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Thermochimica Acta 365 (2000) 119–128

thermochimica acta

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Changes in collagen structure: drying, dehydrothermal treatment and relation to long term deterioration

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Abstract

Collagen is the major component of most connective tissues in animals. The structure of the helix and axial molecular packing within fibrils is well documented. Less is known about the structural alterations that occur on drying and the compounded changes that occur on dehydrothermal treatment. The structural properties of collagen and its supramolecular architecture are of importance in these states, since many of the industrial applications of collagen-based materials involve dried collagen or dehydrothermally treated collagen.

The effects of drying and thermal treatment of collagen can be observed by X-ray diffraction, the changes in the diffraction pattern relate to changes in the axial packing of collagen molecules as dehydration occurs. The meridional diffraction series becomes truncated indicating induced structural disorder, the spreading of diffraction features indicate that the molecular orientation is altered for some of the collagen chains or portions of collagen chains within a fibril.

Dehydrothermal treatment of parchment collagen for up to 24 h reduces the axial periodicity of the collagen fibril from 64.5 to 60.0 nm. Analysis of the X-ray diffraction data shows the possible alterations in molecular packing that may explain the structural changes.

Keywords: Collagen; Fibril; Structure; X-ray diffraction; Heat treatment; Dehydrothermal

1. Introduction

Collagen is an extracellular matrix protein that has an essential role in maintaining the architecture of multicellular organisms as well as having important industrial uses as leather, sutures, implants and prostheses [1]. It is also important as gelatin in the photographic, cosmetic and food industries [2]. The importance of understanding the relationship between collagen structures and thermal treatment is therefore essential in understanding the modulation of collagen molecular properties. The use of thermal treatment may also have a role in determining the relevance of the degradation sustained by thermal denaturation as a means of accelerated ageing of samples such as parchment and leather [3]. This is in order to compare the effects of degradation induced in a number of days to oxidative damage sustained over a number of centuries.

Collagen molecules are characterized by the sequential Gly-XY triplet structure that is a prerequisite for triple helix formation. Although at least 20 collagen types have been characterized [4], type I collagen is the predominant type being found as the main component of skin, tendon, aorta and bone. The type I collagen triplex is a heteropolymer

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0040-6031/00/$ – see front matter
PII: S0040-6031(00)00619-5

consisting of two α1 chains and an α2 chain of over 1000 residues in length. The structure of the triple helix has a rope-like nature, where each collagen chain adopts a left-handed helical conformation and the three strands intertwine with a right-handed super-helical twist.

The mechanical integrity of collagen molecules in tissues is often further enhanced by the association of collagen molecules in a specific fibrillar form. The axial packing of collagen molecules in fibrils is well established, since it can be easily visualized by electron microscopy. The axial molecular packing can be described as a step function repeating at 64 nm intervals comprising of gap and overlap regions [5]. In this, each collagen molecule (of approximately 300 nm length) is staggered relative to its nearest neighbour by 67 nm in the native state or ∼64 nm in the dry state. This leads to a fibrillar form where each collagen molecule has a strong molecular connection with neighbouring collagen molecules and an applied force can be transmitted through a fibril to each collagen molecule. A review of the relationship between the collagen molecule, fibrils and mechanical strength is given by Parry [6].

X-ray diffraction of tissues such as tendon gives rise to a complex fibre diagram corresponding to a number of structural features. The diffraction process gives information that is reciprocally related to distances between features in real space. For example, the repeating helix structure of the polyproline II type helix contains a strong periodic function of approximately 0.29 nm corresponding to the axial rise between amino acids; this corresponds to a large diffraction angle and can only be viewed using short sample to detector distances. The axial molecular packing of collagen corresponds to a 67 nm staggering of molecules and results in a sharp series of X-ray peaks nearly parallel to the fibre axis. The first order of this diffraction has a very low diffraction angle and correspondingly can only be detected using long camera lengths. Since the axial packing of collagen is highly regular, the diffraction produces a series of peaks that correspond to the harmonic series based on a 67 nm periodicity at reciprocal spacings of $\frac{1}{67}, \frac{2}{67}, \frac{3}{67}, \frac{4}{67}$ nm, etc. Therefore, although the first order of diffraction may not be observable in a particular camera geometry, the position of a particular order of the diffraction pattern can be identified and the fundamental periodicity determined. This approach is more pertinent than measuring the position of the first order of diffraction since the error in measurement is reduced.

The advantage of X-ray diffraction is that the intensity terms relating to the structural repeat can be observed in the hydrated state where the fundamental axial periodicity (D) is 67 nm. Up to 140 meridional diffraction peaks have been observed in the hydrated state, showing the high degree of crystallinity along a fibril axis [7]. The use of X-ray diffraction has allowed the molecular packing structure of collagen to be relatively well defined as a microfibrillar structure that contains specific molecular kinks [8].

