Effect of Hydrogen-Bond-Breaking Reagent (Urea) on the Dimensional Stability of Rat Tail Tendon (RTT) Collagen Fiber

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ABSTRACT: Influence of hydrogen-bond-breaking reagents such as urea on shrinkage temperature, isometric tension, swelling behavior, tensile strength, and percentage extension of native rat tail tendon (RTT) were examined. The swelling behavior was observed with polarizing optical microscopy and scanning electron microscopy. The results show that the lyotropic swelling increased the width of the fiber and was associated with the action of urea on the collagen fiber. Hydration properties led to significant variations in the swelling phenomenon. Lyotropic swelling produced opaque, limp, and flaccid fibers that did not change appreciably in length. The melting behavior and the swollen fascicles were clearly seen in scanning electron micrographs of 3 and 6*M* urea-treated RTT. The reduction in the dimensional stability of native RTT collagen fiber on treatment with urea demonstrated the role of secondary structure in the dimensional stabilization of collagen. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 84: 975–982, 2002; DOI 10.1002/app.10262

Key words: rat tail tendon; urea; hydrogen bond; polarizing optical microscopy; scanning electron microscopy; swelling

INTRODUCTION

It is known that the various intermolecular interactions are influenced by ionic strength, pH, solvent dielectric, hydrogen-bond-breaking reagents, and covalent crosslinks introduced by the chemical species in collagen.[1–4] The major forces stabilizing the collagen triple helix are hydrogen bonds.[5–8] The unfolding of proteins is most commonly brought about by either heat or the addition of urea or guanidinium chloride. Thermal folding can be monitored by differential scanning calorimetry. The influence of hydrogen bonding and hydrophobic and electrovalent salt interactions on the enthalpy of activation and energy of activation were already reported.[9] It is known that urea is capable of breaking the hydrogen bonds in a protein molecule.[10–13] The effects of urea and acids on connective tissue were first described many years ago. The dimensional stability of collagenous tissues has been examined under various environmental conditions to elucidate the role of intermolecular forces in stabilizing molecular forces that lead to thermomechanical stability. The thermally induced structural transitions in the fibrous collagenous network leads to shrinkage processes. The thermomechanical stability of collagenous tissues and fibers has been extensively investigated.[14–18] The organization of collagen fibers has been studied with optical microscopy and transmission and scanning electron microscopy (SEM).[19–25] The reduction in the dimensional stability of native rat tail tendon (RTT) collagen fiber by urea is better explained in terms of the breakdown of intermolecular hydrogen bonds. A systematic study was undertaken to examine the effect of urea on the dimensional stability of RTT collagen fiber. In

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Table I Thermomechanical Properties of Native RTT in Water and Various Concentrations of Urea

Native RTT in	$T_s \pm 1$ (°C)	I_t (MPa)[a]	$T_t \pm 1$ (°C)
Water	64	0.2 ± 0.1	67
1*M* Urea	57	0.35 ± 0.08	67
3*M* Urea	51	0.6 ± 0.2	57
6*M* Urea	41	0.95 ± 0.2	51

[a] $M \pm$ SD of six determinations.

this investigation, the effect of urea on the shrinkage temperature (T_s), isometric tensile (I_t) tensile strength and percent extension, swelling behavior, polarizing optical microscopy, and SEM of RTT collagen fiber are presented.

EXPERIMENTAL

Sample Preparation

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

Solution Preparation

Urea used in the experiments was of analar grade. Urea solutions (1, 3, and 6*M*) were prepared on a weight basis. The collagen fibers were equilibrated for 30 min in the respective solution before each experiment.

Hydrothermal Isometric Tension (HIT) Experiments

HIT experiments were carried out an Instron testing machine (model 1112). A liquid cell container was kept on a heater, whose input supply voltage was adjusted to get a required heating rate of 3°C/min. The fibers were immersed in 1, 3, and 6*M* urea solutions in the liquid cell with one end attached to the frame and the other end at-

