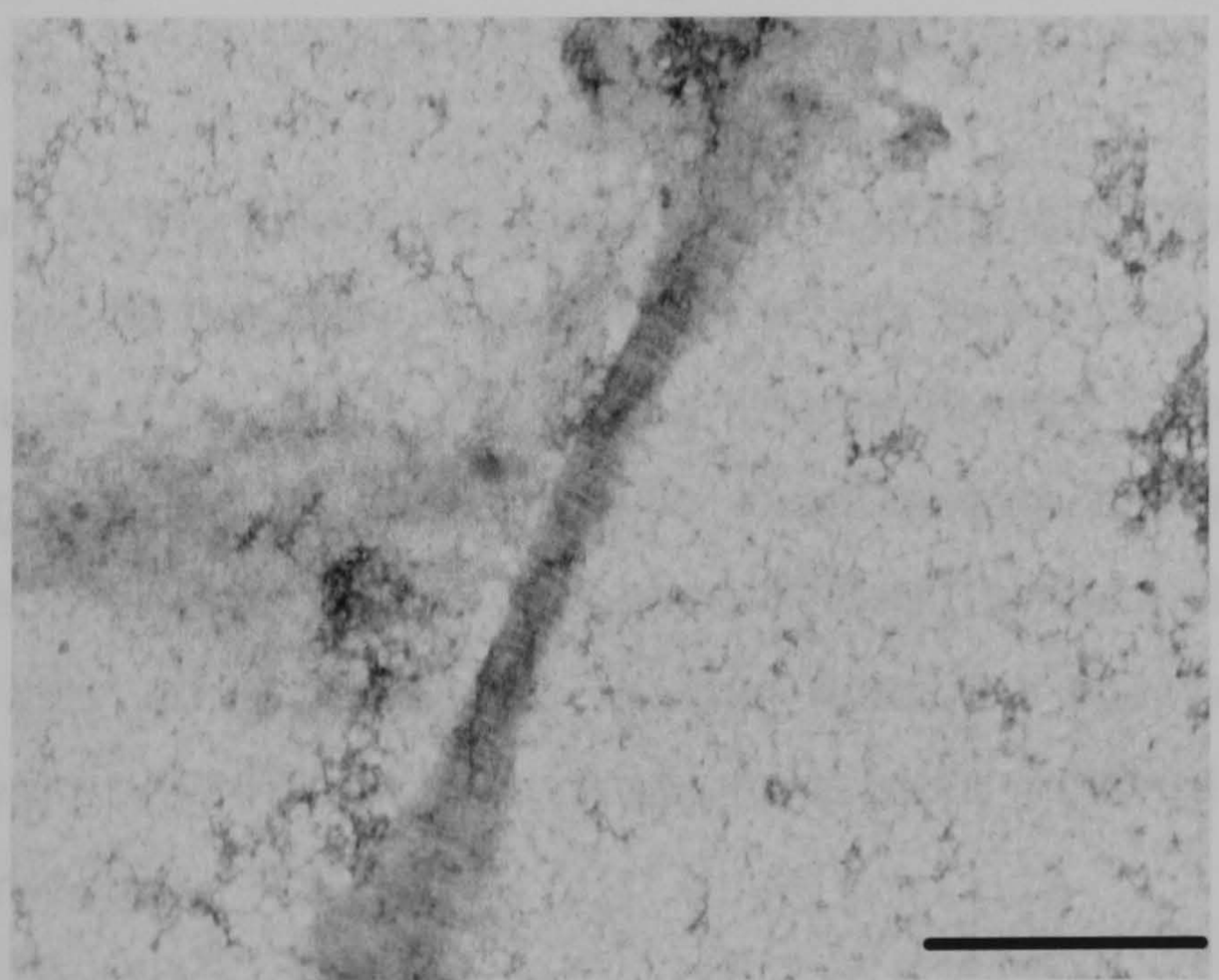


Figure 8.3 A *Dumbbell* type fibril from the Anglo-Scandinavian cow tibia from York. The scale bar is 500 nm.

Whilst it is probable that many of the bone samples used in this preliminary study had gone through a heating event prior to burial, this is unlikely to have been the

case for either the human rib or dog tibia. In these cases the skeletons where still articulated at the time of excavation. The paucity of completely unaltered fibrils observed in these bone samples (see Figure 8.1) confirms the results of the burial experiments, namely that cooking is not the only factor in the degradation of collagen fibrils in bones which have been exposed to a period of burial.

A further difference observed in the archaeological samples was that fibrils appeared to be lighter with less defined edges than modern and forensic counterparts discussed thus far. This difference might simply be an artefact of the staining preparation. However this phenomenon was ubiquitous among the archaeological samples, but was seldom seen in the modern samples. Archaeological and modern samples were often prepared at the same time and the difference in the appearance of the fibrils was still apparent, suggesting that this is a genuine phenomenon.

It is not very easy to convey the difference between archaeological and modern samples on the TEM images because the digital camera compensates and increases the contrast; as a result faint images appear dark. The problem could be overcome by disabling this *gain* function and manually adjusting the contrast but this has proved very time consuming with not particularly good results. For capturing images of these archaeological fibrils still photographs have proved the best method.

The reason for the difference in appearance of the archaeological collagen fibrils is unclear but as the heavy metal stains adhere to the charged side chains on the outer surface of the fibrils it suggests that these sites have (i) been blocked by the strong binding of an alternative lower-density cation, probably from the environment, (ii) that charge has been diffused by the interaction between the collagen and other molecules in the bone or soil (e.g. so-called humic substances) or (iii) that the side chains have been modified to reduce the binding of the uranyl stain.

The use of a strong chelating agent (EDTA) to demineralize the bone makes the first explanation less likely as this will compete aggressively for divalent cations common in the soil. However, as Al^{3+} is very abundant in soils, is insoluble at neutral pH and has a lower affinity for EDTA than divalent ions such as Ca^{2+} this may be a candidate for the effect observed. As to the second explanation, whilst it is reported that humic substances will bind to collagen (van Klinken and

Hedges, 1995) C:N ratios do not suggest that archaeological collagen contains much additional carbon. As to the third explanation, the most common sidechain modification will be the deamidation of asparaginyl and glutaminyl residues (to aspartyl and glutamyl residues respectively). The increase in number of carboxyl groups on the collagen fibril should increase not decrease the amount of staining, although it would reduce the clarity of banding. It is therefore not clear what factors cause this 'diffusing' of the fibril when contrasted with modern preparations.

8.3 Archaeological case study

Sorting the butchered from the boiled

Abstract

Mild heating (100°C,1hr) does not lead to detectable changes in any biochemical parameter yet measured. However during cooking this is exactly the type of temperature regime that a bone would be subjected to. This means that there is a wealth of evidence from bones in the archaeological record that have been cooked, but which have not reached a temperature that will induce charring, and therefore go undetected. In a previous paper (Koon et al., 2003), we used a TEM based approach to investigate changes in the organization of the collagen fibril as it is heated, using bone from heating experiments and short term burials. The results of this and subsequent work have shown the surprising finding that collagen is actually extremely prone to damage. However in bone the presence of mineral matrix protects the collagen and helps maintain a record of the accumulation of damage within the fibril. The damage can be observed once the mineral is removed, as an unpacking of the fibril structure. This unpacking is very sensitive to temperature and therefore with appropriate visualization methods, the degree of alteration can be used to infer cooking.

In this paper the visualization technique was tested in a blind study of bovine bone from the Anglo-Scandinavian site of Coppergate, York. The purpose of the study was to determine if the method could discriminate between bones believed, on the basis of zoo-archaeological and spatial evidence, to be butchered and those believed to be cooked.

8.3.1 Introduction

Bones can go through a number of taphonomic processes between the death of the animal and final deposition into the burial environment. In the first instance, this is likely to involve primary butchery such as the removal of head and feet and disarticulation of the carcass. Perhaps then there would be further butchery into smaller more manageable pieces and then some form of cooking before finally arriving on the dinner plate. When the food waste is discarded, bones may be exposed to carnivore activity and sub-aerial weathering before burial. In this study an attempt was made to distinguish between bones which have gone through the cooking step from bones deposited after a previous stage. Features such as; fragmentation patterns, cut-marks and charring, are used to indicate food processing (see Chapter 2). Charring is the only one of these features however, that directly implies cooking, yet is unlikely to be present if bones were boiled or covered by flesh. What the other features do provide is evidence of human intervention. This zoological evidence, along with the patterns of butchery and the spatial distribution of bones on a site, are used to classify the faunal remains into one of the food processing stages. If it is possible to differentiate between cooked and uncooked bone by looking at the state of the collagen, these classifications could be proved or disproved.

