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Influence of hydrogen bond, hydrophobic and electrovalent salt linkages on the transition temperature, enthalpy and activation energy in rat tail tendon (RTT) collagen fibre

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Abstract

The influence of hydrogen bonding, hydrophobic and electrostatic interactions on the thermal stability of rat tail tendon collagen fibre has been studied using differential scanning calorimetry (DSC) and hydrothermal isometric tension (HIT) experiments. The reagents used to study these effects are urea (hydrogen bonding), aqueous alcohols (hydrophobic) and 0.02 M Tris-maleate buffer at pH 4–8 (electrostatic interactions). The peak temperature, enthalpy changes and energy of activation for collagen to gelatin transition are computed using DSC. The peak temperature and enthalpy changes decrease with increasing concentrations of urea, increasing chain length of alcohol and decreasing pH. The shape of the isometric tension curves of the collagen fibres provide information on the crosslinking of collagen fibre while the extent of relaxation after maximum tension is indicative of thermally stable crosslinks. © 1999 Published by Elsevier Science B.V. All rights reserved.

Keywords: Differential scanning calorimetry; Hydrothermal isometric tension; Hydrogen bonding; Hydrophobic and electrostatic interactions; Rat tail tendon

1. Introduction

Collagen, the connective tissue protein is stabilised by the interplay of vast number of intra- and intermolecular forces. The role of hydrogen bonds in the stabilisation of collagen triple helix has been discussed earlier [1–4]. The charged groups inside the microfibril contributes for electrostatic interactions and are important in defining the intra-microfibrillar structure while the excess of hydrophobic residues occur at a greater concentrations on the outside of the microfibril are therefore significant in inter-microfibrillar packing [5]. Privalov emphasised the role of enthalpy and hydrogen bonding in the stabilisation of collagen triple helix [6]. The various lyotropic reagents which break hydrogen bonds are known to affect the thermal stability of collagen [7,8]. Ionic interactions between the oppositely charged centers are long range forces and are important in the aggregation of collagen molecule to form insoluble fibrils. However, acid–base association of amino and carboxylic acids can be expected to affect the stability of ionic bonds [9–11]. High angle X-ray diffraction studies have been made on collagen fibres as a function of pH and ionic strength to investigate the influence of electrovalent interactions on the structure and stability of the collagen molecule [12]. In aqueous

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environment, repulsive forces between water and nonpolar side chains can be expected to favour the ordering of water structure as well as intermolecular assemblies in collagenous tissues. The vital role of hydrophobic forces in the transition of soluble to fibrillar forms of collagen has been discussed earlier [13–18].

It is known that the intermolecular forces and the long range order influence the melting point of the crystalline substances. In the case of collagen which is influenced by large number of intermolecular forces, sufficient thermal energies are needed to overcome such forces. There are several methods available for examining the physical state of collagen in solution [19–21]. But these methods are not applicable to measure the changes in the fibrous tissue. Hydrothermal isometric tension (HIT) and differential scanning calorimetry (DSC) provide a better understanding of the role played by collagen triple helix denaturation. HIT experiments under various environmental conditions have been discussed earlier [22–27]. Denaturation of collagen is an endothermic reaction which occurs at a very slow rate under equilibrium conditions. Several authors have analysed the endothermic helix to coil transition by DSC in soluble collagen or in the fibrous form and have determined the corresponding enthalpy changes and temperature of collagen to gelatin transition [28–33]. In this present investigation the influence of urea, aqueous alcohol and 0.02 M Tris-maleate buffer at different pH, on the transition temperature, enthalpy, energy of activation and hydrothermal isometric tension associated with collagen to gelatin transitions in rat tail tendon (RTT) collagen fibre have been studied.

2. Materials and methods

2.1. Sample preparation

Collagen fibres, (tendons) were teased out from tails of six month old male albino rats (Wistar strain), thoroughly washed and stored at -20°C until used.

2.2. Hydrogen bond breaking reagent

Urea used in the experiments was of analytical grade. 1, 3 and 6 M urea solutions were prepared on a weight basis. The collagen fibres were equilibrated in urea solution before each experiment.