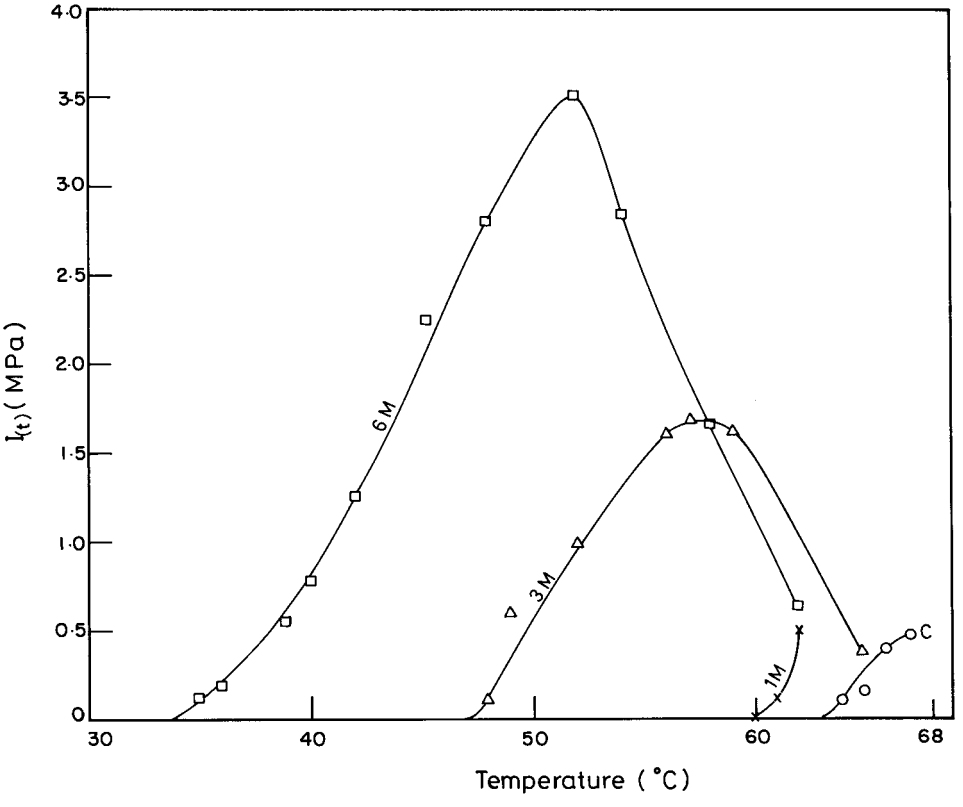

Figure 1 HIT curve for native RTT in water and 1, 3, and 6*M* urea solutions.

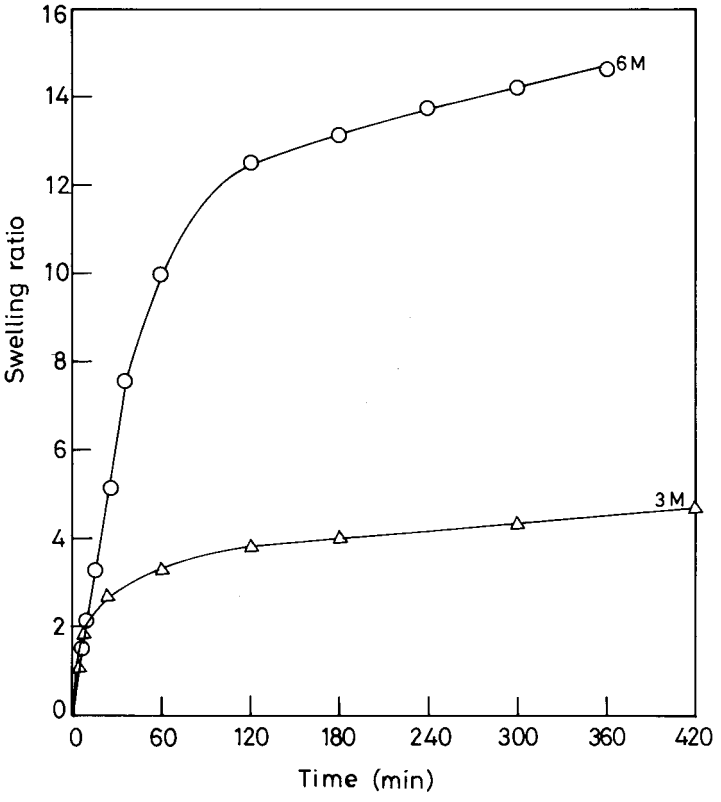

Figure 2 Swelling curve for native RTT in 3 and 6*M* urea solutions at 298K.

tached to the load cell. The T_s and tension were continuously recorded. The HIT behavior of native RTT in various environmental conditions was already reported.[9]

As the fiber was held at constant length during the experiment, the temperature at which the tension began to increase was recorded as the T_s. The temperature at which the tension (which increased progressively with shrinkage) reached the maximum was called the T_t. The corresponding tension was defined as the I_t.