The conformation of collagen molecular packing in the dehydrated state has been studied to a lesser extent than the fully hydrated tissue. However, it could be said that information from electron microscopy is effectively an examination in the dehydrated state [9]. The main effect of drying has been the observation that the axial repeat decreases from 67 nm to the region of 64 nm. The parameters of the collagen helix were in part determined by the use of X-ray diffraction of stretched dehydrated tendon samples [10]. However, the molecular packing in the dehydrated state is less well resolved. Conventional X-ray diffraction has the advantage that the diffraction data correspond to several thousand fibrils being observed at any one time, changes in the diffraction data therefore are inherently statistically significant in comparison to examining single fibrils.

The dehydrothermal treatment of collagen (typically 120°C dry heat for up to 24 h) marks a progression from the air dried state of fibrillar collagen. In this treatment, more water is driven off from the collagen molecules and also there are changes induced in the character of amino acids of the collagen chain that may result from oxidative damage or crosslinking [11]. Dehydrothermal treatment has been used to examine the changes in the strength and solubility of collagen-based products for the biopharmaceutical industry, and also as a means of developing regimens for accelerated ageing. One application of this is in the examination of the ageing of historic parchment manuscripts, where oxidative damage and molecular cleavage may have been occurring over a number of centuries.

The purpose of this study is to examine the changes in molecular packing that occur on the drying and dehydrothermal treatment of collagen. This allows a better understanding of the changes that may occur relating to the axial molecular packing of collagen on drying and subsequent thermal treatment, and also to assess the suitability of dehydrothermal treatment as a suitable technique for accelerated ageing.

2. Materials and methods

2.1. Samples

A variety of samples were examined by X-ray diffraction. Rat tail tendon samples of typical diameter 200 μm were dissected from 3-month-old Wistar rats and maintained in a physiological buffered saline. Samples were air dried (for 24 h as isolated tendons at room temperature), some were used for dehydrothermal treatment. Parchment samples of new and ancient parchments, mostly bookbindings from the Royal Library, Denmark, were a gift from Dr. Larson of the Danish School of Conservation. The unaged parchment sample was produced from calf skin, employing methods used in the 17th century by Z.H. de Groot, Rotterdam, the Netherlands. Each sample used in dehydrothermal treatment was heated in an oven for time periods of 15, 30 min, 2, 4 and 24 h at 120°C. At least three samples of each source material and treatment were produced and examined. Rehydration of samples was conducted by immersion overnight in distilled water followed by air drying for 24 h.

2.2. X-ray diffraction

X-ray diffraction patterns of rat tail tendon and parchment samples were obtained at the Daresbury Laboratory Synchrotron (CLRC), UK, on beamlines 7.2 (in fibre diffraction mode, wavelength 1.448 Å) and 2.1 (small angle fibre diffraction, wavelength 1.54 Å). The sample to detector distance was 0.2 and 6.0 m, respectively. Data sets were recorded on a variety of detector systems. At the SRS 2.1 a gas wire detector, and at the SRS 7.2 a Marresearch scanner. Each sample was placed in a sample cell with mica windows that facilitated maintenance of hydration in the case of wet samples. The diffraction time was 2 min in both cases, this ensured that the ambient temperature variation was minimal.

2.3. Data treatment

Rat tail tendon is often used as a means of calibrating small angle X-ray diffraction cameras. This was not deemed to be appropriate in this case since the samples themselves contained collagen. Calibration of the small angle camera was therefore performed by accurately measuring the sample to detector distance and measuring the angle subtended by the ninth order of diffraction intensity determined in each case by using Bragg's law. This gave a value of 67.2 nm for the *D* repeat of wet rat tail tendon. The high angle X-ray camera used the diffraction spacing from a sample of wax (spacing 0.414 nm), as performed routinely in protein crystallography. Background subtraction of the empty diffraction cell and correction for detector efficiency were made. Diffraction patterns were analysed with the use of in-house computer software that defined the beam centre and measured peak positions to determine the changes in fundamental molecular periodicity and diffraction peak integral. The use of the programs is documented by Purslow et al. [12].

3. Results and discussion

3.1. X-ray diffraction and collagen structure

X-ray diffraction can provide information about the molecular packing of collagen. The principal changes of interest here are the effects on the axial molecular packing of the collagen molecules in a fibril. The highly regulated molecular organization along a fibril axis ensures that in the native hydrated state, the molecular packing results in a quasi-crystalline lattice structure [13]. The axial projection of the repeating electron density profile corresponds to a series of sharp reflections that can be observed on the meridian of the X-ray fibre diagram. The position of each meridional reflection corresponds to the length of the axial fundamental periodic unit of collagen packing. The intensity of the reflections corresponds to the electron density profile within the repeating unit. By examining changes in the position of meridional

diffraction peaks and their intensity, changes in the fundamental periodicity and electron density profile can be monitored.