2.3. Preparation of aqueous alcoholic mixtures

Alcoholic solvents viz., methanol, ethanol and *n*-propanol used were of analytical grade. They were used without further purification. The collagen fibres were equilibrated in aqueous-alcoholic media with alcoholic proportions varying as 1, 7, 10 and 20 mole% [34,35].

2.4. Hydrogen ion concentration

The pH of the medium was adjusted using Tris-maleate buffer. The collagen fibres were equilibrated in 0.02 M Tris-maleate buffer at different pH. The pH of the solution was measured using glass electrode. The ionic strength was maintained constant throughout the experiment. Since in the pH range of 4–8 there are no detectable changes in the microscopic dimensions of collagen fibre, the hydrogen ion concentrations have been maintained in this pH range [12].

2.5. Differential scanning calorimetry (DSC) studies

The rat tail tendon (RTT) collagen fibres were air dried and the dry weight of the RTT fibres was recorded using Mettler balance with 10 μg accuracy. Known amount of RTT (generally 1–2 mg) was immersed in the experimental solutions viz., 1 and 3 M urea, 1, 7, 10, and 15 mole% of methanol, ethanol and *n*-propanol and 0.02 M Tris-maleate buffer at pH 6–8 for 24 h. The soaked samples were blotted uniformly and hermetically encapsulated in aluminum. Initially base line (a run without sample and reference materials) was optimised to obtain nearly a flat background. Then the temperature and enthalpy values were calibrated using indium and zinc. These measured values agree with standard values reported in literature within 1% [36]. During programmed heating, both the holders are ramped in a controlled manner so that the temperature difference ΔT, between them is maintained zero. Whenever a reaction of any sort involving change in enthalpy or specific heat is taking place at a particular temperature then the temperature of the sample would lag behind or

lead over the reference holder. The samples were placed in a differential scanning calorimetric cell of a Perkin-Elmer DSC-7 instrument. The heating rate was maintained constant at 6°C/min. The peak temperature T_p for the collagen-to-gelatin process was recorded and enthalpy changes for this transition were computed using standard methods. After the DSC curve has been obtained, it was fitted to equation

$$d\alpha/dT = \beta k_0 e^{-E_a/RT}(1-\alpha)^n \quad (1)$$

where α is the degree of chemical reaction, n the order of the reaction, k the reaction rate, and β is the proportionality constant. This equation represents the theoretical shape of the DSC curve that is determined in PC series, DSC standard program. The resultant values of k_0, E_a and n are calculated.

2.6. Hydrothermal isometric tension (HIT) experiments

Hydrothermal isometric tension experiments were carried out in an Instron testing machine model 1112. Using appropriate load cells in the range of 0.1 – 500 kg could be measured. The sensitivity of the load cells was 2% at the maximum range. A liquid cell container was kept on the heater whose input supply voltage was adjusted to get a required heating rate of 3°C/min. The fibres were immersed in the respective solutions in the liquid cell with one end attached to the frame and the other end attached to the load cell. The shrinkage temperature and tension were continuously recorded.

As the fibre is held at constant length during the experiment, the temperature at which the tension begins to increase is recorded as the shrinkage temperature (T_s). The temperature at which the tension (which increases progressively with shrinkage) reaches the maximum is the T_t. The corresponding tension is defined as the isometric tension (I_t). 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 and this is used to calculate the isometric tension [35,37].

3. Results and discussion

3.1. Differential scanning calorimetry studies

3.1.1. Role of hydrogen bonding

Thermogram of native RTT swollen in water, 1 and 3 M urea solutions are given in Fig. 1. It is an endothermic process. The shrinkage temperature is 61.5°C and 53°C in 1 and 3 M urea respectively. The half width of the DSC curve is 1.5°C for 1 M urea and the corresponding value is 2.9°C for 3 M urea. In other words, the peak is broader when the concentration of urea is 3 M. The peak profile is very much dependent on urea concentration. Multiple phases are likely for phase transition at higher urea concentrations. Since collagen is stabilised by numerous hydrogen bonds of varying strength, it is probable that depending on the urea concentration wide ranging structural alterations are likely. The thermodynamic parameters for native RTT swollen in water, 1 and 3 M urea are given in Table 1. It is relevant to point out that the enthalpy change associated with phase change for native RTT in water medium is 47 J/g. This is in good agreement with previously reported values in water medium [38–40]. The peak temperature and the enthalpy change for the phase change decreases as the concentration of urea increases from 1 to 3 M.