Swelling Ratio

The dry weight of the native RTT fibers was recorded with a Mettler balance of 10-μg accuracy. The same fiber was swollen in respective solutions for 10, 30, 45, 60, 120, 180, 240, 300 min, and so on, and the weight was noted. The ratio of the swollen weight to the dry weight gave the swelling ratio. Plots were made for the swelling ratio against time for the fibers swollen in 3 and 6*M* urea solutions.

Polarizing Optical Microscopy

The swelling behavior of native RTT was studied with a Jenaval 250-C-F microscope with transmitted light mode with a polarizer and analyzer. The fibers were immersed in a liquid cell, which was kept under the eye piece. The swelling behaviors of the fibers were photographed without changes in orientation at different time intervals of 0, 10, 30, 60, and 180 min.

SEM

RTT collagen fibers were allowed to swell overnight in 1, 3, and 6*M* urea solutions. Then, the RTTs were fixed with Karnovsky fixative and dehydrated through graded series of ethanol and dried. The RTTs were mounted on a stub with a colloidal suspension of silver and coated to a thickness of about 20 nm with a coating material like gold. An Edwards E-306 coating unit was used for sputtering gold onto the sample at a current of 30 mA at 15 kV for a period of 4 min. The samples were then inserted into the specimen chamber of a Cambridge Stereoscan S-150.

Tensile Strength and Extension

Tensile strength measurements were made with a liquid cell attachment designed for the Instron testing machine (model 1112) and a 0–500g load

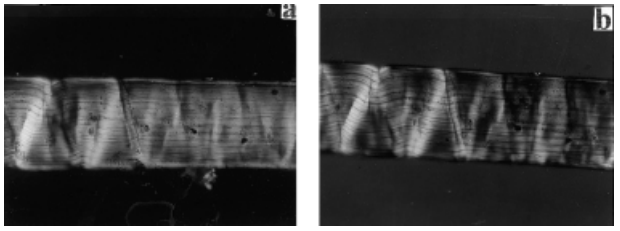

Figure 3 Polarizing optical micrographs of native RTT swollen in 1*M* urea solution at (a) 0 and (b) 24 h. Optical magnification 25×.

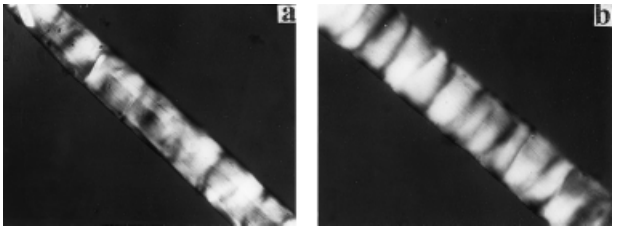

Figure 4 Polarizing optical micrographs of native RTT swollen in 3*M* urea solution at (a) 0 and (b) 24 h. Optical magnification 25×.

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. The wet diameter of the RTT fibers were determined with an optical microscope with filar micrometer. The tensile strength and percentage extension of native RTT in 1, 3, and 6*M* urea solutions at 298K were studied. The stress–strain characteristics of native RTT in water and 1, 3, and 6*M* urea solutions at 298K were plotted.

