Effect of pH on Dimensional Stability of Rat Tail Tendon Collagen Fiber

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ABSTRACT: The organized molecular structure of collagen is related to its dimensional stability. The dimensional stability of collagen arises from the interplay of various intermolecular forces such as covalent, hydrogen bonding, electrostatic interactions, hydrophobic interactions, London or van der Waals forces, and weak interactions. A structure–function relationship exists in collagen. Electrostatic interactions play an important role in dimensional stabilization. The dimensional stability of rat tail tendon (RTT) collagen fiber is affected by the change in the net fixed charge on the molecule as a function of pH. Thermal and mechanical properties are dependent on molecular and lattice orders. The pH dependence of thermal shrinkage, isometric tension, differential scanning calorimetry, swelling behavior, tensile strength, and percent extension and stress relaxation behavior are studied in 0.02*M* Tris-maleate buffer at pH 4–8. The observed experimental results provide compelling evidence that electrostatic interactions play an important role in the dimensional stability of RTT collagen. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 75: 1577–1584, 2000

Key words: rat tail tendon; shrinkage temperature; activation energy; electrostatic interaction; isoelectric point

INTRODUCTION

The collagen molecule is stabilized by the interplay of a vast number of intra- and intermolecular forces involving different types of bonds. The molecular stability of collagen is known to draw strength from covalent, electrovalent, hydrogen bond, van der Waals and London forces, and weak interactions.[1–3] The collagen amino acid sequence shows the importance of electrostatic interactions in specifying the molecular D stagger.[4–6] Salt bridges are formed in the protein molecule by the interactions of oppositely charged protonated amino and ionized carboxyl groups. Ionic interactions between oppositely charged centers are long-range forces and are important in the aggregation of the collagen molecule to form insoluble fibers.[7–9] The effect of environmental perturbants like pH and temperature in collagen fibrils considerably alters the overall stability.[10–17] High angle X-ray diffraction experiments are used to interpret the effects of pH and ionic strength on the structure and stability of collagen fibrils.[18] Rat tail tendon (RTT) represents a relatively pure form of type I collagen. Several chemical substances comprise tendons among which are collagen, polysaccharide, chondroitin sulfates B and C, hyaluronic acid, various proteoglycans, cellular materials, and water.[19] Collagen fibers exhibit their native structures only within a certain range of conditions. The phase transitions from the native helical-ordered (crystalline) state to a randomly coiled (amorphous) state is called denaturation. This transition may be influenced by various parameters. One of them is pH. In our present investigation the pH dependence of RTT

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Table I Thermomechanical Properties of Native RTT in 0.02*M* Tris-Maleate Buffer at Different pH

pH	T_s (°C)	I_t (MPa)	T_t (°C)
8	63 ± 1	0.25 ± 0.08	65 ± 1
7	61 ± 1	0.15 ± 0.05	64 ± 1
6	58 ± 1	0.1 ± 0.02	62 ± 1
5	51 ± 1	0.08 ± 0.02	55 ± 1
4	—	—	—

The values are the mean ± standard deviation of six samples; T_s, shrinkage temperature; I_t, maximum isometric tension; T_t, temperature at maximum tension.

collagen on thermal shrinkage and isometric tension, differential scanning calorimetric studies, tensile strength and percentage extension, swelling properties, and stress relaxation behavior are discussed.

MATERIALS AND METHODS

Sample Preparation

RTT collagen fibers in the native insoluble form were chosen in order to relate the results to physiological conditions of the protein *in vivo*. The tendons were tested soon after the removal from the tail. Eanes and Miller used the tail tendons without further treatment to study the effect of covalent crosslinking on the X-ray diffraction properties.[20] Collagen fibers were taken out of the tails of 6-month-old male albino rats (Wistar strain). The area of cross section of the fibers was determined by taking the diameter of the fiber using a filer micrometer attached to the optical microscope in the wet condition. The shrinkage temperature was checked before each experiment to make sure that no degradation or structural changes had taken place.