This study was set up as a blind test to determine if the novel TEM based approach, developed as part of this thesis, could be successfully used to identify archaeological bone which has been cooked, but which has not reached a temperature high enough to cause charring. Previous attempts using mineral and protein changes (Roberts et al., 2002) and Differential Scanning Calorimetry (Nielsen-Marsh et al., 2000a) have proved too crude to detect these subtle changes.

A TEM based approach had previously been used to look at factors affecting the toughness of meat by heated collagen from the tendinous sheath of the longissimus dorsi muscle of mature sheep (Snowden and Weidemann, 1978). Later Richter (1986) used the same approach to examine the effect of boiling on fish bone collagen. More recently the technique has been refined and used to identify roasted mammal bone in heating experiments and from experimental burials (Koon et al., 2003). The aim of this study was to determine if the same technique could be applied to archaeological material.

8.3.2 Materials and methods

8.3.2.1 Criteria for selection of materials

The experimental work for this thesis had highlighted some potential complicating factors for identifying cooked bone. Species and age-at-death may have an effect on the fibril stability and hence the degree of alteration observed in a bone sample. Also as mentioned above diagenetic processes may mask the effects of cooking. In order to minimize these problems, the following criteria for the bones was used in the archaeological study:

1) bones should be from the same species

2) bones should be well preserved

3) bones should have a similar burial history (environment and length of burial)

4) bones should include several examples of suspected 'cooked' (although without evidence of charring) and 'uncooked' material.

Looking at a number of bones from the same species and of a similar level of preservation would provide a baseline of the extent of damage to be expected from an uncooked bone. Bones which showed more damage than this baseline would most likely have been through a cooking treatment prior to burial. Well preserved bones were chosen to provide the best chance for success, the idea being that if the technique was unsuccessful here, it would not be applicable to archaeological material in general. The case study was set up as a blind test, coded samples of bones from the same site and similar contexts were provided by Professor O'Connor (University of York).

8.3.2.2 Materials used in study

Bovine bones from eight contexts were provided for analysis; no information was given about the bones except the context numbers on each set of bones. From this material three or four bones were chosen from each context. These included intact and fragmented humeri, tibia and metapodials.

8.3.2.2.1 Sample preparation

Samples were taken from the mid-shaft where possible. For bones that were broken, samples were taken from the shaft away from the fracture site. In some cases the samples could be removed directly using a small water-cooled saw. When bones were too big sections were removed using a larger band saw. As the band saw produces a lot of heat, the water cooled saw was then used to remove the first 3 mm of bone from each side previously sawn with the band saw. The samples were then broken into small shards (≤3 mm largest dimension) using a hammer. The shards were demineralized using the EDTA method which is described in section 3.3.2.2 and the samples were then prepared for TEM analysis (section 3.3.4.2). No discrimination was made between the outer and inner portion of the bone as it was felt, based upon previous experience, that this would overly complicate the method. Whilst removing the outer surface might have the effect of removing the areas that are most likely to be diagenetically altered, it might also remove the areas which would most strongly to have been affected by heat.

The bones displayed unusual patterns of staining when viewed in cross-section, usually seen as a yellowish-cream 2.5Y 8/4 outer and inner rim either side of a central reddish-brown region (7.5YR 5/6) (see Figure 8.4.) or a blackish-brown (2.5YR 3/1) rim with a yellowish-cream central region.

Figure 8.4 Photograph of a cross-section through one of the bones from Coppergate showing the staining pattern.

A portion of the inner dark section and outer light section was taken from one sample and analysed by TEM. The results showed little difference between the

different coloured portions indicating that whatever has caused the staining did not affect the integrity of the collagen fibrils and it was not necessary to distinguish between stained areas when taking samples.

8.3.3 Results and discussion

8.3.3.1 Results of the TEM analysis

The results of the TEM analysis are shown on the ternplot in Figure 8.5. Each point represents the observed state of the collagen fibrils for each sample. As described in Chapter 3 the state of the collagen fibrils in a bone sample is estimated by counting and classing 100 fibrils on a TEM grid as; *Unaltered*, *Beaded* or *Dumbbell*. This process is repeated on a further grid then the average of the two results is used to produce the ternplot value. Points with the same symbol belong to the same context.

In general the samples are situated to the right hand side of the plot which means that there was a lack of undamaged fibrils in the bones from this site. In fact in the sample with the most *Unaltered* fibrils, only 23% of the fibrils were in this state. More than half of the samples had fewer than 5% *Unaltered* fibrils. Just under half of the samples displayed greater than 50% *Beaded* alteration and in half of the samples more than 50% of the fibrils observed were in a *Dumbbell* state. The samples appeared to fall into two distinct groups. The first of these clustered in the top corner of the ternplot. Samples from contexts 19739, 19644, 19643 and one sample from 28728 made up this group, characterized by having a majority of *Beaded* fibrils. The second group, made up of samples from contexts 30352T, 30352H, 32105 and 27555, had few or no *Unaltered* fibrils and generally contained greater than 50% *Dumbbells*. These fibrils were therefore clearly more damaged. Since all the bones were of approximately the same age and came from similar contexts, it was concluded that this latter group contains cooked bones samples whereas the former does not.

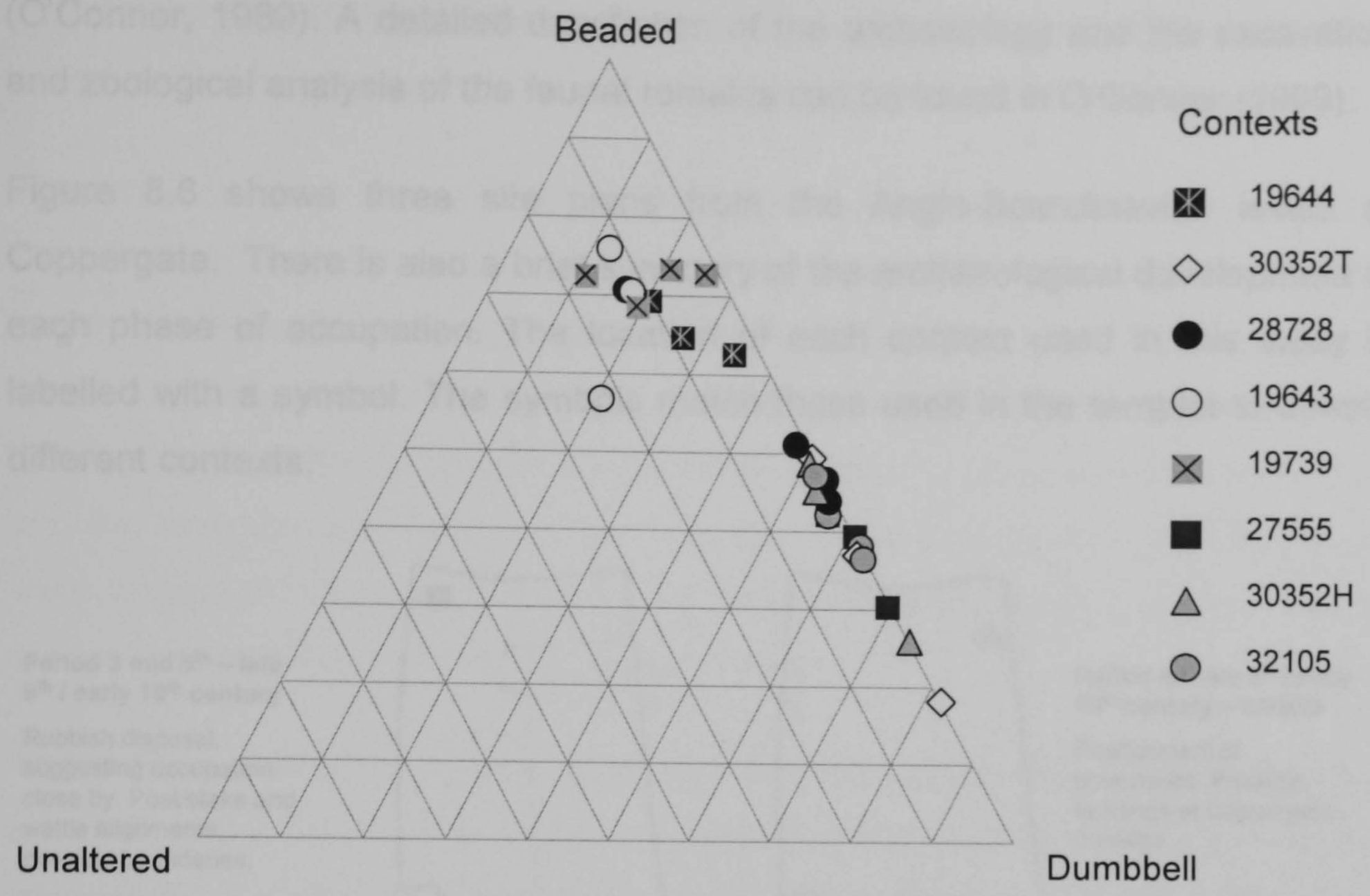


Figure 8.5 A ternplot showing the results of the archaeological case study. Each point represents the proportion of *Unaltered*, *Beaded* and *Dumbbell* type fibrils observed by TEM from each sample (the points represent average values from two analyses of 100 fibrils). Points with the same symbol come from the same context.