Stress Relaxation Experiments

The stress relaxation behavior of native RTT in water and 1, 3, and 6*M* urea at 298, 313, 318, 323, and 328K was studied with an Instron testing machine (model 1112). The fiber (gauge length = 1 cm) was strained up to 20% strain at the rate of 0.5 cm/min; thereafter, the strain was maintained constantly, and the stress decay was monitored for 3 h with the temperature fixed. 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 with 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 with Nonlinear least-squares fit with a Hewlett-Packard work station and standard packages.[26] This was explained elaborately elsewhere.[27,28]

RESULTS AND DISCUSSION

HIT

The T_s and I_t of native RTT were examined in aqueous media containing various concentrations of urea in the range of 0–6*M,* and the data are presented in Table I. There was a significant decrease in the T_s values with increasing concentration of urea, and the I_t values correlated inversely. The breakdown of intermolecular crosslinks was expected to lower the T_s and reduce interfiber cohesion. The observed trend in the T_s data can be reconciled in terms of the breakdown of intermolecular hydrogen bonds. The HIT curves for native RTT in water and 1, 3, and 6*M* urea solutions are given in Figure 1. The increase in isometric force with an increase in urea concentration may have been due to the penetration of urea into the crystalline region, and the degraded crystalline region would have produced an increase in force due to the increase in entropy of the randomly coiled collagen.[29]

Swelling Ratio

The swelling ratio of native RTT fibers was observed at 3 and 6*M* urea concentrations and is given in Figure 2. The first-order behavior for swelling was observed. The rate of swelling in-

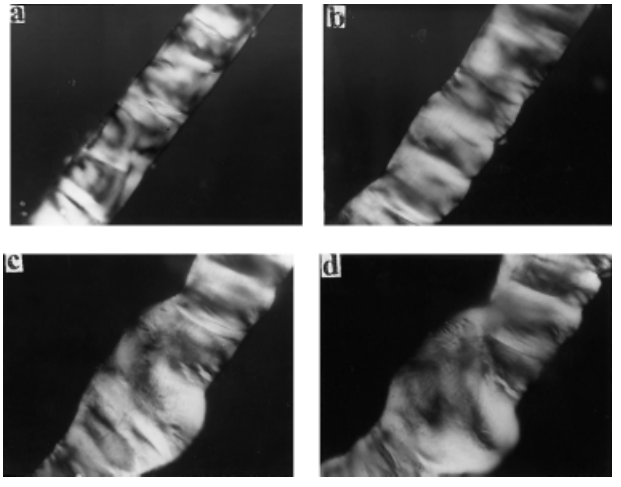

Figure 5 Polarizing optical micrographs of native RTT swollen in 6*M* urea solution at (a) 0, (b) 30, (c) 120, and (d) 180 min. Optical magnification 25×.