The data presented here contain information about the effects of drying and heating both rat tail tendon and parchment samples. Changes in the molecular packing that relate to changes in the orientation of the collagen molecule can be detected more easily by X-ray diffraction in tendon samples, since the rat tail tendon sample contains collagen fibrils that are well orientated parallel to the fibre axis. The inherent nature of the parchment samples is that they contain fibrils in a wide range of orientations since the source material (skin) is a feltwork structure designed to withstand forces in the plane of the tissue. It is therefore almost impossible to investigate the more subtle changes in molecular orientation that may occur on heat treatment; however, the changes in the axial packing and the intensity profile of diffraction peaks can be monitored. The possible application of heat treatment to simulate accelerated ageing in a manner similar to the processes that occur with time in historic parchment requires the use of control parchment in the experiments.

3.2. Structural changes in dry collagen

In the transition from the hydrated state to a dry state, there are four principal changes that occur:

1. The X-ray diffraction data from the dried tendon revealed a meridional diffraction pattern that presented up to 34 meridional diffraction peaks (Fig. 1). The diffraction series was therefore truncated in comparison to the number of reflections that can be observed in the wet state, where up to 140 meridional reflections have been observed. This change was probably due to increased variability in the molecular packing resulting from the dehydration process. This manifests in the attenuation of diffraction intensity with increasing diffraction angle [14].
2. The fundamental periodicity of the collagen was also observed to change from 67.2 to 64.7 nm upon air drying. The change in the fundamental periodicity does not appear to be entirely dependent on the shortening of the pitch of the collagen helix and axial sliding of molecules is likely as dehydration occurs.
3. The changes in the diffraction intensity profile indicated that the electron density profile within the repeating axial unit cell of a fibril has altered. In the dry state, small angle diffraction produced a series of diffraction peaks, where those with the highest intensity corresponded to the periodic distribution of charged amino acids within the collagen fundamental periodic structure [15]. In the hydrated state, the low angle reflections correspond to the step function related to the projected density of the quarter staggered molecular packing where a missing segment of the unit cell is replaced by water [16].
4. On drying, the X-ray diffraction peaks have been observed to broaden (with diffraction angle) in the direction perpendicular to the meridian (see Fig. 1). The exact molecular process that accounts for this is poorly understood. A finite broadening of all reflections would be expected if the radius of the fibril was to decrease. Examination of elastoidin by X-ray diffraction indicated that a phase change in molecular packing occurs upon dehydration, this study indicated that the molecular direction of the collagen chains, or portions of collagen chains, changed upon dehydration [17]. An effect similar to this is observed in rat tail tendon samples upon dehydration. The meridional diffraction pattern is spread along a series of lines rather than arcs, these indicate that the fibrillar structure contains a variety of molecular orientations where the fundamental periodicity is also altered. In the dry state, a number of meridional reflections are no longer located parallel to the fibre axis, indicating that portions of the collagen chains deviate by $21.4^{\circ}(\pm 2.0^{\circ})$ from the fibre axis. Such suggestions have already been made by Bear [18] and an alternative explanation given by Tomlin and Ericson [19]. In the light of our more advanced knowledge of collagen molecular packing, the most likely candidate for such a region would be the centre of the gap region where a molecular shear may occur after molecular collapse on dehydration. If the central gap region of $0.4D$ adopts a tilt in azimuth of 21° to the fibre axis, then this alone would result in a change of the fundamental periodicity from 67.2 to 65.1 nm. A schematic diagram of the molecular packing in wet and dry collagen is shown in Fig. 2.

