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THE INTERACTION OF CONNECTIVE TISSUE WITH AQUEOUS UREA

II. RATE ANALYSIS OF INFLUENCE OF UREA CONCENTRATION, TEMPERATURE AND pH*

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SUMMARY

It is proposed that dilute aq. urea interacts with collagen waters of hydration resulting in dehydration of the polypeptide chains. Isometric force is elevated transiently, and the rate-limiting step is believed to be diffusion of urea into the tendon. On this basis, a relationship from cylindrical-diffusion theory was applied to force–time data, and linearity between initial F^2 and time was accepted as confirming the hypothesis.

Conc. urea caused the force-velocity ($\Delta F/\Delta t$) of degrading tendons to follow an expression involving the initial force-velocity ($(\Delta F/\Delta t)_c$), the terminal force-velocity ($(\Delta F/\Delta t)_r$), the rate constant (K_{cr}), and time (t):

$$\log.\left\{\frac{\Delta F}{\Delta t}-\left(\frac{\Delta F}{\Delta t}\right)_r\right\}=\log.\left\{\left(\frac{\Delta F}{\Delta t}\right)_c-\left(\frac{\Delta F}{\Delta t}\right)_r\right\}-K_{cr}t$$

Lowering the solution temperature to 12° inhibited relaxation, and the F–t curves obeyed a rate law which can be deduced from the above equation by setting $(\Delta F/\Delta t)_r$ equal to zero. Reheating to 40° induced relaxation which followed a new equation, $1/F(\Delta F/\Delta t) = A + Bt$; this could not be deduced from the above rate law. Solution pH had a pronounced influence on relaxation rate. At pH 5.7 the relaxation rate was rapid, but at pH 8.5 it was retarded. Rate of relaxation changed proportionally in between these limits of pH.

This rate analysis shows the empirical laws followed by the F–t data of collagen-enriched tissues undergoing chemical degradation. Since conc. aq. urea primarily causes or allows hydrogen-bond rupture to occur, these expressions provide a way to follow this type of bond-scission in collagen.

INTRODUCTION

Prolonged maintenance and high magnitude of force attending the isometric melting of aging tendons requires the presence of intermolecular bonding. It has been sug-

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gested[1,2] and re-emphasized[3] that crosslinks indeed are a vital part of the macromolecular structure of collagen. Furthermore, pretreatment of young tendons with formaldehyde to introduce crosslinks[4,5] changes them in a way that closely resembles their measured changes with aging[6]. Since it is a likely possibility that chemical modification of aging connective tissue does occur *in vivo*[7], development of ways to detect effects of crosslinking is a necessary undertaking.

Elastic moduli of polymers and shape of their stress–strain curves depend upon the degree of crystallinity, the extent of amorphous structure, and the presence of crosslinks. Collagen in tendons has both amorphous and crystalline regions of structure as well as crosslinks, but there is no adequate theory of stress–strain behavior for collagen-enriched tissue[8]. Consequently, the elucidation of how aging introduces crosslinks into connective tissue cannot be determined by this procedure.

It is possible, however, to remove the crystalline order of collagen simply by heating a hydrated sample to a certain critical temperature[9]; when lyotropic agents are present, this temperature is reduced considerably. The stress–strain data of melted collagen then shows that it is rubbery[10,11], because it has low energy and high entropy of stretching. If one could now consider the elasticity of the completely amorphous collagen of connective tissue, it should be possible to determine the crosslinking density from application of theory. However, the extensive flow of connective tissue which occurs under applied load (especially for young tendons) precludes this possibility.

To circumvent these difficulties, it is proposed here that attention be given to velocity of isometric force which is generated and diminished during the chemical attack on connective tissue by a lyotropic agent. This paper deals with the results of such a rate analysis that utilizes conc. aq. urea (pH 7.0 and 40°) as a lyotropic agent.

METHODS

Rat-tail tendons from 12–14-month-old rats (Sprague–Dawley) were used again in these experiments, and their preparation and handling were the same as described previously; see Part I. Isometric force was measured, and it was used to detect the effect of interacting tendons with dilute and conc. aq. urea.

