

# Effect of crosslinking agents (basic chromium sulfate and formaldehyde) on the thermal and thermomechanical stability of rat tail tendon collagen fibre

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## Abstract

The role of covalent and coordinate covalent crosslinks on the thermal and mechanical properties of rat tail tendon (RTT) collagen fibre has been studied. The tendons were tanned with formaldehyde (HCHO) and basic chromium sulfate (BCS). Thermal properties of the crosslinked fibres were studied using shrinkage temperature, hydrothermal isometric tension behaviour and differential scanning calorimetry (DSC). Mechanical properties were analysed using tensile strength and stress relaxation behaviour. The results show that BCS tanned RTT exhibits a marked increase in the peak temperature and enthalpy changes when compared to both native and HCHO tanned RTT. This may be due to a net increase in the number of intermolecular crosslinks arising from both electrovalent and coordinate covalent interactions during BCS tanning. After treatment with urea there is a decrease in the peak temperature and the enthalpy changes of BCS tanned RTT fibres suggesting alterations in the secondary structure of collagen are possible even after tanning. An increased long range order is seen in the case of chromium tanned fibres in comparison to HCHO treated analogue. © 2000 Elsevier Science B.V. All rights reserved.

*Keywords:* Formaldehyde; Basic chromium sulfate; Shrinkage temperature; Differential scanning calorimetry; Change in enthalpy

## 1. Introduction

Skin matrix is primarily composed of triple helical collagen units, the fibrillar arrangement of these units is stabilised by various non-covalent and covalent intermolecular crosslinks [1–4]. Although non-covalent and covalent crosslinks do afford molecular stability, the long-term preservation of skin has required a chemical intervention by means of tanning agents.

Introduction of intermolecular covalent crosslinks between collagen molecules is responsible for the mechanical and thermal stability of the fibrous network [5–7]. In chromium tanning, the collagen fibrils of a hide become crosslinked by coordinate covalent bonds between bi- or polynuclear chromium ions and carboxylate side chains of the protein. Chromium sulfate and chromium chloride have been used in tanning studies. The crosslinking reaction of basic chromium sulfate (BCS) with collagen is known [8–12]. The aldehyde tannage is done usually with formaldehyde with the reaction occurring at  $\epsilon$ -amino groups of lysine and an elaborate scheme has been given earlier [13–20].

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There are number of methods available for examining the physical state of collagen in solution. But those measurements are not applicable for fibrous tissue itself. Differential scanning calorimetry (DSC) is a useful technique to characterise collagen with various crosslinking agents. Crosslinks cause an increase in the melting temperature because they decrease the entropy of melting transition [21]. DSC has also been applied to the study of the denaturation process which occurs in biological macromolecules such as collagen [22–30]. The isothermal measurement of rate of collagen denaturation, measured continuously using calorimetric method were used to determine rate constants for collagen denaturation in tendons. We have developed a method to calculate rate of relaxation for rat tail tendon (RTT) at various temperatures under different environmental conditions using stress relaxation experiments [20,31]. In this present investigation, the role of covalent and coordinate covalent crosslinks on thermal and thermomechanical properties of RTT have been studied.

## 2. Materials and methods

### 2.1. Sample preparation

Collagen fibres were teased out from the tails of 6-month old male albino rats (Wistar strain). The RTTs were used for testing 15 min from the sacrifice of the animal. The diameter of the fibres in wet condition was measured using a filar micrometer attached to an optical microscope. Area of cross section of the fibres was calculated from the diameter assuming a cylindrical shape for the fibre. Areas of cross section were compiled from the average of diameters measured at least in five locations along the length of the fibre.

The RTTs were tanned with 1% BCS at pH 3.2 and 2, 4, 8 and 10% formaldehyde (HCHO) solutions at pH 5 and 8 for overnight.