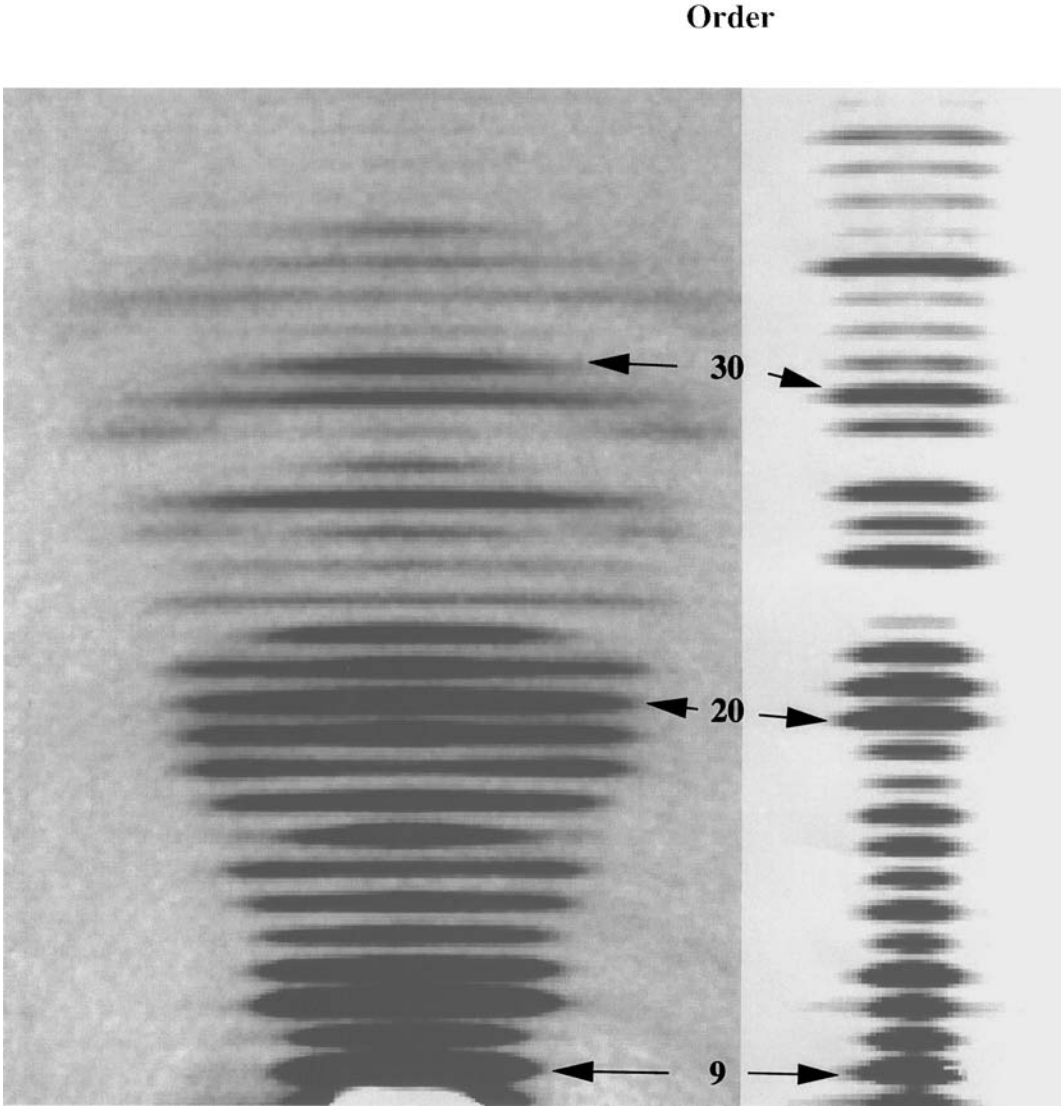

Fig. 1. Hydrated (right) and dried (left) rat tail tendon diffraction pattern recorded on beamline 7.2 of the SRS Daresbury Laboratory. The meridional diffraction peaks are shown from orders 9 to over 34 in both cases. The truncation of the diffraction (compared to the wet state) is probably due to the introduction of static disorder within the axial unit cells. The broadening of the diffraction peaks is due to a population of chains adopting a tilt of about 21° to the fibre axis. The hydrated tendon sample also demonstrates the tilt of the *c*-axis of the unit cell within a fibril this has a smaller magnitude of about 5°. The intensity profile of the meridional series is clearly different, and the position of corresponding reflections has changed due to the changes in the fundamental periodicity upon drying.

3.3. X-ray diffraction and changes in dehydrothermal treatment

The process of dehydrothermal treatment begins with collagen-rich materials in the air dried state. The changes that occur with such treatment have to be regarded as either an extension of the drying effects outlined above, or that a new mechanism of altering collagen molecular packing occurs, see Fig. 2. It has been suggested by Bigi et al. [20] that the energies associated with the stability of the fibrillar structure are far smaller than those associated with