3.1.2. Effect of aqueous alcohols

DSC curves of native RTT swollen in 10 mol% of methanol and *n*-propanol are given in Fig. 2. It has been reported that the transition temperature for the phase change varies with mole percentage of different alcohols and the chain length of the alcohols. The onset temperature is the same for 1 and 15 and 1 and 7 mole% of methanol and ethanol respectively. The onset temperature is shifted when the medium contained *n*-propanol [35]. The thermogram is broader as the chain length of the alcohol increases as seen from Fig. 2. It is believed that hydrophobic forces influence intra and intermolecular interactions in collagen which are weakened in the presence of nonpolar groups from the solvent environment [41–43].

3.1.3. Influence of hydrogen ion concentration

Experimental curves of native RTT swollen at pH 8 and 6 are shown in Fig. 3. Differential scanning

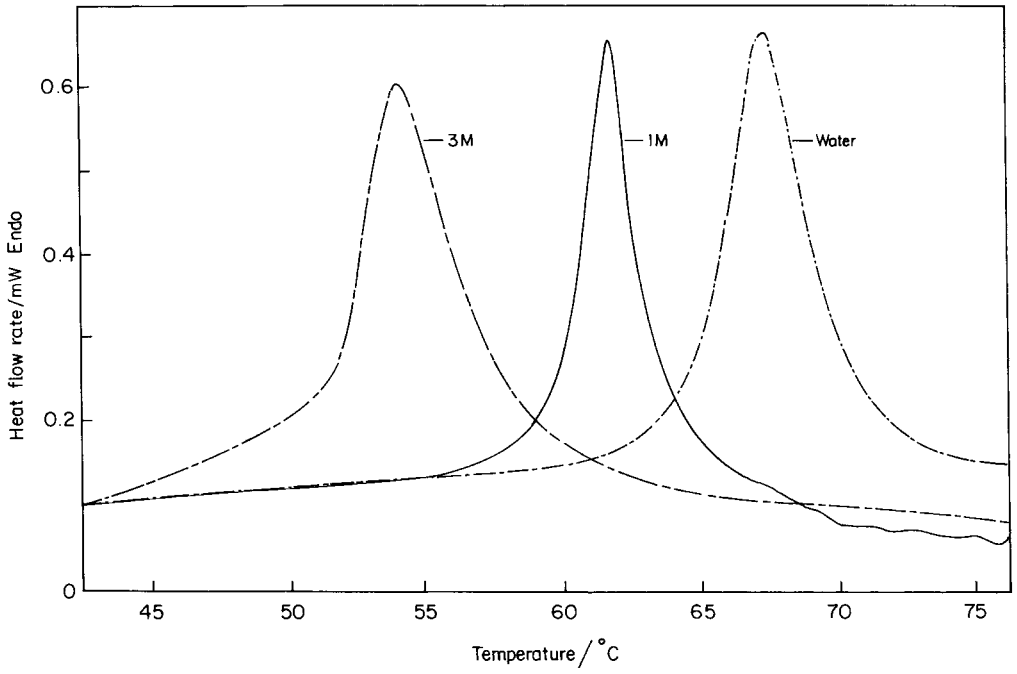

Fig. 1. DSC curve of native RTT swollen in water, 1 and 3 M urea solution.

calorimetric studies show that the peak temperature for the shrinkage process for native RTT at pH 8 is 68°C and 65.7°C at pH 6. Curve of native RTT have been obtained in water medium in the absence of any electrolyte and is given in Fig. 1. The peak of DSC curve has been observed at 67°C in the absence of any buffer with the pH being in the range of 7–8. It is interesting to compare the enthalpy changes associated with the phase changes for native RTT swollen in 0.02 M Tris-maleate buffer at pH 6 and 8. The values estimated are 24.5 and 21.3 J/g respectively. At or near isoelectric point of collagen, collagen is expected to be less extensively solvated. Therefore, work needed to overcome protein–water interaction is the lowest at the isoelectric point. The observed DSC data at different pH values are explained in terms of