Solution Preparation: Hydrogen Ion Concentration

The pH of the medium was adjusted using Tris-maleate buffer. Collagen fibers were equilibrated in 0.02*M* Tris-maleate buffer at different pHs. The pH of the solution was measured using glass electrodes. The ionic strength was maintained constant throughout the experiments. Ripamonti et al. reported that the collagen fibers show osmotic swelling at low ionic strength.[18] However, in the pH range of 4–8 there were no detectable changes in the microscopic dimensions of collagen fibers, so the hydrogen ion concentrations were maintained in this pH range.

Hydrothermal Isometric Tension (HIT) Experiments

The HIT experiments were carried out in an Instron model 1112 testing machine. A liquid cell container is kept on the heater whose input supply voltage is adjusted to get a required heating rate of 3°C/min. The fibers were immersed in 0.02*M* Tris-maleate buffer at pH 4–8 in the liquid cell with one end attached to the frame and the other free end attached to the load cell. The tension generated was continuously recorded. The isometric stress were calculated on the basis of the initial diameter of the wet fibers.

Differential Scanning Calorimetric Studies (DSC)

The weighed (dry weight) samples of RTT were immersed in 0.02*M* Tris-maleate buffer at pH 4–8 for 24 h. The soaked samples were blotted uniformly and encapsulated in an aluminum hermetic scale pan. The pan containing the samples was placed in a heating block housed in a DSC cell mounted in a Perkin Elmer DSC 7 main con-

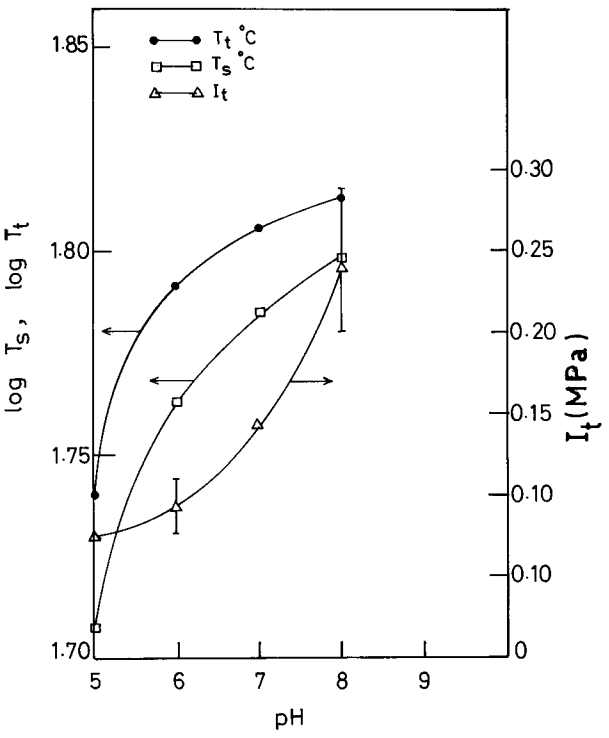

Figure 1 Plots of log T_s (°C), log T_t (°C), and I_t as a function of pH.

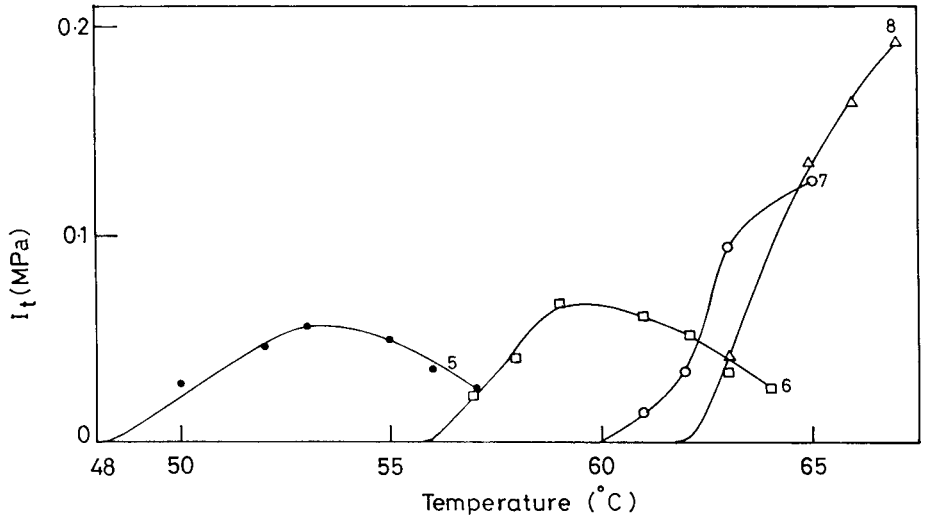

Figure 2 A hydrothermal isometric tension curve for native RTT in 0.02*M* Tris-maleate buffer at pH 4–8.