### 2.2. Thermal properties

The hydrothermal shrinkage temperature of the HCHO and BCS tanned RTT fibres were studied using an Instron testing machine (model 1112) with the

standard procedure. The dry weight of the RTT fibres was recorded using a Mettler balance with 10  $\mu\text{g}$  accuracy. Known amount of RTT (generally 1–2 mg) were crosslinked in 1% BCS solution at pH 3.2 and 2% HCHO solution at pH 5 for overnight and BCS and HCHO tanned RTT were swollen in water and 6 M urea solution for a period of 24 h. The soaked samples were blotted uniformly and hermetically encapsulated in aluminum pans. The samples were fused in a differential scanning calorimetric cell of a Perkin-Elmer DSC-7 instrument. The temperature and energy scales were calibrated effectively using the manufacturer's instructions with indium as standard, applying different temperature calibrations for each scanning rate. The energy scale calibration did not change with scanning rate. The heating rate was maintained constant at 6°C min<sup>-1</sup>. The peak temperature  $T_p$  (in °C) and the enthalpy changes  $\Delta H$  (in J g<sup>-1</sup>) associated with the phase change for the shrinkage process for native, HCHO and BCS tanned RTT fibres before and after urea treatment were studied. The method of calculation of activation parameters has been explained elsewhere [32].

### 2.3. Tensile strength and extension

Tensile strength measurements were made using a liquid cell attachment designed for an Instron testing machine model 1112 and 0–500 g load cell. The sensitivity of the load cell was 2% at the maximum range. The specimen length was 1 cm and the extension rate used was 0.5 cm min<sup>-1</sup>. The tensile strength and percent extension of crosslinked RTT in water medium at room temperature were calculated.

### 2.4. Stress relaxation behaviour

The stress relaxation experiments were carried out in an Instron testing machine model 1112 with a liquid cell container for HCHO and BCS tanned RTT fibres in water medium at 298, 313, 323, 333 and 353 K. The fibre was strained up to 20% strain level, thereafter the strain was maintained constant and the stress decay was monitored for 3 h. From the load-time graph, the values of  $\sigma/\sigma_0$  as a function of time have been plotted. Treatment of relaxation data and activation energy calculations for crosslinked RTT fibres were discussed previously [20].

### 3. Results and discussion

#### 3.1. Thermal properties

Thermally induced structural transitions in the fibrous collagenous network of skin and other connective tissues lead to shrinkage. The hydrothermal shrinkage temperature ( $T_s$ ) for HCHO crosslinked RTT fibres (for varying amounts of HCHO) at pH 5 and 8 are presented in Table 1. The results show that even at low concentration namely 2%, the maximum hydrothermal stability is achieved at pH 5 and 8. The enhanced hydrothermal stability of HCHO tanned RTT is due to new crosslinks formed and consequent changes in the tertiary structure of collagen. In the case of HCHO tanning there is a general belief that the dimensional changes occurring beyond shrinkage temperature may be partially reversible [33]. This is unique in the hydrothermal stability of tanned RTT fibres.

In order to examine the role of secondary structure in the hydrothermal stability of tanned RTT fibres, HCHO tanned RTT fibres are tested in urea solution. It is known that urea is capable of breaking hydrogen bonds in collagen molecules [34–36]. The shrinkage temperature of HCHO tanned RTT fibres in varying amounts of urea solutions are given in Table 2. The decrease in hydrothermal stability of HCHO tanned RTT in urea solution seems to be related to forces implicating the stability of secondary structure.

Table 1

Hydrothermal shrinkage temperature of native and formaldehyde (HCHO) crosslinked RTT at 2, 4, 8 and 10% at pH 5 and 8<sup>a</sup>

Specification	$T_s \pm 1$ (°C) <sup>b</sup>	$T_t \pm 1$ (°C) <sup>c</sup>
Native RTT in water	64	67
HCHO crosslinked RTT at pH 5		
2% solution	84	>98
4% solution	82	>98
8% solution	83	>98
10% solution	84	>98
HCHO crosslinked RTT at pH 8		
2% solution	84	>98
4% solution	84	>98
8% solution	84	>98
10% solution	86	>98

<sup>a</sup> Mean values  $\pm$  S.D. of six determinations.

<sup>b</sup>  $T_s$ : shrinkage temperature.

<sup>c</sup>  $T_t$ : temperature at maximum tension.

Table 2

Hydrothermal shrinkage temperature for formaldehyde (HCHO) crosslinked RTT fibres at 2, 4, 8 and 10% solutions at pH 5 in water, and 1, 3, 6 and 8 M urea solution<sup>a</sup>

	$T_s \pm 1$ (°C) (for HCHO crosslinked RTT at pH 5)			
	2%	4%	8%	10%
Formaldehyde crosslinked RTT in				
Water	84	82	83	84
1 M urea	82	81	83	82
3 M urea	77	80	81	82
6 M urea	77	79	79	80
8 M urea	77	79	79	80

<sup>a</sup> Mean values  $\pm$  S.D. of six determinations.