8.3.3.2 The contexts

The contexts were revealed to have all come from the same site, 16-22 Coppergate, York. The site, situated at the present day street line of Coppergate and sloping down towards the river Foss, was first occupied during the Roman period. The contexts used in this study came from well-stratified Anglo-Scandinavian occupation levels dated to the mid 9th – late 10th century. In the earliest Anglo-Scandinavian levels there was evidence of possible glass-working and pit digging and at the present day street frontage end of the site a series of post/stake and wattle alignments were present. The alignments eventually became boundaries for tenements which were used as houses and workshops. The tenements had long narrow backyards which were covered with pits which were backfilled with domestic debris (O'Connor, 1989). The excavation of these occupation levels produced a large quantity of well preserved and closely datable animal bone. Whilst a variety of species were identified (including fish, birds, pig, sheep, horse, other mammal and amphibian bones) there was a predominance of cattle bone at the site representing a heavy reliance on beef and dairy products

(O'Connor, 1989). A detailed description of the archaeology and the excavation and zoological analysis of the faunal remains can be found in O'Connor (1989).

Figure 8.6 shows three site plans from the Anglo-Scandinavian levels at Coppergate. There is also a brief summary of the archaeological development at each phase of occupation. The location of each context used in this study is labelled with a symbol. The symbols match those used in the ternplot to denote different contexts.

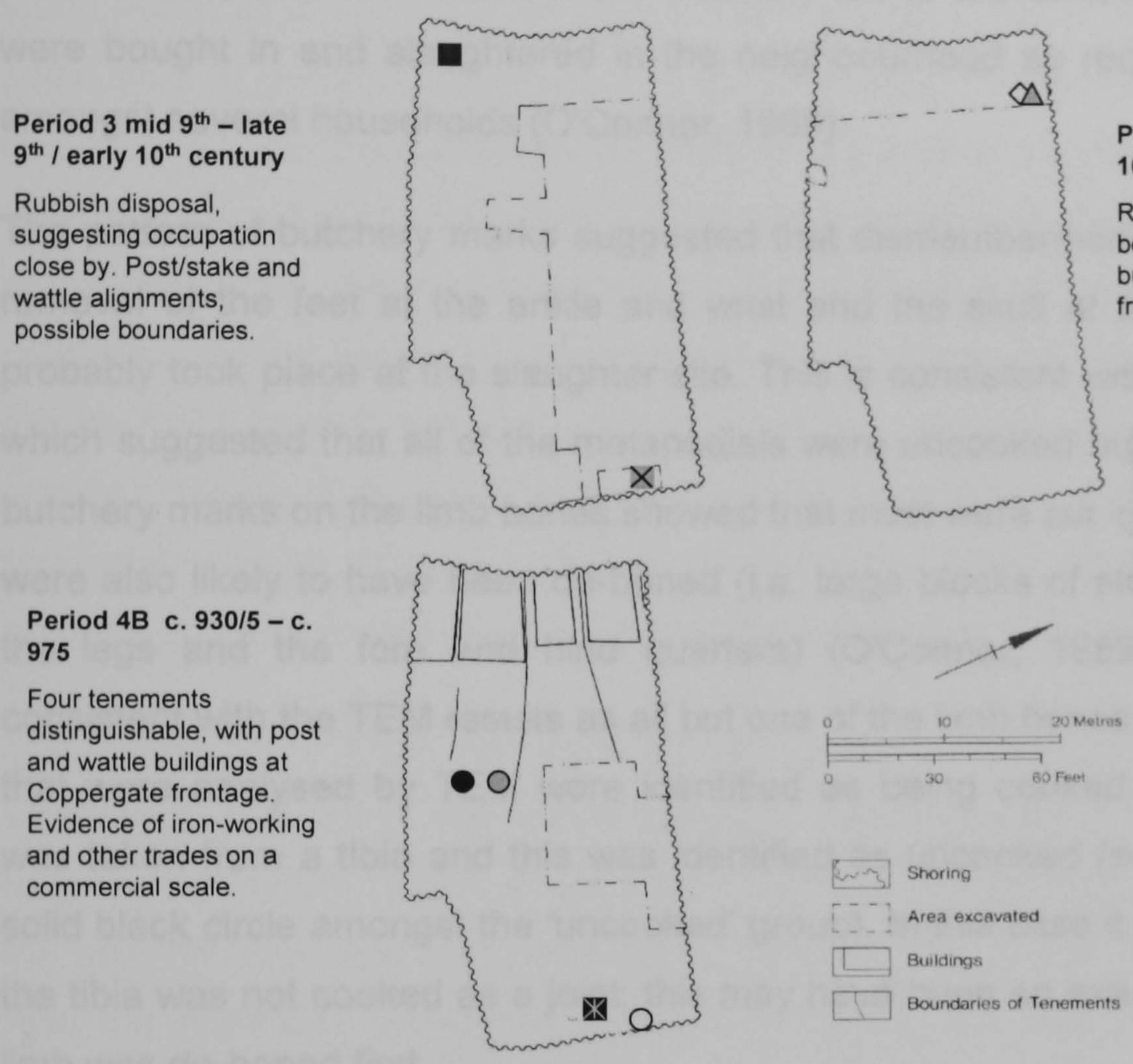


Figure 8.6 Period site plans for 16-22 Coppergate, York showing the location of the contexts from which the samples were obtained. The symbols for each of the eight contexts correspond to the symbols used in Figure 8.1 to distinguish groups of samples from the same context. (The site plans and summaries of archaeological development are from O'Connor, 1989, Table 38 p140, Figure 22 p 144-145).

8.3.3.3 Comparison of 'cooked and uncooked 'bone determined by TEM analysis with zooarchaeological interpretation

It was revealed, based on zoological analysis, that the bones from contexts 19644, 19643 and 19739 were believed to have been uncooked; whereas bones from contexts 30352T, 30352H, 27555, 28728 and 32105 had been identified as

those most likely to have been cooked. The zoological interpretation had been based on butchery marks, skeletal element and location (O'Connor *pers. comm.*).

The analysis of butchery marks on the cattle bones suggested that the carcass was dismembered into smaller pieces on the Coppergate site. There was found to be no disproportionate representation of the parts which would have been removed during the initial butchering (e.g. head, feet) or of the bones most likely to have been retained through the butchery process to the domestic hearth. This and the unsystematic nature of the butchery led to the conclusion that animals were bought in and slaughtered in the neighbourhood as required and shared amongst several households (O'Connor, 1989).

The pattern of butchery marks suggested that dismemberment involved first the removal of the feet at the ankle and wrist and the skull at the neck, and this probably took place at the slaughter site. This is consistent with the TEM results which suggested that all of the metapodials were uncooked butchery waste. The butchery marks on the limb bones showed that most were cut into joints but some were also likely to have been de-boned (i.e. large blocks of steak removed from the legs and the fore and hind quarters) (O'Connor, 1989). Again this is consistent with the TEM results as all but one of the limb bones (tibia and humeri) that were analysed by TEM were identified as being cooked. The odd sample was taken from a tibia and this was identified as uncooked (see Figure 8.5, the solid black circle amongst the 'uncooked' group). In this case it would appear that the tibia was not cooked as a joint; this may have been an example of where the limb was de-boned first.

Whilst there was little difference in the spatial distribution of butchered cow bone within the site there was a slightly greater frequency of complete or largely complete (>50%) long bones at the riverward end of the site away from the street frontage, with a greater degree of butchery and fragmentation seen on bones closer to the habitation areas. The TEM results showed that all of the bones which were furthest from habitation areas (at the riverward end of the site) were uncooked, whereas all but one of the bones close to the houses were cooked.

8.3.3.4 Other factors which could have caused the differences between the two groups:

The separation of the TEM data into two discrete clusters reflects both the skeletal element and spatial distribution on the site. All the bones analysed from

the tenement areas were tibia and humeri and all were identified by TEM as cooked. All the bones from the riverward end of the site were metapodials and all were identified as uncooked. Whilst the most obvious reason for the two clusters is that one represents cooked bone and the other uncooked it is worthwhile to discuss other possibilities for the separation.