sole. The heating rate was maintained constant at 6°C/min. The peak temperature, T_p (°C), for the collagen to gelatin transition and the change in enthalpy for this transition were noted.

Swelling Properties

The dry weight of the native RTT fibers was recorded using a Mettler balance of 10-μm accuracy. The same fibers were swollen in respective solutions for different times and the weight was noted. The ratio of the swollen weight to the dry weight gave the swelling ratio.

Tensile Strength and Percent Extension

The tensile strength measurements were performed at an extension rate of 0.5 cm/min in the

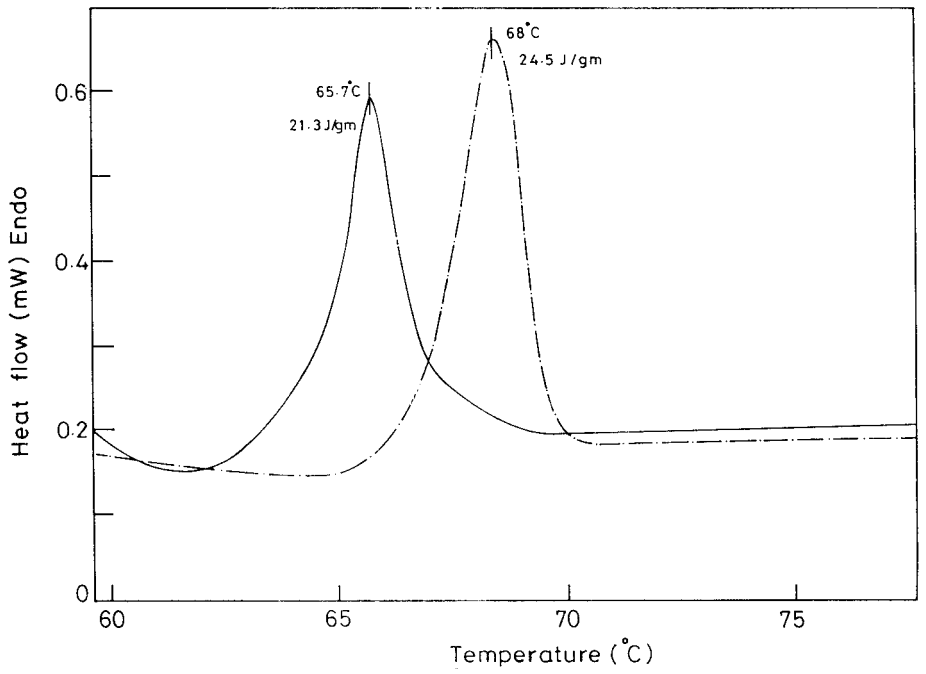

Figure 3 A thermogram of native RTT fibers swollen in 0.02*M* Tris-maleate buffer at pH 6 and 8.