Table 1
Thermodynamic parameters of native RTT under various environmental conditions (mean ± standard deviation of five determinations)

Native RTT in	T_p (°C)[a]	ΔH (J/g)[b]	E_a (kJ/mol)[c]
Water	67 ± 1	47 ± 2	1180 ± 25
1M urea	61.5 ± 1	60.5 ± 3	1351 ± 30
3M urea	53 ± 1	39 ± 1	1096 ± 23
1 mol% methanol	65 ± 1	57 ± 3	1855 ± 39
1 mol% ethanol	65 ± 1	55 ± 4	1621 ± 28
1 mole% *n*-propanol	68 ± 1	54 ± 3	952 ± 20
0.02 M Tris-maleate buffer at pH 8	68 ± 1	25 ± 2	2062 ± 30
0.02 M Tris-maleate buffer at pH 6	65 ± 1	21 ± 1	1605 ± 20

[a] T_p Peak temperature for collagen-gelatin transition.
[b] ΔH Enthalpy.
[c] E_a Activation energy for the phase transition.

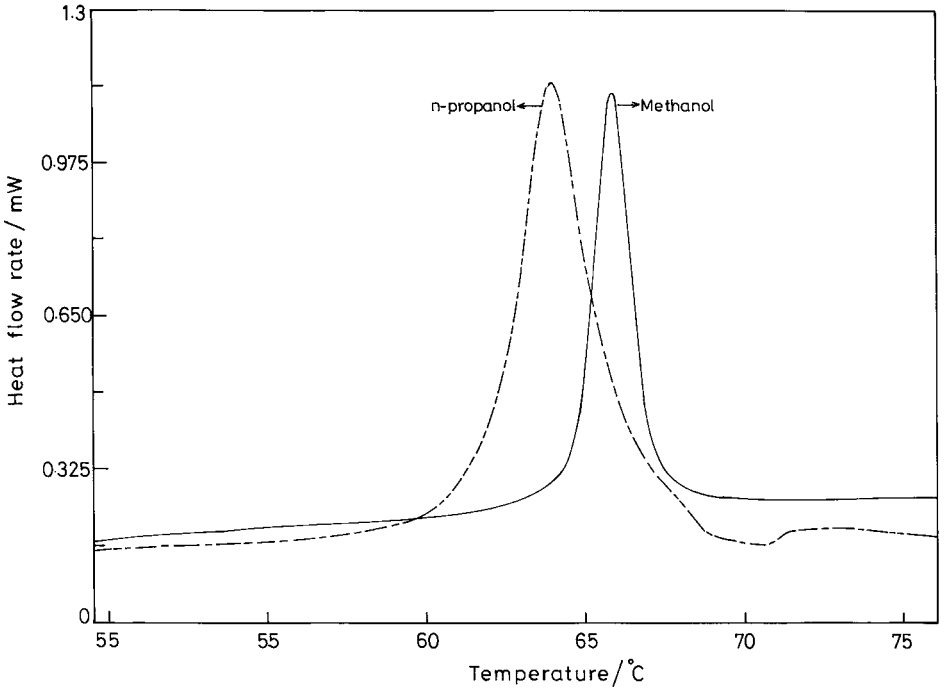

Fig. 2. DSC curve of native RTT swollen in 10 mole% of methanol and *n*-propanol.