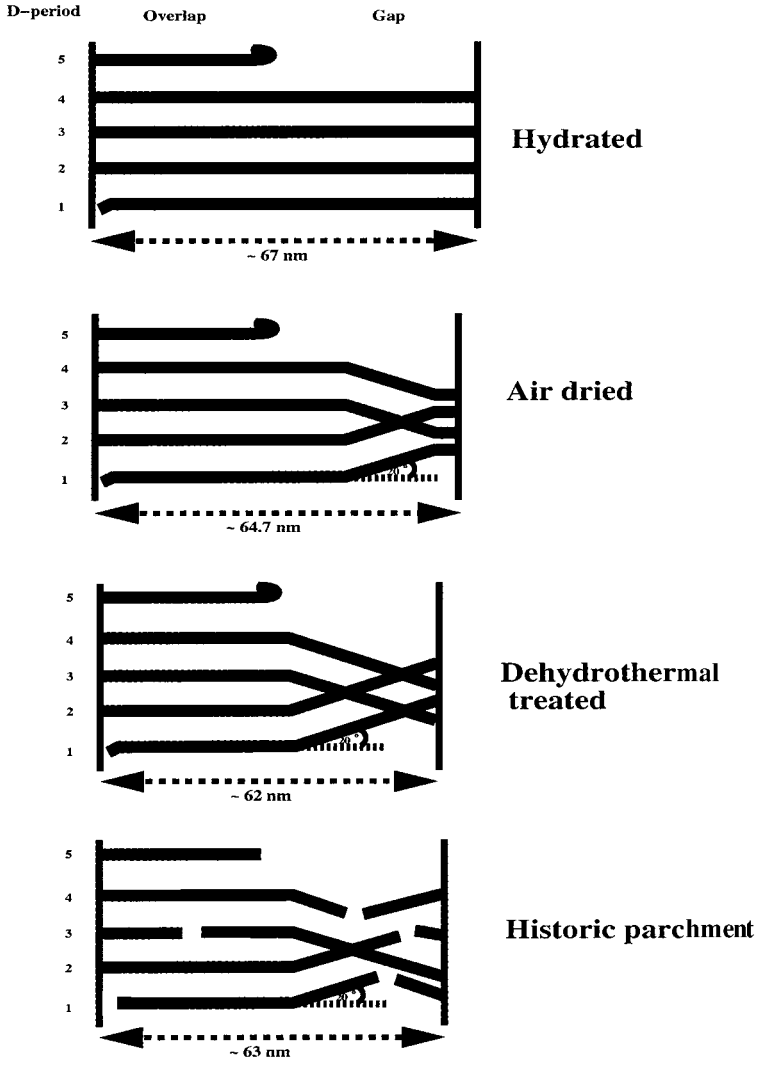

Fig. 2. Diagrammatic representation of the *D* periodic stagger in wet, dry and dehydrothermally treated collagen fibrils. Each of the five lines represents a collagen triplex of approximately 234 amino acids in length. The fifth segment is shorter than the four preceding segments and results in axial packing that contains a high density overlap region with a lower density gap region. The changes in the *D* periodic repeat can in part be accounted for by the tilting of molecular chains in the gap region upon hydration. This effect is extended in the dehydrothermal process. The approximate values of the *D* periods observed experimentally are also shown.

intramolecular H bonds. Therefore, the principal effects of dehydrothermal treatment will be to modulate the fibrillar packing prior to modulation of the helical structure.

Rat tail tendon subjected to dehydrothermal treatment at 120°C exhibits a shortening of the *D* period without a significant change in the intensity profile. The *D* periodic changes are summarized in Table 1. The fundamental periodicity is decreased but without drastically altering the axially projected electron density distribution within the unit cell. Furthermore, the tilt angle of the collagen chains did not deviate sensibly from 21.4° with heat treatment. If the portion of the *D* periodic unit that contains tilted collagen chains is increased, then part of the changes in the *D* period can be accounted for. In the air dried state, it is

Table 1
D periodic values of fundamental axial periodicity in collagen samples in nanometre[a]

Time	Tendon (untensioned)	Parchment	Parchment periodicity after rehydration	Historic parchment (mean value 12 samples)
0 (dry)	65.1	64.5	64.3	63.0 (±0.8)
30 min	63.1	62.8	–	
1 h	62.9	62.1	–	
2 h	62.4	61.7	–	
4 h	62.0	61.0	63.1	
24 h	61.8	60.0	61.9	

[a] The fundamental periodicity of the control tendon and parchment is different due to the fact that parchment contains a mixture of type I and type III collagen this produces a shorter fundamental periodicity although the molecular basis for this is not understood. The results shown are the mean measurement of the *D* periodicity from three samples in each case.

possible that only the central portion of the gap region has adopted a tilted conformation. The effect of progressive dehydrothermal treatment is to increase the portion of tilted chains until it extends over the whole of the gap region. The higher packing density within the overlap region probably limits the process. The projected *D* period length can be determined by estimating the fraction of *D* period that is tilted and then determining its projected contribution to the overall *D* periodic length. After dehydrothermal treatment, if the extent of the tilted region is increased to 0.6*D*, the *D* period would decrease to 64.4 nm. These figures correspond well with tendon samples that were held under approximately 2% extension during the dehydrothermal process. However, untensioned samples decreased their *D* periodic values to below those that could be accounted for by simple changes in the molecular tilt of chains in the gap region. This may relate to changes in the pitch of the collagen helix. It should be noted at this point that the fundamental periodicity of parchment (65.5 nm in the wet state) is shorter than tendon, and the *D* period values after dehydrothermal treatment are lower.