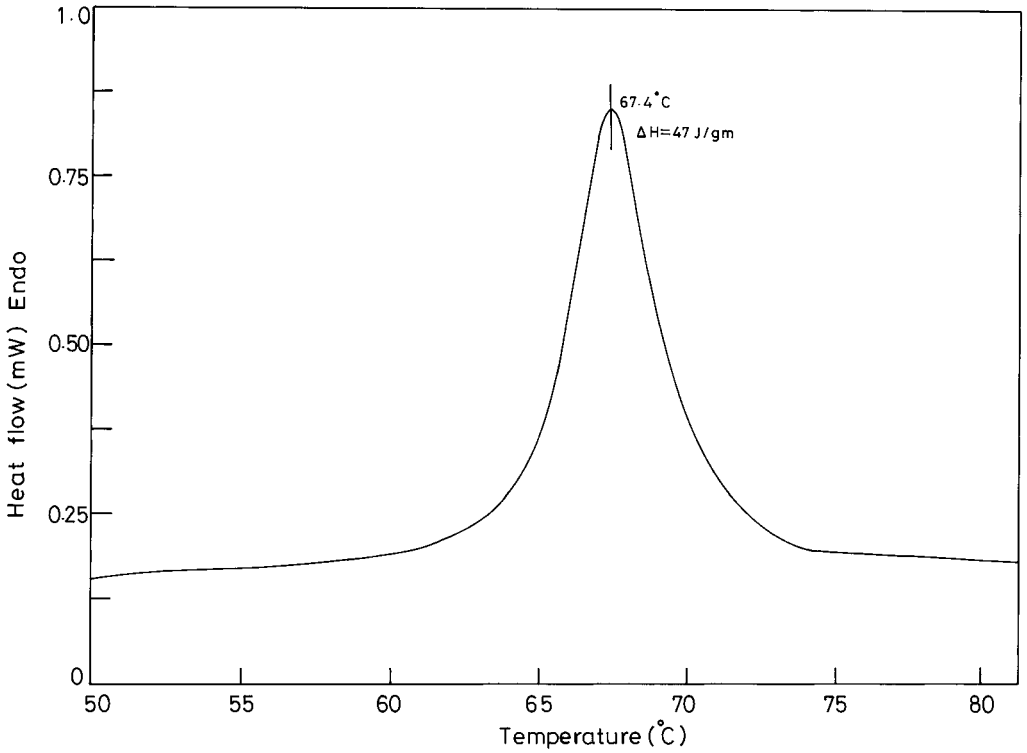

Figure 4 A thermogram of native RTT swollen in a water medium.

Instron model 1112 testing machine using the liquid cell container. The tensile strength and percentage extension were calculated for native RTT in 0.02*M* Tris-maleate buffer at pH 4–8 at room temperature.

Stress Relaxation Experiments

The stress relaxation behavior of native RTT in 0.02*M* Tris-maleate buffer at pH 4–8 at 298, 313, 318, 323, and 328 K was studied using an Instron model 1112 testing machine. The fiber was strained up to 20% strain at the rate of 0.5 cm/min; thereafter the strain was maintained constant and the stress decay was monitored for 3 h with a fixed temperature. From the load time graph the plot of σ/σ_0 (where σ is the stress at time t and σ_0 is the initial stress) against time was plotted. The rate of relaxation was computed using eq. (1),

$$\sigma/\sigma_0 = A_0 e^{-k_1 t} + B_0 e^{-k_2 t} \quad (1)$$

(where A_0 and B_0 are the preexponential factors and k_1 and k_2 are the fast and slow relaxation rate), and a nonlinear least squares fit using an HP workstation and standard packages.[21] This is explained elaborately elsewhere.[22,23] It is highly probable that the system may be much more complex than a single relaxation process and perhaps could be a multiphasic system. In order to verify this, the stress relaxation behavior was treated as biphasic in nature. An Arrhenius plot of the temperature dependence of the rate constant is nonlinear. The rate constant data are better treated using eq. (2),[4]

$$k = AT^m e^{-E_0/RT} \quad (2)$$

where A, E_0, and T are the temperature independent constants and E_0 is the activation energy at absolute zero. The values of ln A, m, and E_0 were computed using a nonlinear least squares fit and an HP workstation with standard packages.[21] The activation energy at various temperatures can be calculated using eq. (3),

$$E_a = E_0 + mRT \quad (3)$$

where E_a is the activation energy at a given temperature.