The cell assembly of the Instron testor was modified slightly, but preliminary conditioning of samples was executed the same way as before. All buffers were checked for pH with a Beckman Model-G pH meter, and stated pH values were those of the solutions prior to reacting with tendons. Since the ratio of conc. solution volume: tendon mass was about 100 ml:5 mg, there was sufficient buffering capacity to maintain constant pH even though molar concentration of buffer salt was low (0.02 M).

To conduct rate studies with dilute (0–2 M) urea solutions, it was found advantageous to reduce the size of the fluid compartment surrounding the tendon. The cell was easily modified by attaching a 1.00-ml capacity tuberculin syringe and a 6-in No. 22 gauge needle. A tendon was folded into the shape of a U, the bend was inserted through the narrow tip-end of the syringe, and the protruding free ends were bent back over the tip. The length was adjusted so that the folded tendon was completely within the syringe. Silicone grease was placed around the tip of the syringe to prevent leaking, and the needle hub was forced over the protruding ends to make a tight seal. The shaft of the needle was inserted through a hole previously made into

an off-vertical direction of the stopper, and finally it was immobilized by threading through the jaws of the lower fiber clamp. The plastic cell was installed on the rubber stopper as before, but the inner well was now used as a constant-temperature water bath. A constant fluid level was maintained by using a Sargent water circulator and leveller. Small-bore plastic tubing was slipped over the protruding end of the 6-in needle, and the end of it was attached to a short needle. Solutions preheated to the desired temperature were admitted to the tuberculin syringe–tendon assembly via the protruding needle. Rapid exchange was possible, and this construction proved satisfactory for the measurements reported.

RESULTS

Transient force (F) which developed following the rapid addition of 2.0 M urea is shown plotted against reaction time in Fig. 1. Initial force (F_0) was varied from

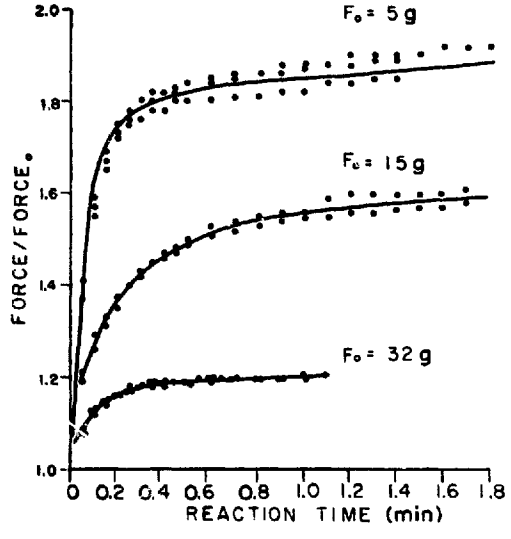

Fig. 1. Transient force ratio (F/F_0) plotted as a function of reaction time for tendons exposed to dilute aq. urea (2 M) at pH 7.0 and 40°. Initial force (F_0) is also shown.

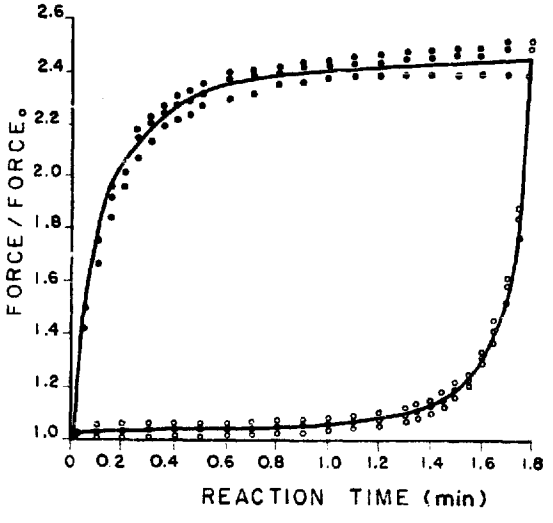

Fig. 2. Dependence of force ratio (F/F_0) on reaction time for addition (●) and dilution (○) of dilute aq. urea (2 M).

5 to 32 g, and it is clear that F/F_0 decreased with elevation of F_0. Subsequent addition of buffer completely regained the initial force, but the F–t curves for addition and removal of urea were not coincidental. Fig. 2 shows a typical plot of F/F_0 *vs.* reaction time where 2 M urea was first added and then diluted by buffer. Good reproducibility is shown by data obtained at 25°, while the spread in reproducibility increased with elevation of temperature to 40°. The shape of the curves obtained at 40° and 25°, however, did not show a significant dependence on temperature. F–t curves were depressed to lower F when concentration of urea was reduced, but otherwise they followed the same general pattern.