In the parchment samples, the main effect of dehydrothermal treatment was the shortening of the axial *D* repeat periodicity from 64.5 to 60.0 nm (see Figs. 3 and 4). This change was progressive with time, but was not found to change significantly after 24 h dehydrothermal treatment (data not shown). In Fig. 4, the intensity profile of orders 6–9 for the control and dehydrothermally treated parchments shows that the peak height and breadth of the control and dehy-

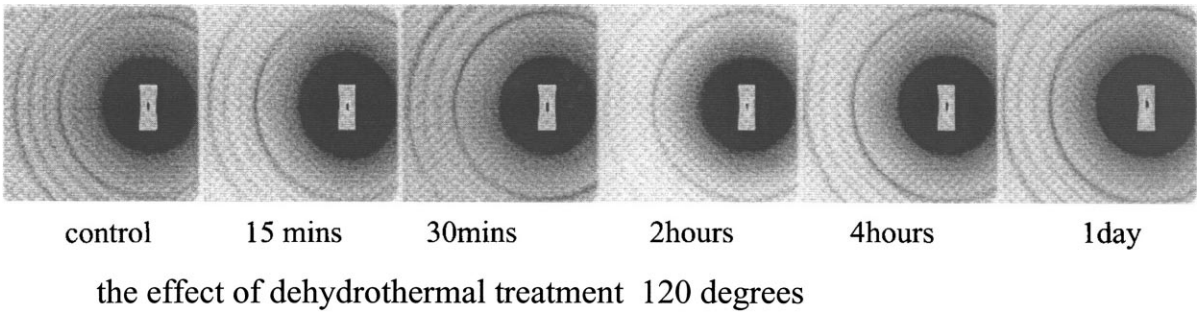

Fig. 3. Changes in parchment spacing with dehydrothermal treatment. Low angle X-ray diffraction recorded on beamline 2.1 of the SRS Daresbury Laboratory reveals the changes in the axial spacing of the meridional diffraction data obtained from the dehydrothermal treatment of parchment. The progressive change of the diffraction spacing to a higher diffraction angle corresponds to a shortening of the axial repeat. This can be seen by the movement of the outer diffraction intensity peak (ninth order) toward the edge of the detector. No significant systematic change was observed in the intensity distribution of the intensities.