8.3.3.4.1 Burial environment:

The results of the burial experiments discussed in Chapter 6 and our previous study (Koon et al., 2003) have shown that the burial environment can cause the same type of damage to collagen fibrils as cooking. This would appear to have occurred to some degree at the site; the bones identified as uncooked show more alteration than is observed in fresh bone. One possible alternative reason for the separation in the TEM results could be burial at the rear of the site afforded better preservation than burial closer to habitation areas. This is unlikely however, all of the deposits at the site were characterized as organic silts and clays which retained water. The soils were of roughly neutral pH and contained high phosphate levels (from bone and excrement). These characteristics would produce an ideal environment for the preservation of skeletal remains.

The factors which determine bone preservation have already been discussed in Chapter 6, but briefly the waterlogging at this site would have (i) produced anaerobic conditions which would inhibit microbial activity and (ii) (combined with the mild pH and high phosphate levels) prevent the dissolution of the mineral. Also the anaerobic conditions and mild pH would not favour hydrolysis of the organic component. Because of the good preservation conditions afforded by the burial environment all the skeletal elements used in this study appeared to be well preserved. The bones were dense and smooth surfaced, as opposed to the porous and powdery texture characteristic of poorly preserved low collagen bone. Also against the argument that differences in burial environment at this site influenced the results, one of the bones which had originated from a deposit close to the houses (denoted by a black circle) showed far less alteration that other bones from the same deposit and a similar degree of alteration as the uncooked bones found at the rear of the site. This suggests that the bones identified as uncooked (all the metapodials and the one tibia) represent the general effects of burial at the site.

8.3.3.4.2 Differential survival of skeletal elements

An analysis of the taphonomic history of the faunal assemblage at Coppergate requires the assessment of the influence of the burial environment on different skeletal elements. As mentioned above, all of the bones that were analysed in this study appeared macroscopically well preserved. The two clusters however do reflect different skeletal elements. The collagen from metapodial bones (and one tibia) was less damaged than from either the humeri or tibia. Whilst this difference is most likely because the latter were cooked, it is important to assess the relative survivorship of different elements.

Degradation in the burial environment is assumed to be density-mediated, a significant correlation between the abundance of a skeletal element and its density in an archaeological assemblage most likely reflects the resistance of different elements to destructive effects within the burial environment (Lam et al., 2003). The fore and hind limb bones were generally well represented throughout the Coppergate site as were the metapodials (see O'Connor, 1989 table 42). In terms of bone density Lam et al. (1999) showed very little difference in the bone density at the mid shaft between the humerus, tibia and metapodial of bovid bones. Using computed tomography (CT scans) they calculated bone mineral density values of 1.10 (n=4 SD .01), 1.14 (n=4 SD .04) and 1.12 (n=4 SD .01) for the humerus, metatarsal and tibia respectively. Although these measurements were taken from *Connachaetes taurinus*, a species of wildebeast so the values are not entirely representative to the bovine species present on the Coppergate site, they do suggest that there is very little difference in the survivorship potential of the three types of elements utilized in this TEM study.

Finally it should be noted that although the metapodials at the Coppergate site were mainly intact, whereas all of the humerus and tibia bones were broken into smaller pieces; three fragmented metapodials were also used in the study and these elements behaved in the same way as the intact metapodials. Therefore the difference between the two clusters cannot simply be a difference between fragmented and intact bones.

8.3.3.4.3 Different degrees of cooking?

The last possibility to consider is that all of the bones were cooked but just to differing degrees. For this to be the case the metapodials would have had to have been cooked for much less time than the humeri and tibia. This seems highly

unlikely because as the feet of a cow would not produce much meat, the metapodials would only be used for making a stock or for grease or glue production, if at all. These procedures would involve boiling the bones for hours if not days therefore the degree of alteration to the collagen fibrils would be far greater than that caused by roasting a joint.

8.3.4 Conclusion

With this TEM technique it is possible to identify intact and partially degraded collagen fibrils from archaeological bone. The case study from York has shown that using this approach within the same site it is possible to distinguish well-preserved archaeological cooked bone from uncooked bone.

Part 4 Discussion

Chapter 9 Synopsis, synthesis, conclusions and future work

9.1 Synopsis of each chapter

From the outset the aim of this PhD thesis was to develop an approach that was able to detect evidence of cooking in bones from archaeological sites. In **Chapter 1** the structure and thermal stability of collagen within bone are discussed. This is in order to provide a necessary background for further discussions about the deterioration of bone collagen both from cooking and following burial. The problems associated with detecting cooked bone are discussed in **Chapter 2**. Here it was concluded that before there is any significant alteration to the physical appearance or chemical composition of a bone, it must be heated to temperatures beyond those which would be achieved during a domestic cooking event. The main techniques available to detect thermal alteration of bone can detect burning but are apparently 'blind' to cooking. A further complication is the similar types of alteration that are observed within bones which have been exposed to weathering and diagenesis, and those which have been cooked. The possibility of using TEM and DSC to look at subtle heat induced microstructural changes in the collagen component of bone are proposed. These techniques have rarely been applied to either cooked or archaeological bone therefore analysis methods and optimized sample preparation protocols were developed, and these are explained in **Chapter 3**.

To gain a clearer understanding of how the collagen fibril structure is degraded by heat, a combined analytical approach (TEM, DSC and XRD) was used to investigate changes in the organization of the fibril which occur over the denaturation transition. The results of this analysis, described in **Chapter 4**, reveal that bone collagen degrades differently to tendon collagen; in the former all lateral order is lost whereas in tendon there still appears to be some degree of order, seen by TEM as strings of electron dense material. It is suggested that this may be caused by differences in cross-linking and may reflect the physiological position and role of these different tissues within the body. In addition there is a high temperature endothermic transition which is observed in bone collagen, it is unclear what causes the transition but it is suggested that it may represent hydrolysis of the polypeptide chains.

Chapter 5 describes a series of heating experiments on bone collagen from modern material analysed by TEM and DSC. As well as determining the potential of these techniques to detect thermal alteration, the roles of mineral and age-related cross-links in stabilizing the fibrillar structure of collagen within bone, are explored. The results suggest that the mineral component plays an extremely important role in preserving the fibrillar structure of collagen and thus the collagen molecule. It was also discovered however that bone collagen accumulates damage during mild heat treatment. This damage is visible, using TEM, once the collagen has been liberated from the mineral 'cage'. The damage, seen as a disruption of the fibril packing, appears to represent the very early stages of collagen fibril collapse. It is observable at temperatures that would be achieved during the roasting of fleshed bone or during boiling, and well below the detection limits of other techniques including DSC, which appears only to be able to detect catastrophic damage. Heating experiments conducted on human samples, which had subsequently been acid demineralized, visualized the affect of age in maintaining the structural integrity of the fibrils. In the older individuals, who presumably had more highly cross-linked collagen, the fibrillar structure was less sensitive to heat damage and swelling. In younger individuals the collagen fibrils displayed extensive swelling and often appeared as flat ribbons; perhaps representing the cross-linked outer sheath of a fibril which has lost its poorly cross-linked and labile inner core.

When dealing with archaeological material it is necessary to take into account processes in the burial environment which might degrade bone. Two key factors responsible for the degradation of bone are soil pH and microbial attack. In **Chapter 6** the results of analysis conducted on bones from experimental burials are presented. The samples came from environments sufficiently corrosive to yield demonstrable alteration to the bone within relatively short time scales. It was found that acidic soils cause significant alteration to the collagen fibrils. Within a given site, however, even in these aggressive environments cooked bone could still be distinguished from uncooked; suggesting that prior heat induced damage made the collagen more susceptible to alteration in the burial environment. The unusual chemistry of peat bog sites makes them of special interest to the study of collagen preservation. The DSC analysis from different collagenous materials within the two peat bog sites was able to give a good indication of the state of the collagen, and its deterioration over time spent in the bog.

DSC analysis also proved useful in one of the two case studies discussed in **Chapter 7**. This work arose from instances in which it was necessary to determine the state of the collagen component within important skeletal material. The techniques and knowledge obtained from this research were applied to address two specific forensic problems; DSC along with TEM were able to predict the likelihood of DNA extraction from MIA skeletal remains which had been treated with mortuary chemicals; TEM analysis was able to identify heat-treated bones from the Hamann-Todd skeletal collection. The TEM technique was pushed further when it was applied to well-preserved archaeological bone, discussed in **Chapter 8**. Whilst all the material studied displayed some collagen damage the results of the analysis provided a convincing argument that TEM has the potential to identify cooked bone from archaeological sites.