To more critically evaluate this rapid force-elevation, fast recordings of F–t data were obtained at 50 in/min in 2 and 5 M urea. The effect of changing temperature on 5 M urea interaction also was obtained, and the findings are shown in Fig. 3. These data reveal that interaction of dilute urea with tendons causes a rapid elevation of

force. However, force is elevated more rapidly by adding conc. urea, but a second process follows (slow force-elevation) which characterizes Step 2.

If the interpretation of Step 1 (Part I) is correct, elevation of force by dilute urea should be limited by diffusion of urea into the tendon. One can test this hypothesis if it is further accepted that force depends directly upon the concentration of urea which diffuses into the tendon. Mathematical analyses for cylindrical diffusion have been worked out[12] and Eqn. 1 is an expression for the quantity of solute (Q_t) which

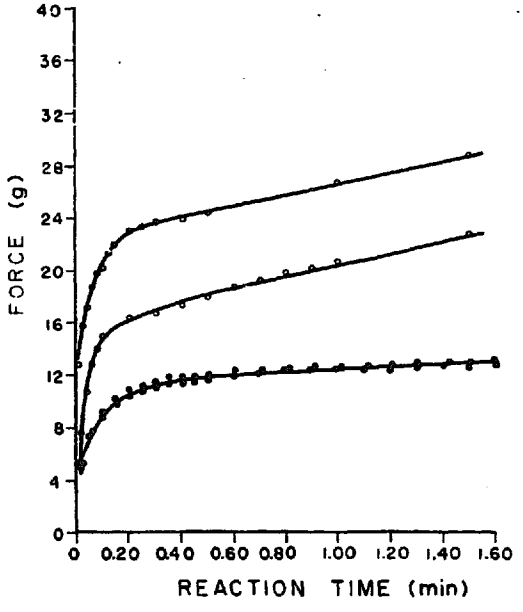

Fig. 3. Fast recording of force–time data for interaction of dilute (2 M (●) at 40°) and conc. (5 M (○) at 20° (upper curve) and 40° (lower curve)) urea with tendons.

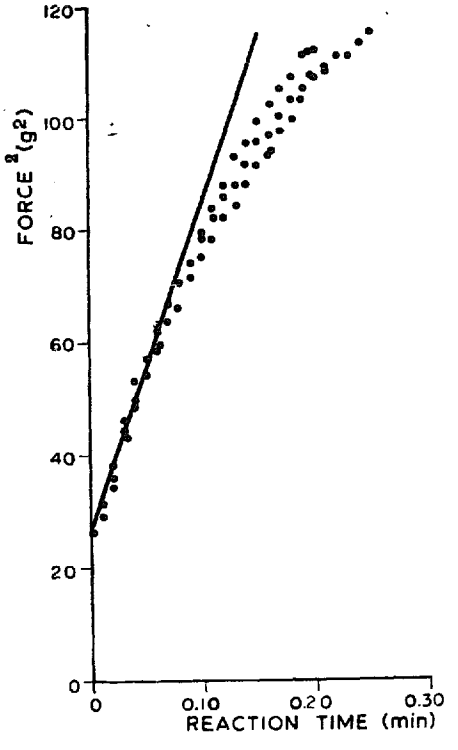

Fig. 4. Diffusion analysis of force–time data of dilute urea (2 M) interacting with tendons. Initial slope of F^2 *vs.* time plot is shown by heavy line.

has permeated a cylinder of radius r in terms of the maximum quantity of urea attainable (Q_∞), diffusion coefficient (D), reaction time (t) and Bessel constants (α_n).

$$\frac{Q_t}{Q_\infty} = 1 - \frac{2}{r^2} \Sigma \frac{1}{\alpha_n^2} e^{-D\alpha_n^2 t} \qquad (1)$$