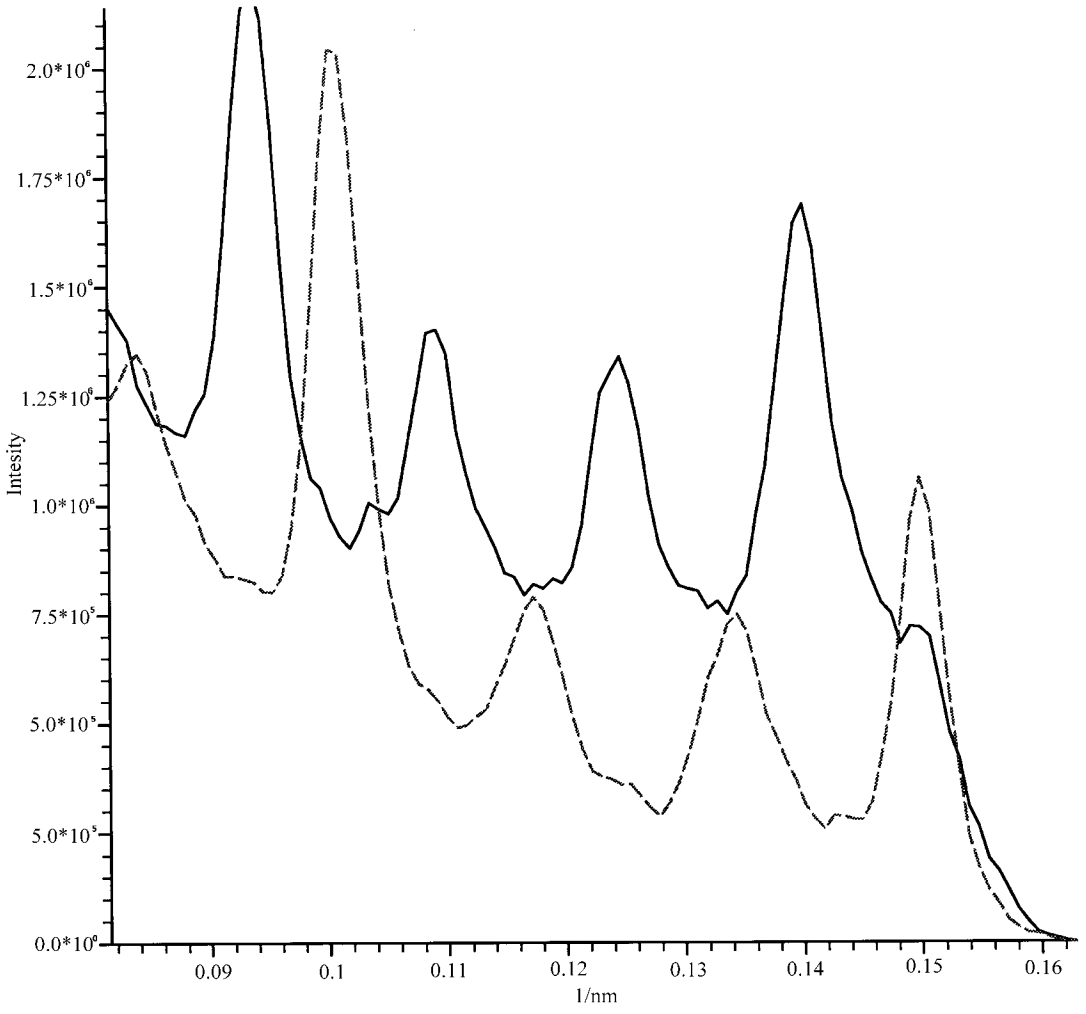

Fig. 4. Detail of change in spacing observed in the meridional diffraction pattern of parchment. The diffraction orders 6–9 are shown. The overlaid meridional intensity peaks of untreated parchment and dry parchment that has been treated for 24 h at 120°C. The periodicity of the fundamental axial repeat has changed from 64.5 to 60.0 nm as judged by the position of the ninth order of diffraction.

drothermally treated samples are not significantly different. The nature of the sample precludes the ability to make any analysis of changes in the chain direction within the sample as a function of dehydrothermal treatment since the diffraction is nearly isotropic. It is possible to assume that the basis for shortening of the *D* period in parchment has a similar structural basis to that in tendon.

The fundamental periodicity generated after 24 h dehydrothermal treatment was lower than that observed in the historic parchment samples, where the effect of ageing did not produce a fundamental periodicity below 62 nm, and where the mean fundamental periodicity of 12 historic bookbinding samples was 63.0 nm. The mean value for historical parchment therefore resembles the periodicity of a control parchment sample treated for approximately 1 h by dehydrothermal treatment. Variation in the *D* period of historic parchment may also be due to the anatomical location of the source material and also the exact species and age of animal at death.

The reversibilty of the effects of induced changes in the *D* period was also examined by rehydrating the sample and then returning to the air dried state. This

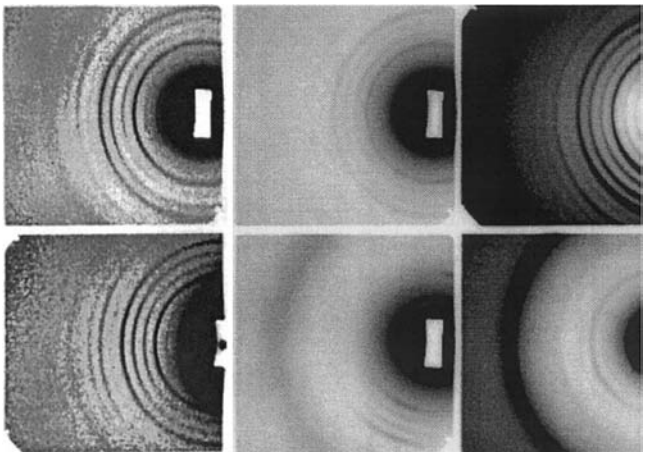

Fig. 5. A sample of diffraction patterns obtained from historic parchments on beamline 2.1 at the SRS Daresbury Laboratory. The diverse nature of historic parchment is indicated by the quality of diffraction from different samples taken from the School of Conservation sample bank, Copenhagen. The fundamental periodicity of the samples was close to 62.7 nm in each case. The control reference sample is the same as in Fig. 3. From bottom left to top right : (1) bookbinding of unknown age from the Royal Library of Denmark; (2) bookbinding from the Royal Library of Denmark data 1641; (3) bookbinding of unknown age from the Royal Library of Denmark; (4) 17th century bookbinding from the Regional Archive, Denmark.

resulted in diffraction patterns where a change in the fundamental periodicity was observed to return toward values obtained from tendon and parchment prior to dehydrothermal treatment; however, the air dried control values were not attained. The effect of rehydrating and redrying the parchment samples did however result in the *D* period values that are in the range of those obtained from historical parchments. The dehydrothermal treatment does not produce a fundamentally different molecular packing, rather it ensures the progression of the molecular shearing and chain tilting that is observed in the air drying process, the compounded effects of changes in the amino acid chemistry [11] may aid stabilization of a distorted conformation.