9.2 Synthesis of findings - the significance of the fibril in the degradation of bone collagen

Following exposure to mild heating (80°C,1hr) damage is observed to accumulate in the collagen fibril. This mild heating regime equivalent to the temperatures a bone might be exposed to during roasting, does not lead to detectable changes in any biochemical parameter yet measured, nor indeed collapse of the collagen structure measurable by DSC. However once demineralized and visualized using TEM, there are clear differences between the heat-treated and unheated bone. The banding pattern begins to be lost in discrete regions of the fibril which appear to expand in diameter and reduce in their longitudinal dimension. After prolonged heating a number of swollen features (*beads*) are observed along the fibril. The fibril then appears to part at these *Beaded* features resulting in short fragments with an intact banded central segment but which are swollen at both ends. It would seem therefore that collagen is degrading by unpacking of the fibril in these *Beaded* regions, which perhaps may themselves be initiated by a single degradation event. Eventually, the unpacked fibril appears to deteriorate to an amorphous (gelatin) phase. The rate that this degradation takes place can be accelerated by exposing the collagen to low pH. This causes considerable swelling and an increase in the proportion of short *Dumbbell* fragments. Conversely, the degradation can be slowed down by the presence of age-related or synthetic cross-links, which reinforce the fibril structure.

This pattern of damage is not solely caused by thermal alteration; the burial environment can cause the same types of alteration in uncooked bone (although to a lesser degree than in cooked material). This pattern of fibril degradation can therefore be used to gain a more general understanding of how bone collagen degrades. This is summarized in a diagram in Figure 9.1.

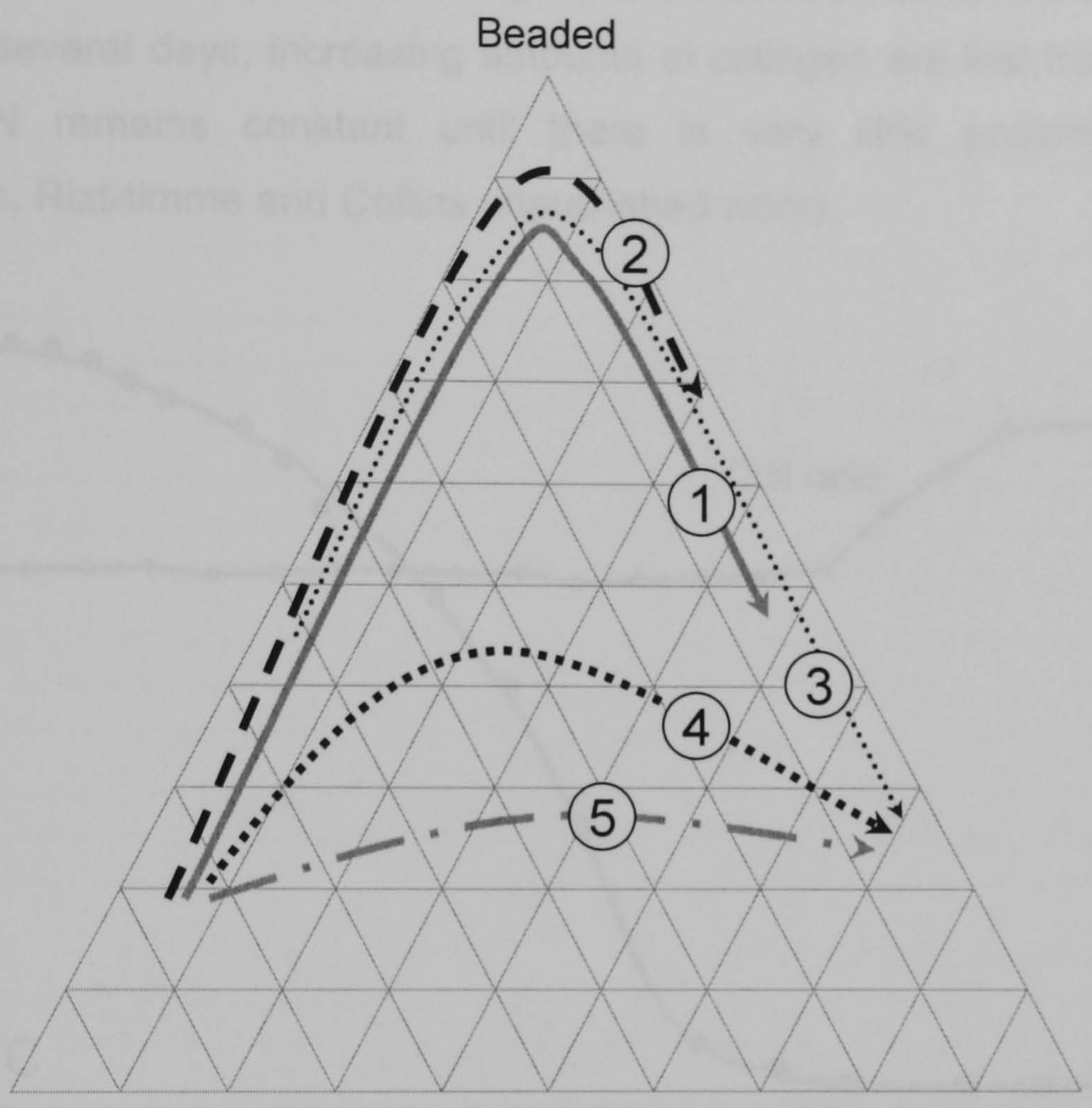


Figure 9.1 Ternplot showing some general predictions of the path that collagen degradation would take depending on the taphonomic history of a bone and the intrinsic stability of the bone collagen fibrils. Route 1 for the degradation for heated bone or bone buried in a neutral soil, then demineralized in EDTA. Route 2 the same as route 1 except this path is for bone samples which are more highly cross-linked therefore the rate of degradation is slower. Route 3 the same as route 1 except this path is just for bones which have been heated prior to burial in a neutral soil, this bone is already damaged and more susceptible to further degradation in the burial environment therefore the rate of degradation is faster than for the unheated buried bone. Route 4 for the degradation of mature heated bone or mature buried bone which has been demineralized in HCl or mature bone which has been buried in an acidic soil. Route 5 the same as for route 4 except for young immature bone as this collagen is less highly cross-linked and therefore more susceptible to swelling and fragmentation into *Dumbbells*.

The degradation of bone collagen, which appears to be governed by the unique packed fibrillar structure of collagen, can account for the unusual characteristics of ancient collagen. Bone collagen can survive for hundreds of years in a relatively unaltered state and thus is used as a reliable substrate for ^{14}C dating and dietary reconstruction based on stable isotopic analysis (Athfield et al., 1999;

Barrett and Richards, 2004; Bocherens et al., 1995; Schoeninger et al., 1983). A C:N ratio of between 2.9-3.6 is used as an indicator of good quality protein (Ambrose, 1990), this ratio has been found in archaeological material of different ages and regardless of organic content (Ambrose, 1990; DeNiro and Weiner, 1988a; Hedges and van Klinken, 1992). This unusual behaviour can also be seen in the artificial aging study, shown in Figure 9.2. Here cow bone has been heated at 95°C for several days, increasing amounts of collagen are lost from the bone yet the C:N remains constant until there is very little protein remaining (Dobberstein, Rizt-timme and Collins unpublished work).