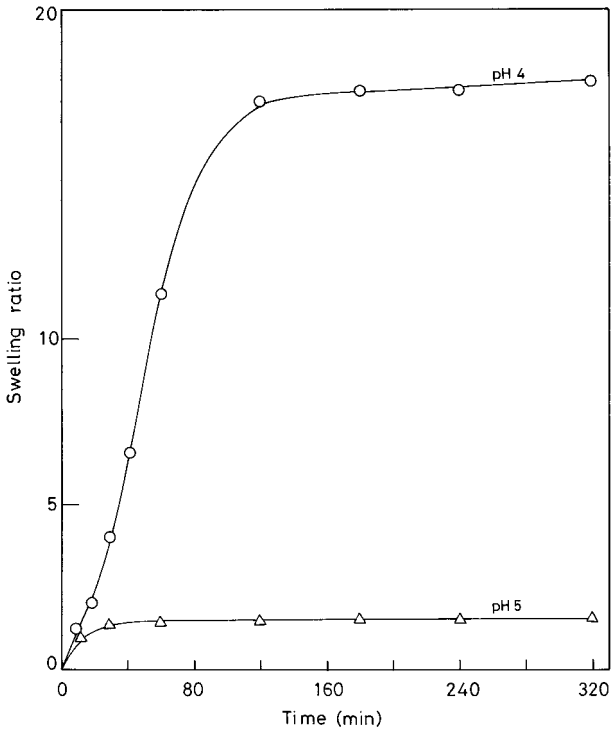

Figure 5 The plot of the swelling ratio as a function of time for native RTT in 0.02*M* Tris-maleate buffer at pH 4 and 5.

RESULTS AND DISCUSSION

Shrinkage Temperature and Isometric Tension

The shrinkage temperature and isometric tension for native RTT were measured in the pH range of 4–8 in 0.02*M* Tris-maleate buffer and the data are given in Table I. The measurement of shrinkage temperatures at pH 4 is not possible due to dimensional destability. It may be observed that below pH 6 there is a significant decrease in shrinkage temperature. 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 the ionizable 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 the 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, °C) with pH is evident from Figure 1. In other words, at the isoelectric point of collagen the ionic forces dominate and provide thermal stability. Under the conditions of the isoelectric point the hydration and swelling of the matrix is less favorable and osmotic forces do not play a significant role. At pH values ≤ 5 the osmotic forces synergize with isometric tension. Low dimensional stability of collagen under the influence of osmotic force was observed.

HIT Curves

The HIT curve for native RTT in 0.02*M* Tris-maleate buffer at pH 5–8 is given in Figure 2. The shape of the isometric tension curves of the collagen fibers provide information on the crosslinking of the collagen fiber.[25–27] Allain et al. found that the hydrothermal isometric tension curves developed by rat skin exhibited different shapes according to the age of the animal.[28] HIT curves of collagen fiber at 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 crosslinks and that is reflected in the HIT curves of collagen fiber at pH 5–6. There is no relaxation after the maximum tension is observed at pH 7 and 8. This may be due to the fact that charge interactions exert a stabilizing influence under isoelectric conditions. Relaxation after the maximum tension was observed at pH 5 and 6. Charge repulsion disrupts the stability of the RTT collagen fiber at lower pH values.

DSC

Thermograms of native RTT swollen at pH 8 and 6 are shown in Figure 3. Our DSC studies showed that the peak temperature for the shrinkage process for native RTT at pH 8 is 68 and at pH 6 is 65.7°C. The DSC studies of native RTT were

Table II Tensile Properties of Native RTT in 0.02*M* Tris-Maleate Buffer at Different pHs

pH	Tensile Strength (MPa)	Extension (%)
8	40 ± 8	48 ± 10
7	39 ± 3	49 ± 8
6	35 ± 8	45 ± 4
5	27 ± 5	38 ± 5
4	18 ± 4	37 ± 6

The values are the mean ± standard deviation of 15 samples.