A more workable approximation, however, has been proposed as an alternative to using Eqn. 1. For a short time after the tendon is exposed to dilute urea, it is proposed that solute enters by diffusing across the nearly planar periphery of its surface. Mathematical formulation of diffusion across a plane is a close approximation of initial conditions for cylindrical diffusion, and during the first 40% of solute transfer, the planar-diffusion equation coincides with the more complex cylindrical-diffusion equation. The much simpler expression resulting thereby is Eqn. 2.

$$\frac{Q_t}{Q_\infty} = 1 - \frac{4}{r}\sqrt{\frac{Dt}{\pi}} \qquad (2)$$

When diffusion has proceeded for a sufficiently long time, however, Eqn. 1 approaches Eqn. 3:

$$\log. Q_t \approx D\alpha_n t \tag{3}$$

One can now evaluate initial and final diffusion coefficients by using Eqns. 2 and 3, respectively.

Fig. 4 shows a plot of typical data obtained by adding 2 M urea to a tendon previously saturated with pH-7.0 buffer at 40°, and under 5 g initial load. The initial linearity of F^2 with time covers about 40% of the increase in load from 5 g to its maximum. The initial diffusion coefficient evaluated from this slope was $5 \cdot 10^{-7}$ cm^2 per sec using a radius that was estimated from the ratio of weight to length and density of 1.5, using the limiting data required by Eqn. 3, a terminal diffusion coefficient of $6 \cdot 10^{-9}$ cm^2/sec was evaluated. These figures should be accepted only as orders of magnitude, but they show a real 100-fold reduction in D as the reaction proceeds.

As shown in Fig. 3, elevation of urea concentration makes Step 1 proceed faster, but it also leads into Step 2 which is considerably slower than Step 1. This period of the reaction was analyzed by plotting force-velocity as a semi-log function of reaction time. Following this there developed an even slower force-velocity, Step 3, but the force now decreases with time. Analysis of this last step showed a linear relationship between $-1/F(\Delta F/\Delta t)$ and t_r (time measured from that of maximum force). These data are shown plotted accordingly in Fig. 5. When F–t data were obtained using small tendons, those having W_0 (weight:length) less than 0.15, it was observed that the overall course of reaction could be expressed kinetically by Eqn. 4.

$$\log. \left\{\frac{\Delta F}{\Delta t} - \left(\frac{\Delta F}{\Delta t}\right)_r\right\} = \log. \left\{\left(\frac{\Delta F}{\Delta t}\right)_c - \left(\frac{\Delta F}{\Delta t}\right)_r\right\} - K_1 t \tag{4}$$