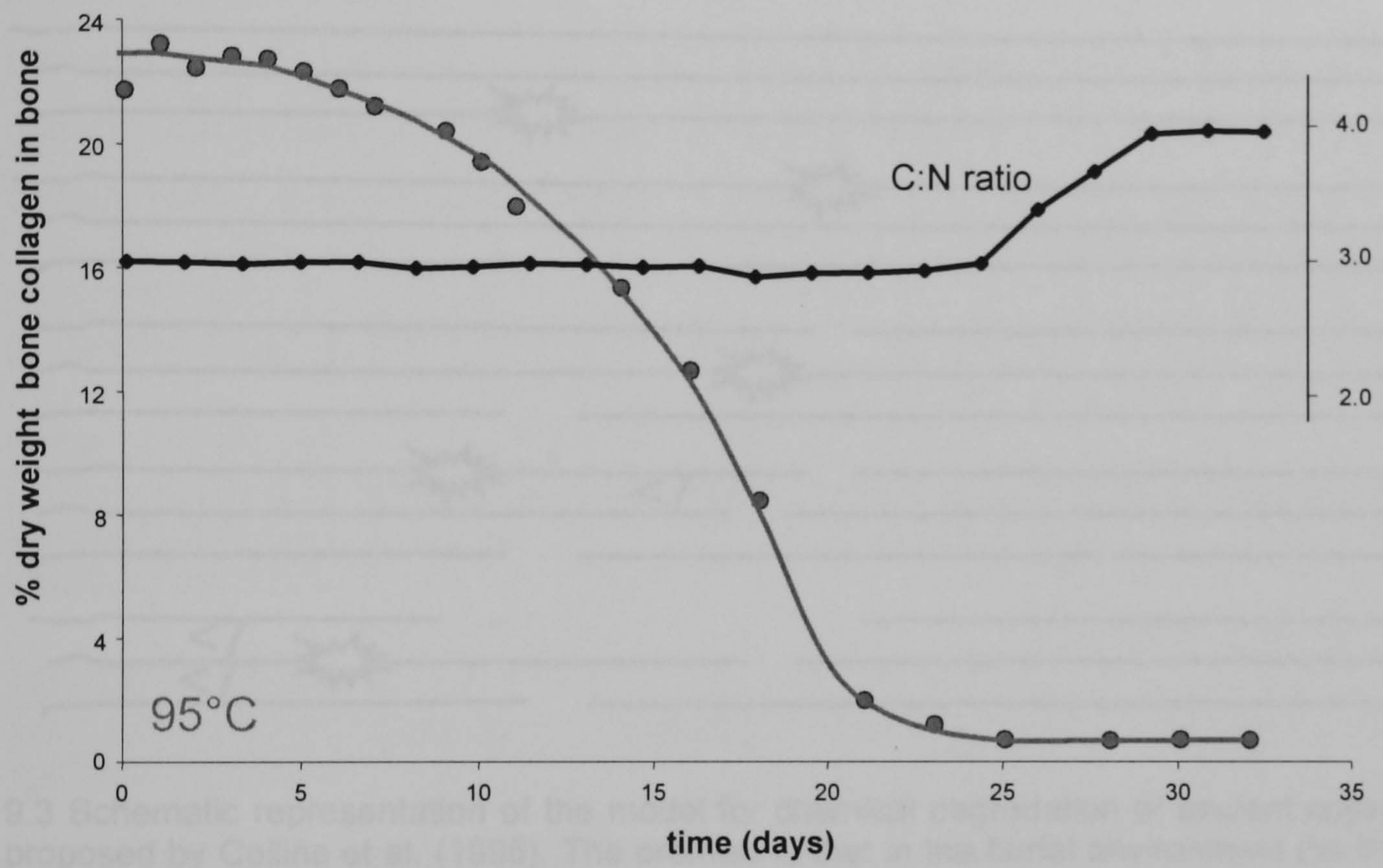
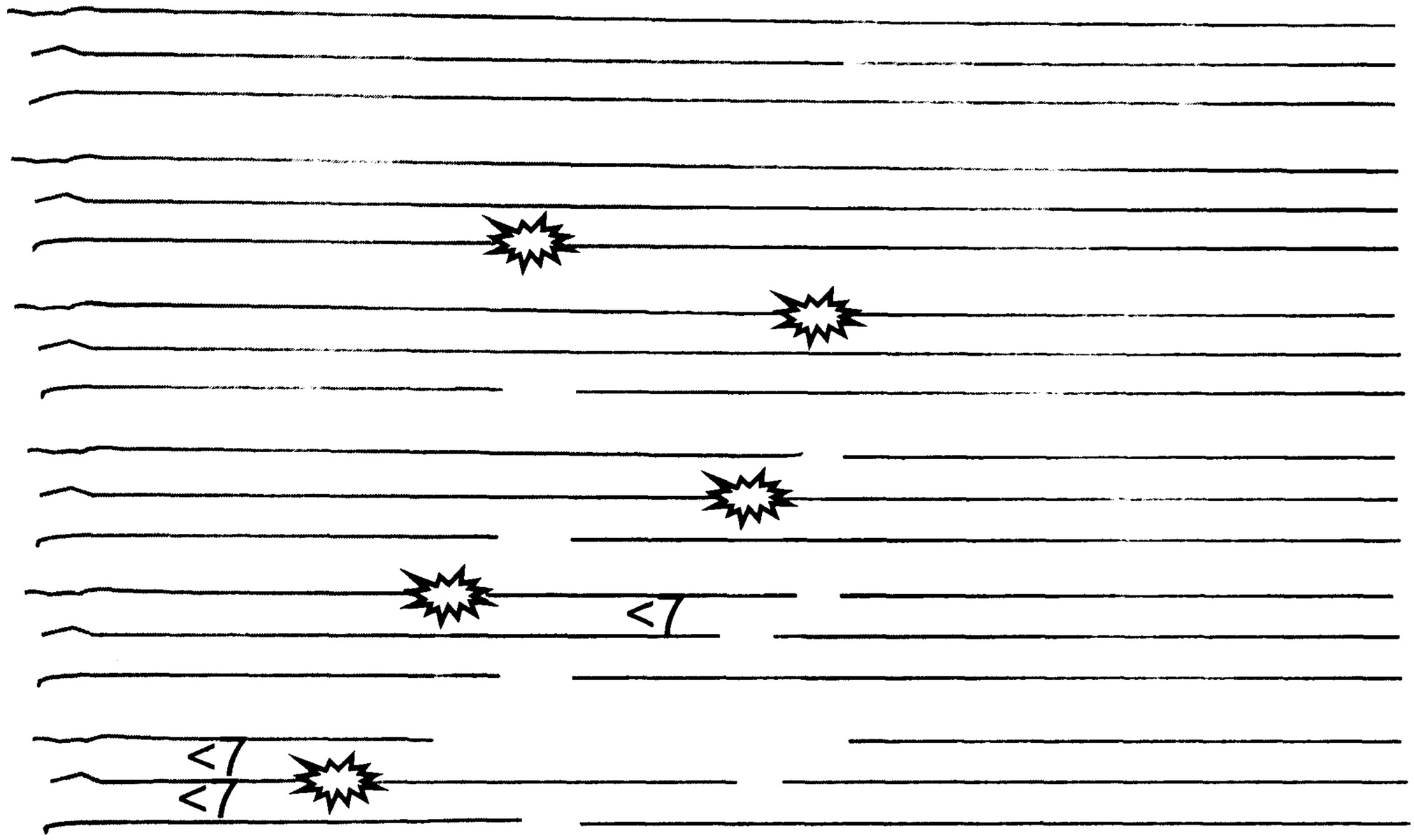


Figure 9.2 Graph showing both the collagen loss from bovine bone heated in water at 95°C for several weeks (shown in grey, left-hand scale) and changes in C:N ratio of the collagen remaining within the bone during this period (shown in black, right-hand scale). The data shows that the C:N ratio (which is used an indicator of the state of degradation of bone collagen) remains unaltered until there is very little protein remaining within the bone. This suggests that whilst collagen is being gelatinized and lost the collagen that is retained within the bone is in an unaltered state. Data from Dobberstein, Rizt-timme and Collins (unpublished work).

The degradation of collagen in bone has previously been described as a random process of gradual chain scission of all triple helices (Collins et al., 1995) (see Figure 9.3). The mechanism is based on the model for polymer degradation and explains the sigmoidal curve seen for experimentally induced collagen loss (Figure 9.2). This mechanism does not however take into account the fact that in bone the collagen molecules are not behaving as individual entities, but are packed within collagen fibrils. As already discussed in Chapter 4, it has been

observed that individual triple helices have melting points close to body temperature and yet the thermal transition in a packed fibril is raised by over 20°C, this despite the extremely high activation energy of the transition (~ 500 kJ mol^{-1}). Work by Miles and Gelashvili (1999) has argued that the temperature of triple helix denaturation is greatly elevated by the close packing of fibrils; which prevent triple helical collapse by limiting the space into which the distorted collagen molecules can expand.



9.3 Schematic representation of the model for chemical degradation of ancient collagen proposed by Collins et al. (1995). The premise is that in the burial environment the three α-chains which make up the collagen molecule become damaged, mainly through hydrolysis of the peptide bonds within the individual chains. The fragmented α-chains are able to remain intact because they are held together by hydrogen bonds between the chains. The molecule remains intact until there are a critical number of hydrogen bonds left holding the structure together (seven H-bonds). After this point the α-chain with less than eight hydrogen bonds connecting it to the rest of the molecule will be lost and the two remaining α-chains will be susceptible to further damage.