Shrinkage temperature of RTT fibres tanned with BCS salt has been examined both in aqueous glycerol and 6 M urea (in water) media. The data are given in Table 3. There is 9°C decrease in shrinkage temperature of BCS tanned RTT fibres when tested in 6 M urea solution as compared with that of BCS tanned RTT in aqueous glycerol. The decrease in the shrinkage temperature of the crosslinked RTT in urea solutions reveal alterations in the secondary structure of collagen are feasible even after tanning.

The measured curves of native, HCHO and BCS tanned RTT fibres are shown in Figs. 1–3. In all the cases a single peak is observed. It is an endothermic process in which a phase transition involving changes in the lattice and long range order are realised.

The peak temperature for the shrinkage processes, the changes in enthalpy and energy of activation associated with the phase change for the native, BCS and HCHO tanned RTT are given in Table 4. The peak temperatures for the shrinkage process for native and HCHO tanned RTT collagen fibres are 67.6 and 74.3°C, respectively. But there is no significant

Table 3

Hydrothermal shrinkage temperature of native and basic chromium sulfate (BCS) crosslinked RTT in water and 6 M urea solution<sup>a</sup>

Specification	$T_s \pm 1$ (°C)
Native RTT in water	64
BCS tanned RTT in glycerol+water	105
Native RTT in 6 M urea solution	41
BCS tanned RTT in 6 M urea solution	96

<sup>a</sup> Mean values  $\pm$  S.D. of six determinations.

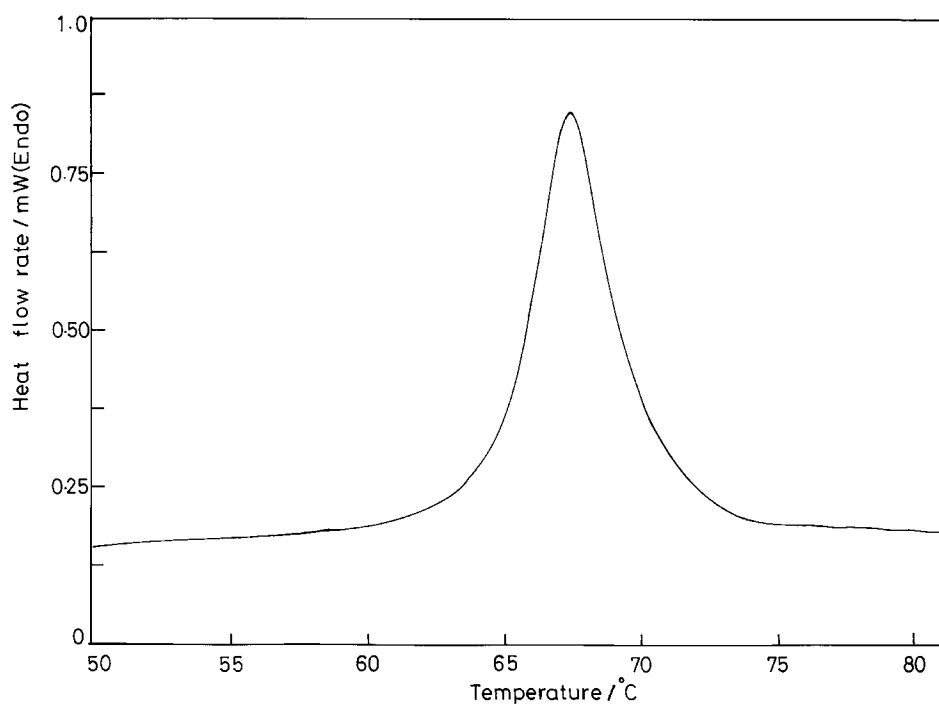


Fig. 1. DSC curve of native RTT swollen in water medium.