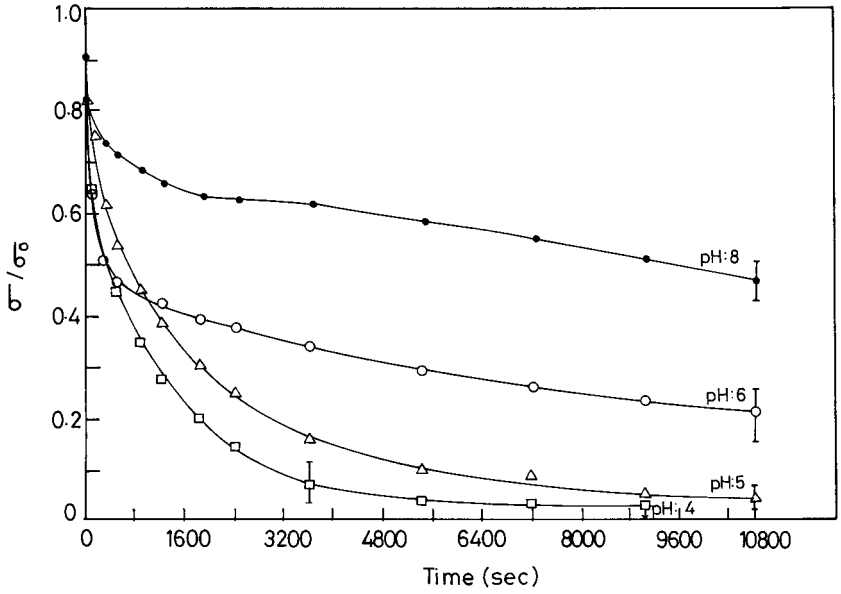

Figure 6 The stress relaxation behavior of native RTT in 0.02*M* Tris-maleate buffer at pH 4–8 at 298 K.

made in a water medium in the absence of any electrolyte. This is given in Figure 4. The DSC peak with the temperature at 67.4°C was observed in the absence of any buffer with the pH being in the range of 7–8. The enthalpy changes associated with the phase change for native RTT in the water medium was 47 (J/gm). These data are in good agreement with previously reported values in a water medium.[29–33] 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. We estimated the values are 24.5 and 21.3 (J/gm), respectively. At or near the isoelectric point of collagen the collagen is expected to be less extensively solvated. Therefore, the work needed to overcome the protein–water interaction is lowest at the isoelectric point. The observed DSC data at different pH values were explained in terms of phase changes associated with solute–solvent interactions and hydration phenomena.

Swelling Behavior

The swelling ratio of native RTT was investigated as a function of time in the pH range of 4–5 in Tris-maleate buffer at 0.02*M*, and the data are given in Figure 5. An order of magnitude difference is observed in the swelling ratio at pH 4 and 5. It is known that at pH values closer to the isoelectric point of collagen the swelling ratio of the tendon is the lowest. Because the acid–base

Table III Fast and Slow Rates (k_1, k_2) of Relaxation for Native RTT in 0.02*M* Tris-Maleate Buffer at pH 6–8 at Various Temperatures

Temp. (K)	pH 8		pH 7		pH 6	
	$k_1 \times 10^3$ (s^{-1})	$k_2 \times 10^5$ (s^{-1})	$k_1 \times 10^3$ (s^{-1})	$k_2 \times 10^5$ (s^{-1})	$k_1 \times 10^3$ (s^{-1})	$k_2 \times 10^5$ (s^{-1})
298	11 ± 0.2	10 ± 5	11 ± 2.5	12 ± 0.6	14 ± 1.7	14 ± 1.5
313	13 ± 7.0	15 ± 1.5	15 ± 1.5	21 ± 3.0	20 ± 1.4	34 ± 2.0
318	14 ± 5.4	25 ± 2.0	18 ± 3.0	36 ± 3.0	32 ± 2.0	57 ± 5.0
323	17 ± 0.8	29 ± 6.0	19 ± 4.0	48 ± 8.0	45 ± 8.0	89 ± 8.0
328	48 ± 1.1	142 ± 5.5	110 ± 9.0	184 ± 10.0	—	—

The values are the mean ± standard deviation of six determinations.

Table IV Fast and Slow Rates (k_1, k_2) of Relaxation for Native RTT in 0.02*M* Tris-Maleate Buffer at pH 4–5 at Various Temperatures

Temp. (K)	pH 5		pH 4	
	$k_1 \times 10^3$ (s^{-1})	$k_2 \times 10^5$ (s^{-1})	$k_1 \times 10^3$ (s^{-1})	$k_1 \times 10^5$ (s^{-1})
298	16 ± 9.0	28 ± 10	36 ± 1.5	46 ± 10
313	42 ± 10	98 ± 5	76 ± 10	184 ± 20
318	53 ± 9.0	112 ± 15	—	—
323	—	—	—	—
328	—	—	—	—

The values are the mean ± standard deviation of six determinations.

equilibrium is rapid, the transport phenomena may limit the rate of swelling of fibers such as RTT.