TEM analysis from fibrils in different stages of denaturation has shown that collagen does not degrade by a random process of chain scission as envisaged by Collins et al. (1995). Rather the mutual protection of the packed fibrils when disturbed perhaps by only a single degradation event, initiate localized reorganization and unpacking of the fibril. The unpacked triple helices being much less stable than their packed counterparts will rapidly denature further to gelatin. Because gelatin is both soluble and, being highly flexible, more prone to hydrolysis, it has a short life span within the bone before it either degrades or

diffuses away. Despite this, in the very same collagen fibril other, not as yet unpacked, regions can display good banding patterns and therefore near intact and highly organized triple helices. Collagen degradation can therefore be seen as an '**all or nothing process'** (see Figure 9.4); once part of the fibril has become unpacked, the damaged molecules within this region will diffuse out of the bone. Within the intact fibrillar regions the collagen molecules will be largely intact and exhibit the high molecular weight, low D/L ratios and the consistent C:N ratios and amino acid profiles that are associated with ancient bone collagen (Ambrose, 1990; DeNiro and Weiner, 1988a; Hedges and Law, 1989; van Klinken, 1999; Wyckoff, 1972). Consequently processes which restrict fibril unpacking such as the presence of numerous intermolecular cross-links or mineral precipitates within and between fibrils will greatly enhance the life span of collagen within a burial environment.

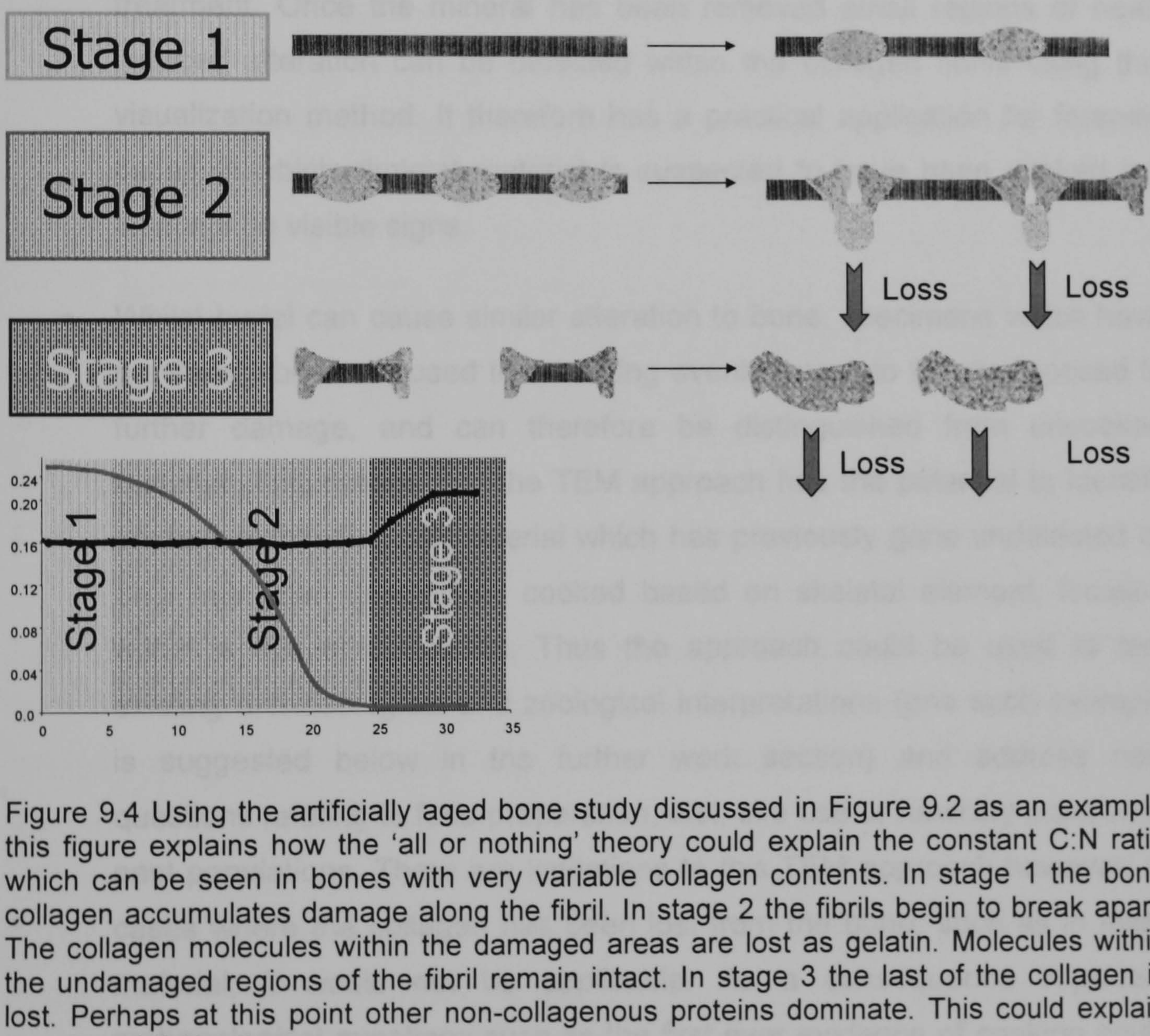


Figure 9.4 Using the artificially aged bone study discussed in Figure 9.2 as an example this figure explains how the 'all or nothing' theory could explain the constant C:N ratio which can be seen in bones with very variable collagen contents. In stage 1 the bone collagen accumulates damage along the fibril. In stage 2 the fibrils begin to break apart. The collagen molecules within the damaged areas are lost as gelatin. Molecules within the undamaged regions of the fibril remain intact. In stage 3 the last of the collagen is lost. Perhaps at this point other non-collagenous proteins dominate. This could explain the altered C:N ratio.

This 'all or nothing' theory for the molecular survival of bone collagen is further supported by a recent observation; CnBr treatment of a range of archaeological

bones, with very variable yields of collagen, produced high molecular weight fragments suggesting the presence of collagen molecules without any significant deterioration (Dobberstein et al., *pers. comm.*). This finding is inconsistent with the Collins et al., (1995) mechanism for collagen degradation, which suggests that collagen molecules would be found with varying degrees of fragmentation depending on how much collagen was left in the bone. The fact that the molecules were intact regardless of the amount of collagen remaining can be readily explained at the level of the collagen fibril by the 'all or nothing' mechanism whereby the collagen molecules are present and intact or else lost to the burial environment.

9.3 Conclusions

- TEM is able to identify bone which has been exposed to mild heat treatment. Once the mineral has been removed small regions of heat-induced alteration can be detected within the collagen fibrils using this visualization method. It therefore has a practical application for forensic cases in which skeletal material is suspected to have been cooked but displays no visible signs.

- Whilst burial can cause similar alteration to bone, specimens which have previously been exposed to a heating event appear to be predisposed to further damage, and can therefore be distinguished from uncooked material. This means that the TEM approach has the potential to identify cooked archaeological material which has previously gone undetected or else has been inferred as cooked based on skeletal element, location within a site or cut-marks. Thus the approach could be used to test existing archaeological and zoological interpretations (one such example is suggested below in the further work section) and address new questions relating to food preparation, diet, site use or funerary practise in past populations. There are limitations to this TEM approach however, in cases where the collagen has been lost from the bone, such as in fossil material, it would not be applicable. As a consequence important archaeological questions such as the first ever evidence of cooking could not be addressed with this technique.

- As well as identifying thermally altered bone the TEM, along with DSC analysis, has also given an insight into early stages of collagen

degradation and the roles that mineral and the collagen fibrils play in stabilizing bone collagen. As a result it has been possible to make suggestions as to how and why collagen molecules are able to survive for thousands of years within bone in a relatively undamaged state.

- The observations of differences in the degradation of bone collagen compared to tendon collagen; of a second high temperature transition in demineralized bone collagen; of apparent age related stability in bone collagen fibrils; and of the presence of flat ribbon-like fibrils are all potentially exciting findings which may have greater implications beyond understanding the thermal degradation of bone collagen. Unfortunately these observations could not be explored in any great detail during this research. Suggestions for further work are given below.

- One area focused on briefly in the thesis is the affect that post-mortem treatments will have on biomolecular preservation. Both forensic and archaeological scientists often rely on the important information that is stored at the molecular level in bone; if this has been altered in some way this will obviously affect the results. TEM and DSC could be used to assess the affect that treatments such as defleshing via heat treatment or embalming have had on the molecular preservation of bone collagen, as illustrated in the two forensic case studies.