In contrast to changes in the fundamental periodicity, the main characteristic of deteriorated historic parchment is the truncation of the meridional diffraction series; four examples showing this effect to different extents can be seen in Fig. 5. This indicates that the organization of the diffracting matter in some historic samples is less than in the native state. The effect is best described by static disorder of the fibrillar structure where the unit cell contents vary throughout the diffracted area. In Fig. 5, some parchment samples contain only faintly visible diffraction above a steep sample derived scattering.

4. Conclusions

The process of dehydration of collagen and subsequent changes in molecular properties on dehydrothermal treatment are complex, and a number of factors may be involved in the alteration of the *D* period. The principal alteration in molecular packing from the hydrated state is probably the collapse of the gap/overlap and the partial shearing of unit cell contents within the gap region upon loss of water. Dehydrothermal treatment completes this structural phase change by reducing the molecular periodicity and may accelerate shearing of the unit cell. The rehydration of the samples and redrying indicate that the alterations that occur on dehydrothermal treatment are in part permanent and this is probably related to the changes in the amino acid cross-linkages and oxidative damage that may have been induced.

The applicability for dehydrothermal treatment in the simulation of accelerated ageing is debatable, the primary effects of deterioration of historic parchment samples is the truncation of the meridional diffraction series. The effect of dehydrothermal treatment shown here is to alter the *D* periodic function without modulating the intensity profile or the extent of the diffraction. Therefore, although dehydrothermal treatment may mimic some of the properties of

deteriorated historic parchment such as oxidative damage of amino acid side chains, the structural changes indicate that this technique is not appropriate to simulate some of the changes in molecular packing and coherence during deterioration.

Acknowledgements

We wish to thank Kurt Nielsen of the Danish Technical University, Lyngby and Andrew Miller of the University of Stirling for helpful advice. We also wish to thank staff at the CLRC SRS Daresbury beamlines 2.1 and 7.2 for technical support.

References

[1] P.J. Klopper, Eur. Surg. Res. 18 (1986) 218.
[2] A.J. Bailey, J. Soc. Leather Technologists Chemists 76 (1991) 111.
[3] P. Richardin, S. Copy, in: R. Larsen (Ed.), STEP Leather Project for the Protection and Conservation of European Heritage, Research Report No. 1, Bjarnholt Repro., Copenhagen, 1994.
[4] C.M. Kielty, I. Hopkinson, M.E. Grant, Connective Tissues and its Heritable Disorders, Wiley/Liss, New York, 1993, 103 pp.
[5] A.J. Hodge, J.A. Petruska, in: G.N. Ramachandran (Ed.), Aspects of Protein Chemistry, Academic Press, London, 1963, p. 289.
[6] D.A.D. Parry, Biophys. Chem. 18 (1988) 195.
[7] J.P. Orgel, T.J. Wess, A. Miller, The in situ conformation and axial location of the intermolecular crosslinked non-helical telopeptides of type I collagen 8 (2000) 137.
[8] T.J. Wess, A.P. Hammersley, L. Wess, A. Miller, J. Mol. Biol. 275 (1998) 255.
[9] K.M. Meek, J.A. Chapman, R.A. Hardcastle, J. Biol. Chem. 254 (1979) 1070.
[10] R.D.B. Fraser, T.P. MacRae, E. Suzuki, J. Mol. Biol. 129 (1979) 463.
[11] S.D. Gorham, N.D. Light, A.M. Diamond, M.J. Willins, A.J. Bailey, T.J. Wess, N.J. Leslie, Int. J. Biol. Macromol. 14 (1991) 129.
[12] P.P. Purslow, T.J. Wess, D.W.L. Hukins, J. Exp. Biol. 201 (1998) 135.
[13] A. Miller, J.S. Wray, Nature 230 (1971) 437.
[14] D.W.L. Hukins, Order and Disorder in Biological Systems, Pergamon Press, Oxford, 1977.
[15] D.J.S. Hulmes, A. Miller, D.A.D. Parry, K.A. Piez, J. Woodhead-Galloway, J. Mol. Biol. 79 (1973) 137.
[16] D.J.S. Hulmes, A. Miller, S.W. White, B. Brodsky-Doyle, J. Mol. Biol. 110 (1977) 643.
[17] R. Chandross, Coll. Rel. Res. 2 (1981) 344.
[18] R.S. Bear, J. Am. Leather Chem. Assoc. 46 (1951) 438.
[19] S.G. Tomlin, L.G. Ericson, Acta Crystallogr. 13 (1960) 395.
[20] A. Bigi, G. Cojazzi, N. Roveri, M.H.J. Koch, Int. J. Biol. Macromol. 9 (1987) 363.