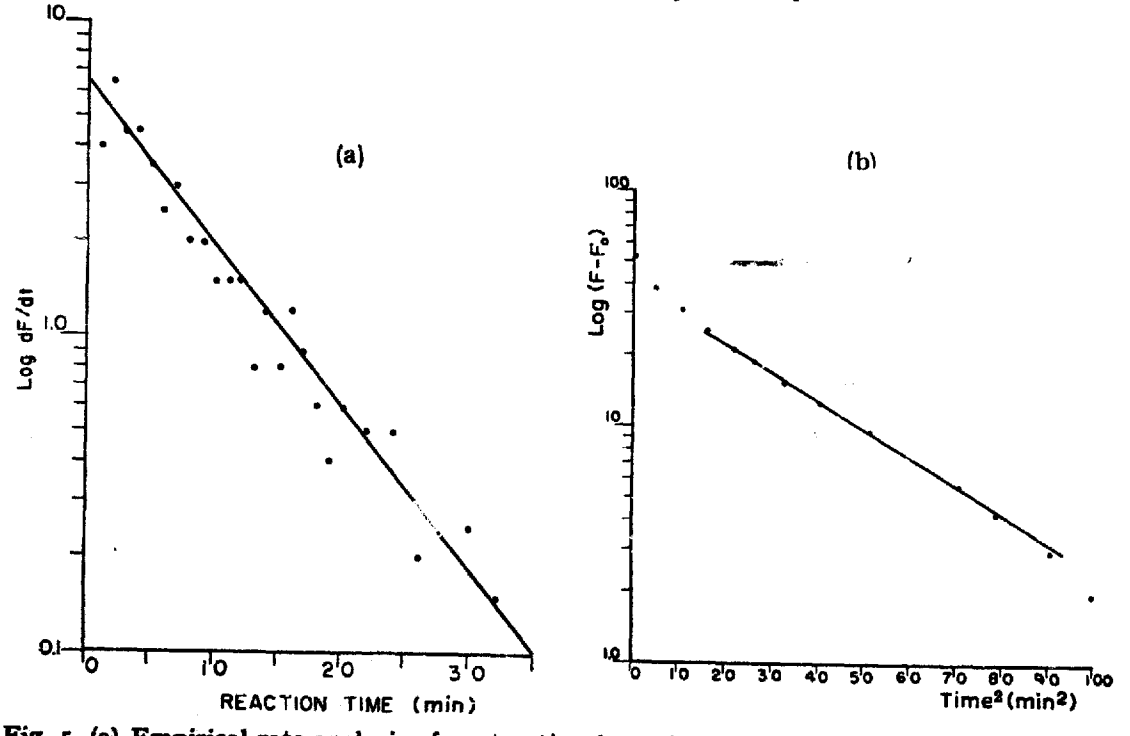

Fig. 5. (a) Empirical rate analysis of contraction force during Step 2, in 8 M urea. (b) Semi-log dependence of net force ($F - F_0$) on second power of relaxation time in 8 M urea. This was obtained by integrating the rate law referred to in the text and demonstrated in Fig. 8, *i.e.*, $1/F(\Delta F/\Delta t)_r \sim t_r$.

When W_0 was higher, the force-velocity data showed a minimum below zero which increased slightly and asymptotically approached a constant relaxation rate $-(\Delta F/\Delta t)_r$. This is illustrated in Fig. 6.

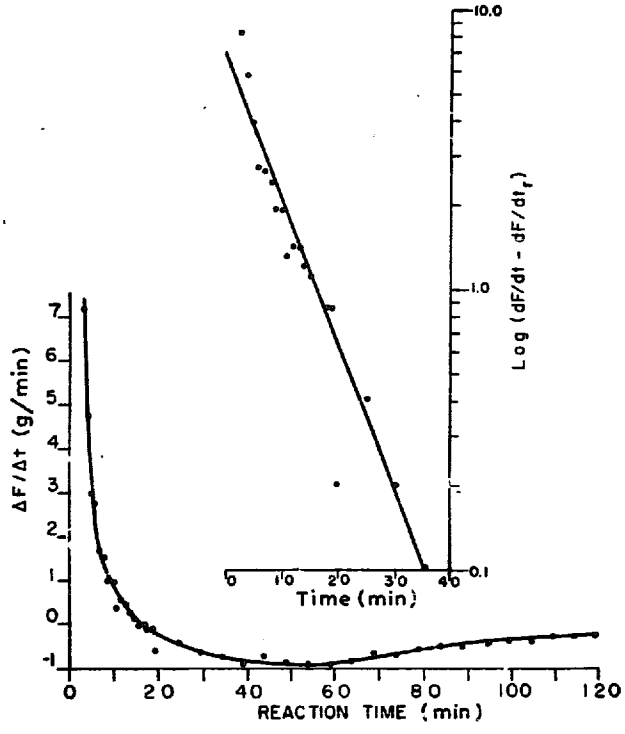

Fig. 6. Rate analysis of force and its dependence on reaction time, in 8 M urea. Inset shows a semi-log function that was obeyed by tendons having W_r less than 0.15 mg/cm.

It is significant to point out at this time that isotonic data of length (L) *vs.* time were observed[5,13] to follow an empirical rate law analogous to the isometric law of Eqn. 4; this is shown by Eqn. 5:

$$\log.\left\{\frac{\Delta L}{\Delta t}-\left(\frac{\Delta L}{\Delta t}\right)_r\right\}=\log.\left\{\left(\frac{\Delta L}{\Delta t}\right)_c-\left(\frac{\Delta L}{\Delta t}\right)_r\right\}-K_2t \tag{5}$$

In addition, a recent series of measurements by ELDEN AND WEBB[14] showed that the periodic extendability ($\Delta L'$) of tendons reacting with conc. urea also followed the same general rate law, *viz.*, Eqn. 6:

$$\log.\left\{\frac{d}{dt}\left(\frac{\Delta L'}{L}\right)-\frac{d}{dt}\left(\frac{\Delta L'}{L}\right)_r\right\}=\log.\left\{\frac{d}{dt}\left(\frac{\Delta L'}{L}\right)_c-\frac{d}{dt}\left(\frac{\Delta L'}{L}\right)_r\right\}-K_3t \tag{6}$$