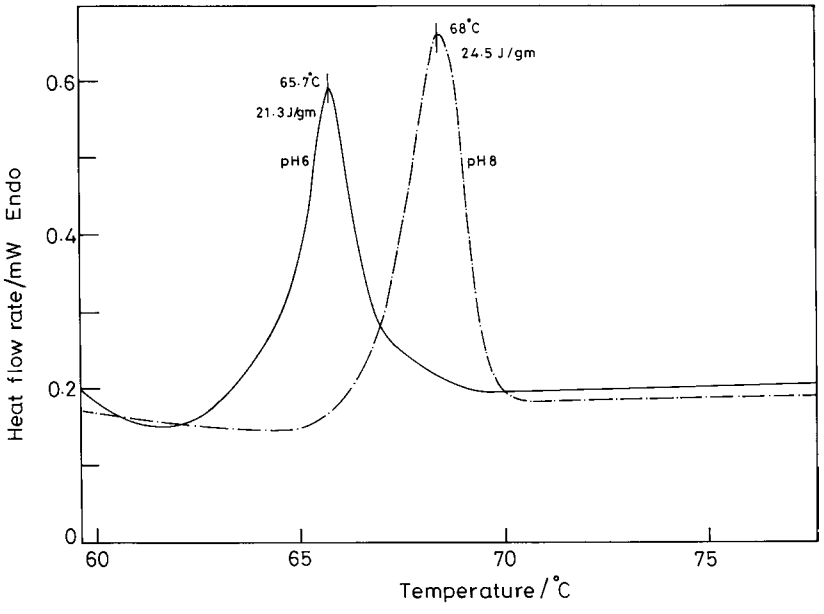

Fig. 3. DSC curve of native RTT swollen in 0.02 M Tris-maleate buffer at pH 6 and 8.

Table 2
Thermomechanical properties of native RTT in water and various concentrations of urea (mean and standard deviation of six determinations)

Native RTT in	T_s (°C)[a]	I_t (MPa)[b]	T_t (°C)[c]
Water	64 ± 1	0.2 ± 0.1	67 ± 1
1 M urea	57 ± 1	0.35 ± 0.08	67 ± 1
3 M urea	51 ± 1	0.6 ± 0.2	57 ± 1
6 M urea	41 ± 1	0.95 ± 0.2	51 ± 1

[a] T_s Shrinkage temperature.
[b] I_t Isometric tension.
[c] T_t Temperature at the maximum tension.

solute–solvent interactions and hydration phenomenon and these are associated with the phase transitions.

3.2. Hydrothermal isometric tension behaviour

3.2.1. Effect of urea

The shrinkage temperature and the isometric tension of native RTT have been examined in aqueous media containing various concentrations of urea in the range of 0–6 M and the data are presented in Table 2. There is significant decrease in the shrinkage temperature values with increasing concentrations of urea while the isometric tension values correlate inversely. The break down of intermolecular crosslinks is expected to lower the shrinkage temperature and reduce the interfibre cohesion. The observed trend in the shrinkage temperature data can be explained in terms of break down of intermolecular hydrogen bonds. The increase in isometric force with an increase in urea concentration may be due to the penetration of urea into the crystalline region and the degraded crystalline region would produce an increase in force due to the increase in entropy of the random coiled collagen [44].

3.2.2. Role of aqueous alcohols

The shrinkage temperature, (T_s) data for native RTT have been correlated as a function of mole percentage of different aqueous alcoholic solvent media in Fig. 4. The variation of T_s of native RTT with mole percentage of methanol and ethanol is marginal. Significant variation in shrinkage temperature data was observed in the case of *n*-propanol. The hydrothermal stability of native RTT reaches the lowest value at 10 mole% of *n*-propanol. The initial decrease in T_s with increase in mole% of *n*-propanol (up to 10 mole%) can be explained in terms weakening of the hydrophobic interactions by alcohol resulting in the reduction of