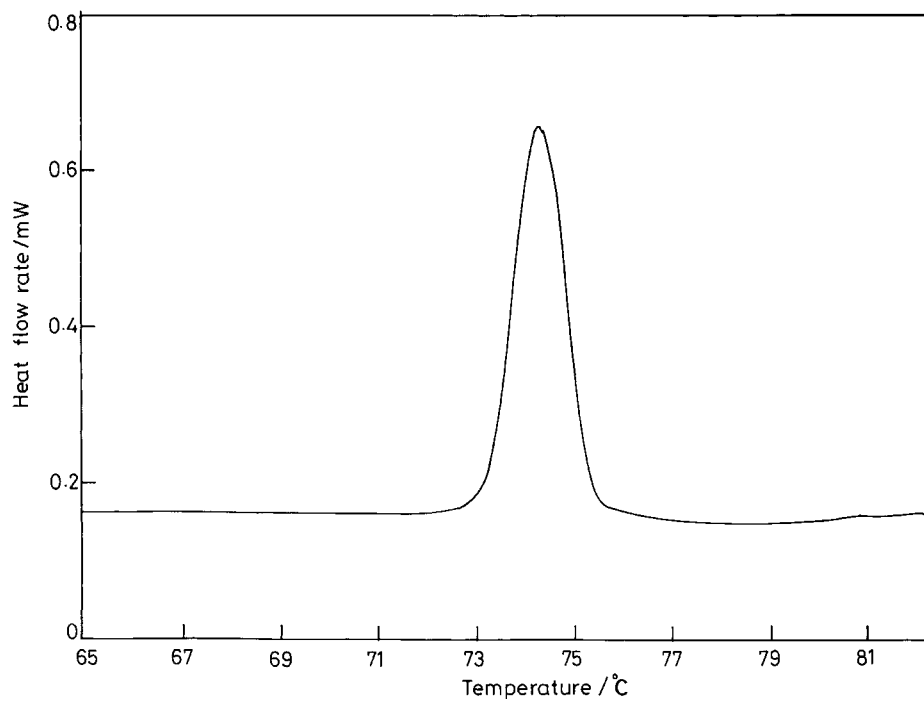


Fig. 2. DSC curve of formaldehyde crosslinked RTT swollen in water medium.

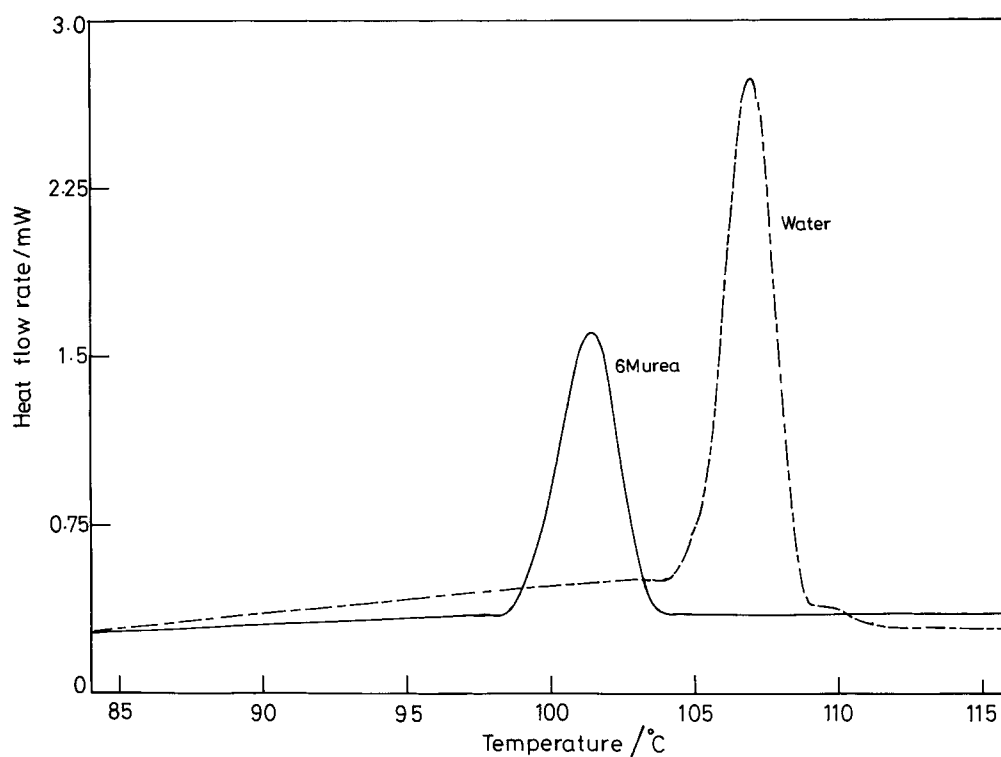


Fig. 3. DSC curve of basic chromium sulfate crosslinked RTT swollen in water and 6 M urea solution.

change in the enthalpy values for the native and HCHO tanned RTT.