9.4 Future work

9.4.1 Collagen transitions

TEM images taken during and after the denaturation transition suggested that tendon collagen (rat tail, bovine Achilles') degrades in a different way than bone collagen (bovine, human) (See Chapter 4). It is unclear whether these differences are caused by the presence of mineral, a different distribution of cross-links or both. One way to try to separate the different contributions of mineral and covalent cross-linking in dictating how the collagen fibril degrades may be to look at mineralized turkey tendon. The same experiment could be conducted on fully mineralized, partially mineralized and non-mineralized portions of the same turkey leg tendon. It has been suggested that as the tendon becomes mineralized, it takes on a bone-like cross-link profile (Knott et al., 1997). Thus one might expect the mineralized portion to degrade in a similar way as the bone collagen over the denaturation transition, whereas the non-mineralized portion

would produce the strings of electron dense material seen in other tendon collagens.

It is still unclear what the high temperature endotherms observed in demineralized bone collagen and in mineralized collagen represent. In Chapter 4 it was suggested that both may represent gelatinization by hydrolysis of the polypeptide chains. Using appropriate kinetic software, it would be possible to determine the activation energy of the high temperature transition using isothermal heating and thereby obtain a clearer idea of what these transitions may represent, because fibril collapse has a much higher activation energy (Ea ~ 500 kJ mol $^{-1}$) than hydrolytic gelatinization (Ea ~ 175 kJ mol $^{-1}$).

9.4.2 Effect of age on bone collagen

The TEM analysis of modern human femur sections with known age at death, suggested an increased stability of the collagen fibrils as a function of age. By subjecting the samples to mild heating and then demineralizing and swelling the collagen fibrils in acid, it was possible to observe differences between the collagen fibrils of young and old individuals. In older specimens there was a build up of fibrils which were less resistant to swelling and thermal alteration. This observation may relate to a decline in the mechanical properties of bone and increase in secondary cross-linking, both of which are known to occur in old age (Li et al., 2003; Wang et al., 2002b). At this point this is simply conjecture, these links would need to be determined by quantifying the cross-links using HPLC (Bank et al., 1997) or hydrothermal isometric tension (Le Lous et al., 1982) and testing the mechanical properties of both the bone and the bone collagen (Wang et al., 2002b; Zioupos, 2001; Zioupos et al., 1999) from these known-age samples. A further step could be to conduct TEM analysis on bones with elevated levels of cross-links due to skeletal pathologies such as diabetes. If it is cross-links which are restricting the collagen fibrils, as suggested in Chapter 5, one would expect the collagen from these specimens to behave in the same way as in the old human bones.

9.4.3 Cryo-TEM to visualize flat fibrils

In Chapter 5 a number of TEM images were shown which suggest that under certain circumstances (young specimens, demineralized in HCl for 14 days) bone collagen can be found as flat ribbons rather than cylindrical fibrils. The problem with examining biological material with the TEM is the unphysiological conditions

used to prepare and stain the specimens, including exposure to heavy metal salts, dehydration and high vacuum. The way in which the ribbons were folding over on themselves provides compelling evidence that these fibrils are in fact flat and not simply a distortion of the TEM image. However the possibility that artefacts introduced during the TEM preparation or analysis could have caused this phenomenon must be eliminated. A way to accomplish this may be with cryoTEM. With cryofixation it is possible to freeze biological material fast enough that ice crystals have no time to form. The vitrified samples can be examined on a TEM with a low temperature specimen stage. This will allow the collagen fibrils to be imaged at as close to their natural hydration level as possible without the need for chemical fixation and staining (Laing et al., 2003).

9.4.4 Burial experiments

Considering that much of the animal bone that is found on archaeological sites is likely to have been cooked, the impact of this taphonomic treatment and its subsequent effect on bone in the burial environment should warrant attention. In this thesis a number of bones from different sites were analysed to investigate differences in the extent of collagen fibril degradation between cooked and uncooked material, but the results are limited due to the lack of appropriate samples. Most of the cooked material from Palace Leas was not analysed because it had been heated for durations far longer than would be needed from a domestic cooking event (Roberts, 2003). The cooked bones from the peat bogs though interesting in their own right, were also cooked for excessive periods (see section 6.4). There are other burial experiments (reviewed in Andrews, 1995; Denys, 2002) but these do not include cooked bone in their assemblages. One exception is the experimental earthwork at Overton Down, UK, but the limitations of this study serve to highlight the need for further research in this area. Part of the earthwork was excavated in 1993, after 32 years, and two bones were examined by Armour-Chelu and Andrews (1994). These included an adult sheep metapodial which had been boiled for one hour prior to burial and an immature sheep metapodial which was uncooked. The comparison of a single cooked and uncooked element would not be sufficient unequivocally to assess the affects for cooking. Furthermore the results of the heating experiments conducted in Chapter 5 suggested that species and age-at-death may affect the rate of degradation of bone collagen.

It is suggested that a set of burial experiments of paired cooked and uncooked bone in different soils should be undertaken (to include, acidic, chalky, neutral, sandy, organic rich, free-draining and waterlogged soils) much as in the series of burial experiments undertaken by Nicholson (1992, 1996 and 1998), with the following additional inclusions:

1) To assess the impact of cooking different heating regimes should be included, such as bones cooked for 1, 2, 3 and 6 hours at 100°C coupled with unheated bones from the same element.

2) To consider variability between skeletal elements, different elements (cooked and uncooked) from the same carcass should be included, such as metapodial, rib and femur.

3) To consider species and age variability mammal bones from different common domestic species (and known age-at-death) should be included, such as cow, pig and sheep.

4) To assess the rate of early diagenesis, sets of bones should be removed after different time periods, such as 1, 3, 6 and 12 years

9.4.5 Archaeological study on Greenland samples

It has been suggested that despite their known contact with the Paleo-Eskimo hunters, the Medieval Norse Pastoralists of Greenland learned nothing from them about fishing or ice-hunting for fish, ringed seals or whales. As the climate worsened, it made maintenance and reliance on domestic animals for subsistence more difficult and ultimately led to the Norse dying out in the fifteenth century (Buckland et al., 1996). Fat and nutrient resources from within bones would have been of great importance to Greenlandic hunter-gathers and pastoralists as neither would have had access to good supplies of carbohydrates. Outram (1999, 2002, 2003) has suggested that evidence for fat exploitation can be used as an indicator for dietary stress. By looking at fracture patterns within bone assemblages from both Inuit and Norse sites he has suggested bone fat exploitation was more exhaustive within the latter and that this shows that these individuals were under greater dietary stress. Interestingly he has also suggested that for both groups land mammal skeletons were exploited for fat, whereas marine mammal bone was virtually never used (Outram, 1999). Elsewhere it has also been suggested that among historical Inuit groups consumption and food

processing were bound by specific rules which involves differences in the treatment of land and marine mammals (Murray, 2000)

The work by Outram is based on the assumption that this material has in fact been boiled and that the fragmentation has not been caused by weathering processes, but rather during the processing of the bones to extract the fat. The TEM approach developed in this PhD research could be used to test these assumptions and determine if it is true that marine mammal bone was not cooked. An extra attraction of this project is that these permafrost bones will be exceptionally well preserved.

9.5 Dissemination of research

List of first author publications, reports and conference abstracts

Koon H.E.C. 2005 Bone of contention *BLC journal* May/June: 9-11

Koon, H.E.C. 2004 Punchbowl: Investigation of MIA remains in order to elucidate their post-mortem history specifically the use of aldehydic cross-linking agents. *Report for the US Armed Forces Institute of Pathology, Department of Defense DNA registry.*

Koon, H.E.C., 2004 The Live-steam defleshing method: TEM investigation to identify the method of sample preparation of material used for the Hamann-Todd skeletal collection. *Report for the Cleveland Museum of Natural History.*

Koon H.E.C., Nicholson R. and Collins M. 2003 A Practical Approach to the Identification of Low Temperature Heated Bone Using TEM. *Journal of Archaeological Science* **30**:1393-1399.

Koon H.E.C., Collins M., O'Connor, T. and Covington T. 2006 Sorting the Butchered from the Boiled. ***Archaeometry***, Laval University Quebec City; ***Association of Environmental archaeology***, University of Exeter.

Koon H.E.C., Collins M. and Covington T. 2005 A new look at unravelling bone collagen. ***V International Bone diagenesis Meeting***, University of Cape Town

Koon H.E.C., Collins M. and Covington T. 2005 Unravelling the Mystery of Collagen Degradation Helps to Reveal the Horrors of the 'Live Steam' Defleshing Method. ***UK Archaeological Science***, University of Bradford.

Koon H.E.C., Collins M. and Covington T. 2004 Fingerprinting the Early Deterioration of Collagen Fibrils and its Application for the Identification of Cooking in Archaeological Bone. ***International Symposium of Biomolecular Archaeology***, Vrije University, Amsterdam

Koon H.E.C., Nicholson R. and Collins M. 2003 A Practical Approach to the Identification of Low Temperature Heated Bone Using TEM. ***UK Archaeological Science*, University of Oxford**.

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