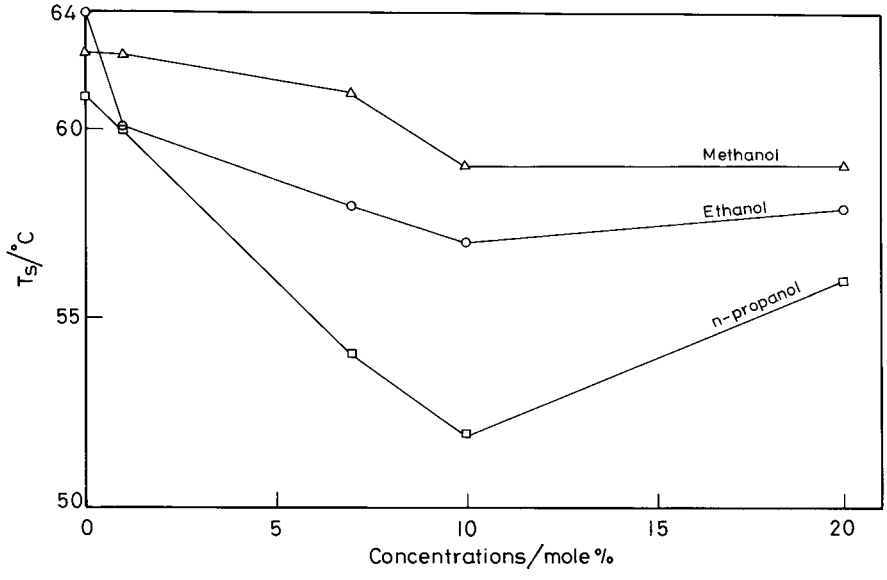

Fig. 4. Plot of shrinkage temperature against concentrations of methanol, ethanol and *n*-propanol.

long range interaction. Since ionic interactions are strongly influenced by the solvent dielectric constants, there are variation in the shrinkage temperature of collagen as the chain length of the alcohol increases. Since solvation of protein in general and the hydrodynamic sphere of collagen in particular are known to be influenced by microviscosities of the solvated protein samples, the observed data need to be discussed in terms of both extent of solvation and interplay of salt bridges. When the partial volume of *n*-propanol increases, there may arise a possibility that the hydrodynamic sphere is altered and desolvation increases. This may partially explain the higher shrinkage temperature of collagen at 20 mole% than at 10 mole%. Another possible explanation is that the hydrogen bonds between the alcohol rich medium and the collagen become weaker, making hydrogen bonds within polypeptide chain comparatively stronger. The shrinkage temperature T_s for native RTT as a function of logarithm of dielectric constant ($\log \varepsilon$) of the alcoholic solvents seem to be an apparent linear relationship at a solvent composition of 10 mole%.

Data of shrinkage temperature (T_s), isometric tension (I_t) and temperature at the maximum tension (T_t) of native RTT in 1, 7 and 10 mole% of methanol and *n*-propanol are listed in Table 3. In the case of *n*-propanol, the maximum isometric tension increases with the concentration of alcohol up to a concentration of 7 mole%. However temperature at maximum tension is less sensitive to the nature of the solvent.

Table 3
Thermomechanical properties of native RTT in 1, 7 and 10 mole% of methanol and *n*-propanol (mean ± standard deviation of six determinations)

Native RTT in	T_s (°C)[a]	I_t (Mpa)[b]	T_t (°C)[c]
Methanol (mole%)			
1	63 ± 1	0.25 ± 0.02	67 ± 1
7	61 ± 1	0.22 ± 0.03	65 ± 1
10	60 ± 1	0.23 ± 0.03	64 ± 1
n-Propanol (mole%)			
1	61 ± 1	0.25 ± 0.03	66 ± 1
7	56 ± 1	0.4 ± 0.04	64 ± 1
10	53 ± 1	0.42 ± 0.15	64 ± 1

[a] T_s Shrinkage temperature.
[b] I_t Maximum isometric tension.
[c] T_t Temperature at maximum tension.

Table 4
Thermomechanical properties of native RTT in 0.02 M Tris-maleate buffer at different pH conditions (mean values of six samples)

Native RTT in	T_s (°C)[a]	I_t (Mpa)[b]	T_t (°C)[c]
pH 8	63 ± 1	0.25 ± 0.08	65 ± 1
pH 7	61 ± 1	0.15 ± 0.05	64 ± 1
pH 6	58 ± 1	0.1 ± 0.02	62 ± 1
pH 5	51 ± 1	0.08 ± 0.02	55 ± 1
pH 4	–	–	–

[a] T_s Shrinkage temperature.
[b] I_t Maximum isometric tension.
[c] T_t Temperature at maximum tension.