Tensile Strength and Percent Extension

The tensile strength and percentage extension of native RTT fibers in 0.02*M* Tris-maleate buffer at different pH conditions at 298 K are given in Table II. The tensile strength and percent extension of RTT fibers at pH 4 and 5 unambiguously show a decrease in tensile strength resulting from acid swelling and interfiber cohesion. The percentage extension is also marginally reduced. Davison discussed the tensile strength data with intermolecular crosslinks.[34] It is tempting to attribute the loss of tensile strength under acidic conditions to the cleavage of acid labile intermolecular crosslinks. In principle, under low pH conditions, which are on the acid side of the isoelectric point of collagen, protonation of —COOH sites in the protein is likely to reduce intermolecular ion–pair interactions between the ionized carboxyl and protonated amino groups. In other words, intermolecular interaction of collagen due to an electrovalent salt bridge is likely to decrease. In addition protein–water rather than protein–protein interactions are likely to favor lowering of the tensile strength.

Stress Relaxation Behavior

The stress relaxation profile of native RTT in 0.02*M* Tris-maleate buffer at pH 4–8 at room temperature is given in Figure 6. The fiber relaxes faster as the pH decreases. This may be due to breaking of crosslinks that tend to hold the fiber from relaxing faster in an acidic medium. The decrease in tension of the fiber is an indication of the dissipation of the mechanical energy during the sliding process.[35] The tendon under steady strain showed a decrease in load measured in the stress relaxation process at pH 6, implying the faster rate of relaxation that sometimes proceeded to the failure of the specimen. This observation suggested that low pH and strain can lead to intermolecular bond and fibril rupture. The decrease in tendon strength at acid pH suggested that cleavage of acid labile crosslinks was responsible for weakening the tissue.[14]

The rates of relaxation computed for native RTT in 0.02*M* Tris-maleate buffer at pH 4–8 at various temperatures are given in Tables III and IV. It is evident from the data that the values of k_2 are more sensitive to the pH of the medium than k_1. There is apparently a linear correlation of the values of k_2 with the pH of the medium. It is known that the p*K* values of protein amino and carboxyl groups lie in the range of 8.5–12.5 and 2.5–4, respectively.[36] This may be due the ionization process of the collagen molecule.

The activation energies for the k_1 and k_2 path at pH 6–8 are listed in Table V. The decrease in pH appears to promote the decrease in activation energy for both k_1 and k_2. The isoelectric point of collagen is known to be 7.5. Therefore, at pH conditions deviating from the isoelectric point acid or alkaline swelling is likely. The swelling phenomenon is influenced by protein–water interactions. The lowering of activation energies at pH 6 for both k_1 and k_2 processes from those in the range of pH 7–8 may be due to the influence of hydrogen bonding and electrostatic interactions.

To summarize, charged pairs stabilize the molecular structure by forming interchain and intrachain dipoles, and it is these charged pairs that are disrupted by lowering the pH and raising the temperature. The pH dependent decrease in dimensional stability is likely to occur due to break-

Table V Activation Energy Values at Absolute Zero (E_0) and E'_0 for Native RTT in 0.02*M* Tris-Maleate Buffer at pH 6–8

	Activation Energy (kcal/mol) Calculated Using	
pH	$k_1 = 10^{-3}$ s^{-1} for E_0	$k_2 = 10^{-5}$ s^{-1} for E'_0
8	26 ± 3.6	31 ± 4.5
7	26.4 ± 5.0	30.6 ± 3.5
6	23.2 ± 1.0	26.5 ± 1.4

The values are the mean ± standard deviation of six determinations.

ing of salt bridges involved in maintaining the native conformation. The experimental approach at the macromolecular level will ultimately yield the fundamental basis of the mechanical properties of the ubiquitous protein collagen.

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