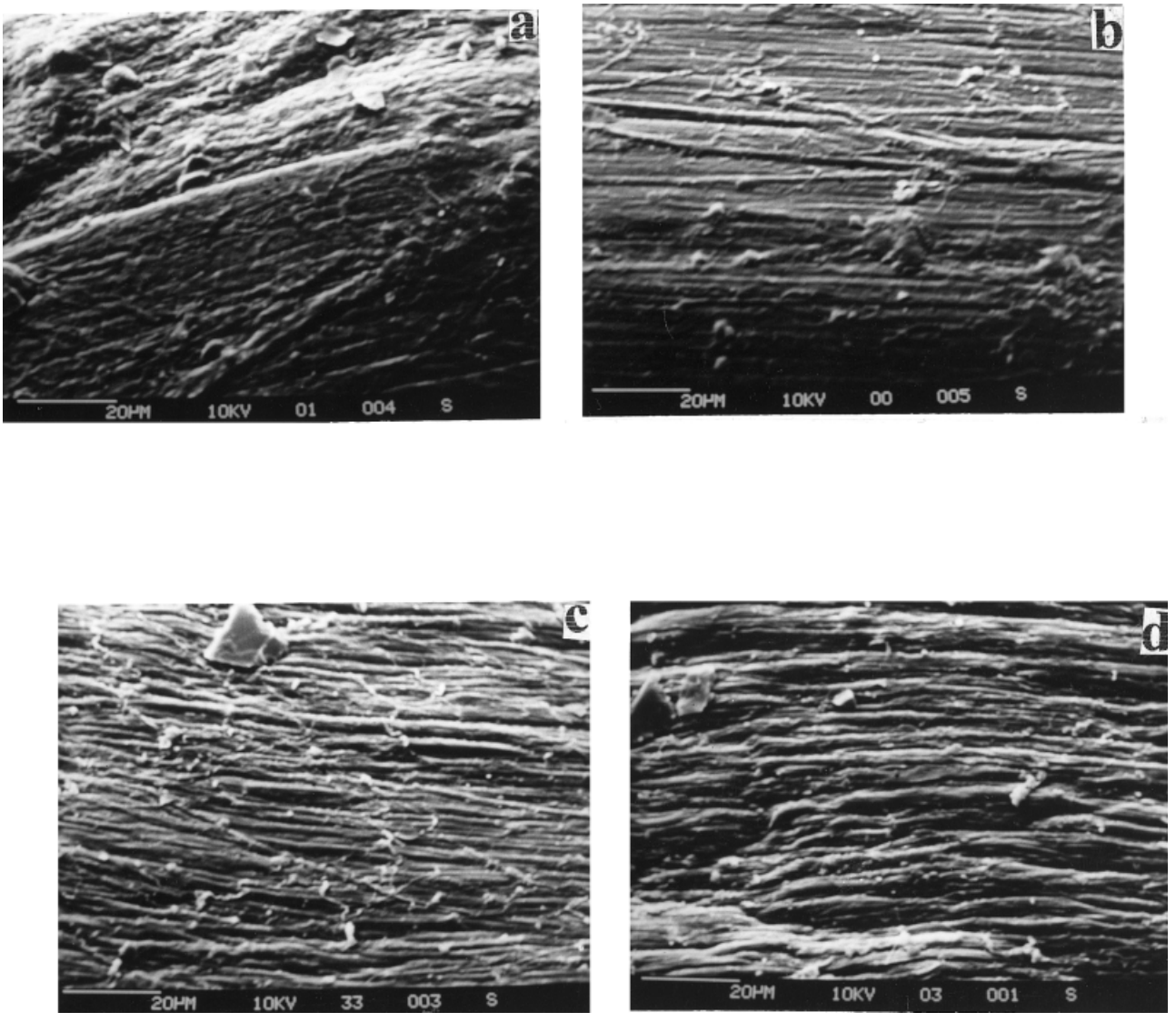

Figure 6 Scanning electron micrographs of native RTT swollen in (a) water and (b) 1, (c) 3, and (d) 6*M* urea solutions.

creased with the concentration of urea. A more than threefold increase in the extent of swelling was observed when the concentration of urea increased from 3 to 6*M*. Lyotropic swelling is believed to be associated with increase in width of the fiber[30] and is associated with the action of urea on collagen fiber. Hydration properties led to significant variations in swelling phenomenon. Lyotropic swelling produced opaque, limp, and flaccid fibers that did not change appreciably in length.

Polarizing Optical Microscopy

Collagen fibers have a wavy configuration at the level of the light microscope that is important for the mechanical function of the tissue. When the tendon is viewed in the optical microscope under crossed polarizers, the wavy fibers produce characteristic banding patterns of bright and dark extinction bands.[31] Polarizing optical micrographs of native RTT swollen in 1 and 3*M* urea at 0 and 24 h are given in Figures 3(a,b) and 4(a,b), respectively. There was no significant change in the micrograph of native RTT swollen in 1 and 3*M* urea solutions when compared to the swelling behavior of native RTT in 6*M* urea solutions at 0, 30, 120, and 180 min, as shown in Figure 5(a–d). Morphological investigations showed that as the concentration of urea and the time of contact increased, major dimensional changes were brought