3.2.3. Influence of hydrogen ion concentration

Shrinkage temperature and isometric tension for native RTT have been measured in the pH range of 4–8 in 0.02 M Tris-maleate buffer and the data are given in Table 4. The shrinkage temperatures (T_s °C) at pH 4 are not possible due to dimensional destability. It may be observed that below pH 6, there is a significant decrease in T_s. This may be partially due to osmotic forces that could lead to acid swelling. Extensive hydration could lead to significant volume changes and rupturing of the matrix structure. Further, protonation of ionisable group may dominate at pH values lower than the isoelectric point. This could well decrease the intermolecular ion pair formation. It is generally observed that the temperature at maximum tension is marginally higher than the shrinkage temperature and is more representative of thermal stability. It may be noted that as pH decreases the isometric tension values decrease. An exponential decrease of isometric tension I_t with pH has been observed. In other words, at isoelectric point of collagen, ionic forces dominate and provide thermal stability. Under conditions of isoelectric point, hydration and swelling of the matrix is less favoured and osmotic forces do not play a significant role. At pH values ≤ 5 osmotic forces act synergetically with isometric tension. Low dimensional stability of collagen under the influence of osmotic force has been observed.

Hydrothermal isometric tension (HIT) curve for native RTT in 0.02 M Tris-maleate buffer at pH 5–8 is given in Fig. 5. The shape of the isometric tension curves of the collagen fibres provide information on the crosslinking of collagen fibre [23]. Allain et al. [26] have found that the hydrothermal isometric ten-

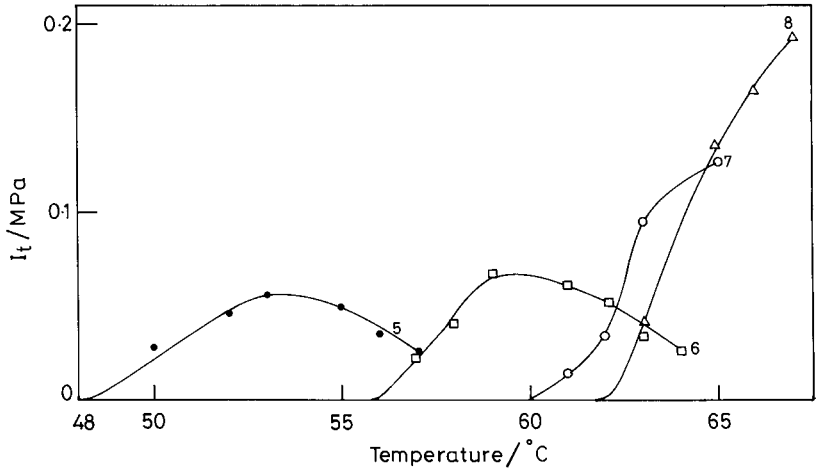

Fig. 5. Hydrothermal isometric tension curves for native RTT in 0.02 M Tris-maleate buffer at pH 4–8.

sion curves developed by rat skin exhibited different shapes which varies according to the age of the animal. HIT curves of collagen fibre in pH 5 and 6 are characteristic of collagen denaturation. The extent of relaxation after maximum tension increases due to the decreasing proportion of thermally stable cross-links and that is reflected in the HIT curves of collagen fibre in the pH range 5–6. There is no relaxation after maximum tension is observed at pH 7 and 8. This may be due to charge interaction exerting a stabilizing influence under isoelectric conditions. There is relaxation after the maximum tension at pH 5 and 6. At low pH conditions charge repulsion disrupts the stability of the collagen molecule.

4. Conclusions

The significant role of hydrogen bonding, hydrophobic and electrostatic interactions on the stability of RTT collagen fibre are well explained using DSC and HIT experiments. The thermodynamic parameters of denatured collagen are important because a significant change in the peak temperature, enthalpy changes, energy of activation and hydrothermal isometric tension for collagen to gelatin transition suggest the importance of collagen stabilisation. A linear relationship has been observed for the peak temperature and enthalpy changes associated with the phase transition in DSC experiments. In the case of HIT curves, the extent of relaxation after maximum tension increases due to the decrease in proportion of thermally stable crosslinks. This trend has been observed in 3 and 6 M urea solutions and 0.02 M Tris-maleate buffer at pH 5 and 6. In conclusion, hydrothermal isometric tension (HIT) and differential scanning calorimetry (DSC) provide better understanding of the role played by collagen triple helix denaturation on the development of isometric tension under the influence of temperature gradient.

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