Measured curves of BCS tanned RTT has been recorded before and after the treatment with 6 M urea and are given in Fig. 3. It is seen that the peak temperature for BCS tanned RTT before treatment with urea is 107°C with an enthalpy value of 67.2 J g<sup>-1</sup> (Table 4). Chromium tanned RTT exhibits

a marked increase in the peak temperature as well as in the enthalpy changes when compared to both native and HCHO tanned RTT. This may be due to a net increase in the number of intermolecular crosslinks arising from both electrovalent and coordinate covalent interactions. Therefore, the rise in melting temperature with crosslinking agent reflects an increase in the average number of crosslinks per molecule. The

Table 4

Comparison of thermodynamic parameters (DSC) of native, formaldehyde (HCHO) and basic chromium sulfate (BCS) crosslinked RTT fibres in water and urea solutions<sup>a</sup>

Specification	$T_p \pm 1$ (°C) <sup>a</sup>	$E_a$ (kJ mol <sup>-1</sup> ) <sup>b</sup>	$\ln k_0$ (S <sup>-1</sup> ) <sup>c</sup>	$\Delta H$ (J g <sup>-1</sup> ) <sup>d</sup>
Native RTT	67	1180±25	415±9	47±1
HCHO crosslinked RTT in water	74	1969±20	474±8	44±0.5
BCS crosslinked RTT in water	107	2000±18	631±10	64±2
BCS crosslinked RTT in 6 M urea solution	101	1422±13	455±15	42±1
Native RTT in 3 M urea solution	53	1096±23	401±7	39±0.5

<sup>a</sup>  $T_p$ : peak temperature.

<sup>b</sup>  $E_a$ : activation energy.

<sup>c</sup>  $k_0$ : rate of shrinkage.

<sup>d</sup>  $\Delta H$ : enthalpy change.

decrease in the peak temperature and the enthalpy changes of BCS tanned RTT fibres after the treatment with 6 M urea solution reveal that the alterations in the secondary structure of collagen are possible even after tanning.

The energy of activation associated with the phase change increases for crosslinked RTT compared to the native ones. The energy that is needed for the conversion of collagen to gelatin transition is more due to the additional crosslinks introduced by BCS and HCHO. The activation energy decreases from 2000 to 1422 kJ mol<sup>-1</sup> in the case of BCS tanned RTT in 6 M urea suggesting that some of the hydrogen bonds were broken in the presence of urea even before heating. Comparing the shape of the calorimetric curve of BCS crosslinked RTT in water medium and 6 M urea solution (Fig. 3) and the corresponding activation energy values suggest that a process with a large activation energy has a sharp peak with high normalised peak height, whereas one that has a low energy of activation displays a broad peak [37].

The rate of shrinkage for native, HCHO, and BCS crosslinked RTT are given in Table 4. The rate of shrinkage  $\ln k_0$  (s<sup>-1</sup>) increases with degree of crosslinking. The rate of shrinking is dependent on the energy of breaking of intermolecular hydrogen bonds and the resulting increase in structural disorder.

Chromium tanned collagen fibre is known to resist thermal shrinkage to relatively higher temperature compared to native fibres and formaldehyde is believed to impart crosslinks of varying thermal stability. The enthalpy changes associated with the phase transition need to implicate the thermomechanical events associated with secondary, tertiary and quaternary structures while the composite enthalpy changes need to derive contributions from the extent of ordering in all the levels of collagen structure.

### 3.2. Tensile strength and extension

Tensile strength and percentage extension of native and formaldehyde treated RTT (with 2, 4, 8 and 10% solution at pH 5) in water at 298 K are given in Table 5. On tanning with formaldehyde, tensile strength increases significantly, until a critical concentration of formaldehyde is reached. Formaldehyde is capable of diffusing into the molecular pore dimensions and participating in fibre splitting. Increase in tensile

Table 5

Tensile strength and percent extension of native and formaldehyde (HCHO) crosslinked RTT at pH 5 at 2, 4, 8 and 10% solutions and tested in water at 298 K<sup>a</sup>

Specification	Tensile strength (MPa)	Extension (%)
Native RTT	58±5	45±5
HCHO crosslinked RTT with		
2%	138±10	55±5
4%	160±15	42±3
8%	165±10	36±5
10%	115±8.0	26±2

<sup>a</sup> Mean values±S.D. of 20 samples.

strength can be interpreted in terms of the number of covalent crosslinks formed during the tanning processes. The bound volume measurements of formaldehyde tanned collagen provides an explanation of increased tensile strength of formaldehyde tanned fibre.