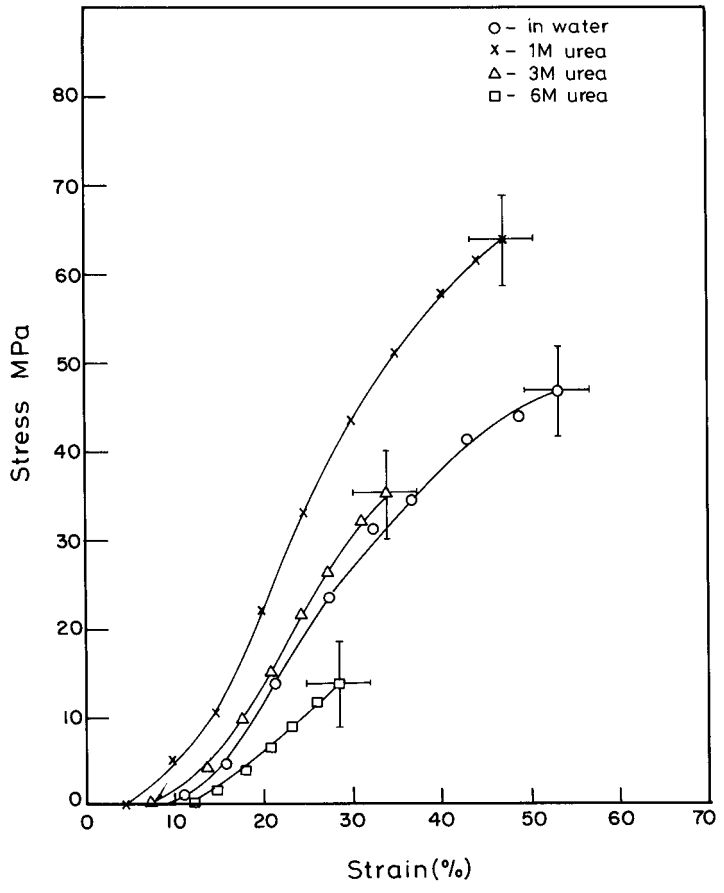

Figure 7 Stress–strain characteristics of native RTT in water and 1, 3, and 6*M* urea solutions at 298K.

about. The diameter of the RTT fiber increased on urea treatment. The collagen fiber swelled in the radial direction and not much in the axial direction. The collagen tertiary structure was probably held together in the longitudinal direction by higher strength more than in the radial direction. The breakdown of hydrogen bonds through molecular interactions could well have caused deaggregation in the macromolecular assembly of collagen. A loss of long-range order, leading to disorganization, was observed in the polarizing optical micrographs of native RTT fibers treated with 6*M* urea. The generation of defect was evident on urea treatment. Such defect seemed irreversible when the exposure was prolonged and concentrations of urea were higher. The lyotropic function resulting from hydrogen-bond breakage and alterations in the secondary structures seemed to reduce long-range order in collagen.

SEM

Kastelic et al.[32] suggested the existence of a structural unit about 200 μm in diameter, which was termed the *fascicle.* They proposed that the fascicle is a roughly cylindrically symmetrical array of crimped fibers, with the plane of the crimp more or less parallel to the surface of the fascicle. The scanning electron micrographs of the native RTT fibers swollen in water, and 1, 3, and 6*M* urea solutions are given in Figure 6(a–d). The melting behavior and the swollen fascicles are clearly seen in Figure 6(c,d). The diameter of the fascicles increased in 3 and 6*M* urea solutions. Nicholls et al.[33] examined RTT with SEM after the removal of the endotendinium by the use of swelling agents and confirmed the presence of planar crimping along the fiber axis when compared with the control. Although the swelling nature of the fibers were clearly seen with SEM, the optical micrographs showed the true swelling nature of the fibers. This may have been due to (1) the swollen fibers being fixed with the fixer (the fixative can also enhance the swelling) or (2) the drying procedures of the fibers giving some artifacts. The presence of numerous defects in the fascicle structure has important implications on the stress–strain relationship of the tendon.

Table II Tensile Properties of Native RTT in Water and 1, 3, and 6*M* Urea Solutions

Native RTT in	Tensile Strength (MPa)[a]	% Extension[a]
Water	56 ± 5	56 ± 5
1*M* Urea	65 ± 10	48 ± 2
3*M* Urea	39 ± 2	35 ± 2
6*M* Urea	14 ± 2	29 ± 3

[a] *M* ± *SD* of 20 determinations.

Tensile Strength and Extension

The effects of urea on the mechanical stability of collagenous matrices were examined by earlier workers.[34,35] The tensile properties of native RTT in water and 1, 3, and 6*M* urea solution at room temperature are presented in Figure 7. When the urea concentration exceeded 1*M,* a significant decrease in the tensile strength was observed, as shown in Table II. It is known that urea is a structure-breaker at higher concentrations (> 2*M*). Because collagen fibers derives significant structural stability through intermolecular hydrogen bonding, a chemical reagent such as urea, which is capable of hydrogen-bond breaking, could well lower the matrix stability. An increase in tensile strength at lower concentrations of urea may seem to suggest increased order when the urea concentration is less than 1*M*. It may be due to a urea-mediated hydrogen-bond network at low concentrations of urea, concomitant with a certain degree of desolvation.