Tensile strength and percentage extension of native and basic chromium tanned RTT in water at 298 K are given in Table 6. The tensile strength of native RTT fibres under the same conditions of temperature vary in the range of 58±5 MPa. These values are nearly the same as those observed for chromium tanned RTT fibre within experimental errors. In other words, chromium tanning does not seem to change the tensile strength of RTT fibres significantly in wet condition.

### 3.3. Stress relaxation behaviour

The viscoelastic behaviour of formaldehyde and basic chromium sulfate tanned RTT fibres has already been reported [20]. The comparison of the stress relaxation profile of native, HCHO and BCS tanned RTT at 298 K in water medium is given in Fig. 4. It is obvious that native RTT fibres relax faster than the

Table 6

Tensile strength and percent extension of native and basic chromium sulfate (BCS) crosslinked RTT in water medium at 298 K<sup>a</sup>

Specification	Tensile strength (MPa)	Extension (%)
Native RTT in water	58±5	45±5
BCS crosslinked RTT in water	60±5	60±4

<sup>a</sup> Mean values±S.D. of 20 samples.

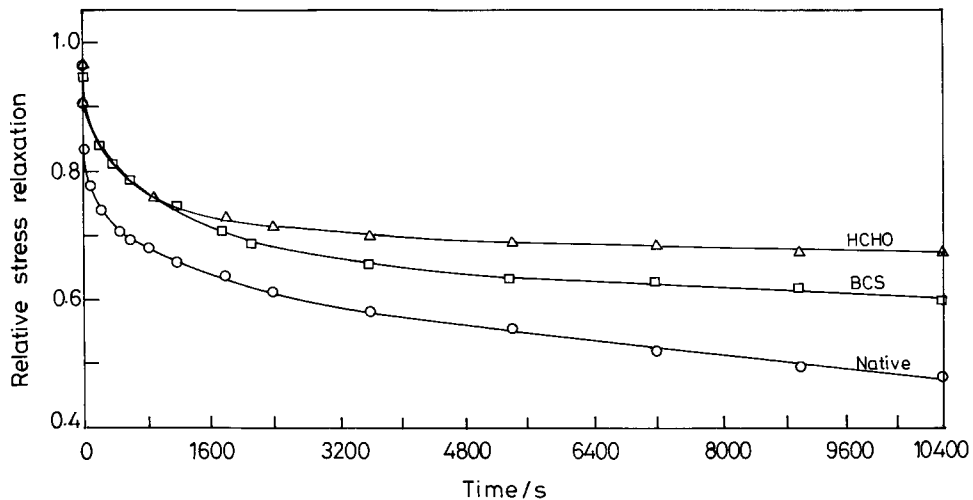


Fig. 4. Stress relaxation behaviour of native, formaldehyde and basic chromium sulfate crosslinked RTT in water medium at 298 K.

BCS and HCHO tanned ones. The additional cross-links introduced by HCHO and BCS tend to hold the fibre from relaxing faster. The thermomechanical stress is higher in HCHO tanned samples compared to the chromium tanned fibre. Deposition of material with a hardness higher than that of collagen fibres may well reduce the thermomechanical and tensile strength.

#### 4. Conclusion

The dimensional stabilisation of RTT fibres due to HCHO and BCS tanning has been investigated in detail. Shrinkage temperature of tanned fibres have been used as an effective guideline for assessing the stability of tanned fibres. On the basis of increase in thermal stabilisation of tanning, a higher level of long range order is invoked in the case of chromium tanned fibres in relation to HCHO treated analogue. A higher ordering in chromium tanned collagen lattice may originate from the ability of chromium to assemble more number of collagen molecules. While thermal stability of a chromium tanned collagen fibre is higher than that of a HCHO tanned specimen, the thermomechanical stress is higher in a HCHO tanned sample compared to the chromium tanned fibre as seen from Fig. 4. Deposition of material with a hardness higher than that of collagen fibres may well reduce the

mechanical properties. The propagation of thermomechanical stress in a fibre bundle like RTT is influenced by a number of factors including the frictional forces in the interfibrillar region. Appreciable changes in the DSC studies suggest the occurrence of conformational change on treatment with HCHO and BCS crosslinked RTT fibres. The activation parameters need to derive contributions from the extent of ordering in all the levels of hierarchical organisation of RTT collagen fibre.

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