Stress Relaxation Behavior

Because the secondary structure of collagen is influenced by both intermolecular and intramolecular hydrogen bonds, the stress relaxation behavior of native RTT in aqueous media containing various concentrations of urea was studied, and the curve is presented in Figure 8. The rates of relaxation (both k_1 and k_2) increased as the concentration of urea increased, as shown in Table III. There was a slight decrease in the rate constant values in 1*M* urea solution. This may have been due to the amide moiety facilitating further ordering of the collagen structure. The increase in the rate constant values as the concentration of urea increased can be explained in terms of a breakdown of intermolecular hydrogen bonds. The stress relaxation decay corresponding to k_1 was more strongly influenced by urea concentration.

CONCLUSIONS

It can be concluded that the fiber lost its integrity when it was swollen in denaturing solution. At a

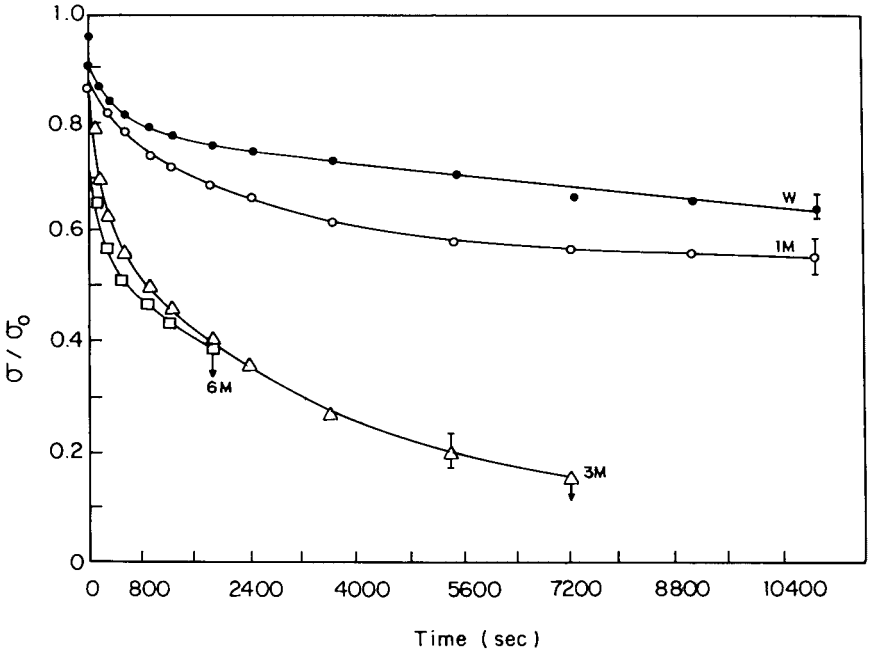

Figure 8 Stress relaxation behavior of native RTT in water and 1, 3, and 6*M* urea solutions.

Table III Rate of Relaxation (Both k_1 and k_2, Fast and Slow) for Native RTT in 1, 3, and 6*M* Urea Solution at Different Temperatures

	1*M* Urea[a]		3*M* Urea[a]		6*M* Urea[a]	
Temperature (K)	$k_1 \times 10^3$ (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	2.4 ± 0.6	2.5 ± 1.0	9.7 ± 0.9	9.0 ± 1.2	18 ± 8.0	39 ± 5.0
313	8.7 ± 1.0	19 ± 8.0	31 ± 3	62 ± 4.0	96 ± 10	159 ± 10
318	26 ± 6.0	22 ± 5.0	39 ± 5.4	118 ± 7	—	—
323	28 ± 5.0	73 ± 10	59 ± 3.0	183 ± 8.0	—	—
328	42 ± 8.0	132 ± 10	—	—	—	—

[a] $M \pm SD$ of five determinations.

low denaturing concentration, the fibers swelled with a rupture of the some of the more accessible interhelical bonds, whereas the less accessible interhelical bonds broke at higher reagent concentrations. Secondary and quaternary structures of collagen implicated a hydrogen-bond network involving (1) intermolecular and intramolecular chains of polypeptides, (2) intermolecular and intramolecular interactions in collagen, and (3) solvent–water interactions. It is known that water plays a significant part in the matrix stability of collagenous structures. At lower concentrations, urea may favor protein–protein interactions.

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