Finally, it has been observed in our laboratory (unpublished observation by WEBB) that the change in force ($\Delta F'$) for fixed change in strain follows the same general rate law for tendons reacting with conc. urea, Eqn. 7:

$$\log.\left\{\frac{d}{dt}\left(\frac{\Delta F'}{F}\right)-\frac{d}{dt}\left(\frac{\Delta F'}{F}\right)_r\right\}=\log.\left\{\frac{d}{dt}\left(\frac{\Delta F'}{F}\right)_c-\frac{d}{dt}\left(\frac{\Delta F'}{F}\right)_r\right\}-K_4t \tag{7}$$

These observations clearly show that length, change in length, force, and change in force follow a systematic course.

Even though force is the dependent variable in these measurements, it can also

be used as an independent variable. Data obtained using this approach are shown in Figs. 7 and 8 thereby aiding in the interpretation of Steps 1, 2, and 3. In one series of experiments, the initial force was raised from 10 to 70 g in increments of 10 g. F–t data were obtained at each new value of F_0, and the results are shown in Fig. 7. Elevation of initial force inhibits the amount of additional net force produced in Steps 1 and 2. Relaxation of force in Step 3 does depend upon force.

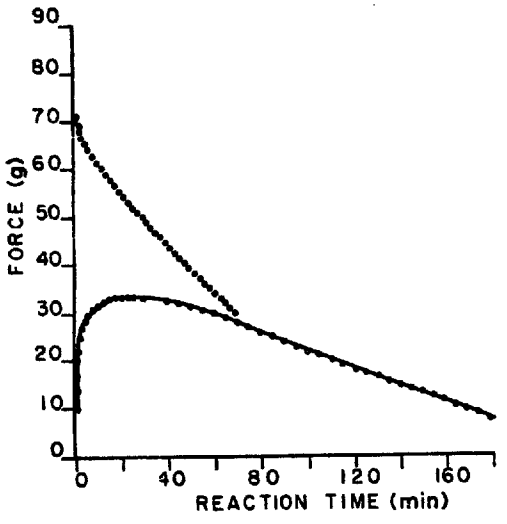

Fig. 7. Influence of initial force (F_0) on isometric force produced by melting tendons, in 5 M urea.

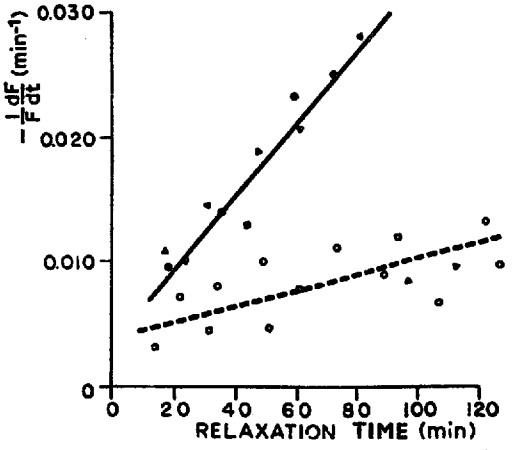

Fig. 8. Influence of force on rate of force-relaxation of melting tendons, in 5 M urea (– – – –) and 8 M urea (———). Circles are controls, triangles are force-reduction, and squares are force-elevation manually introduced periodically during Step 3.

In the second series of measurements, all data were obtained with an initial force of 10 g. A new force was applied, however, only when Step 3 was in progress—not before. The relationship of $-1/F(\Delta F/\Delta t)$ *vs.* t_r, shown in Fig. 5, was used to determine whether or not this new application of force resulted in modification of F–t. Fig. 8 shows that periodically raising and lowering the force during Step 3 does not change the rate law governing F–t data therein. Consequently, force does not influence the velocity mechanism for creeping in Step 3, but it significantly influences the mechanisms of force production in Steps 1 and 2.

Temperature had a pronounced influence on rate of relaxation, and Fig. 9 shows that 12° completely inhibited Step 3. While doing so, it allowed Step 2 to develop a higher F_{max}. F–t data obtained at 20° and 30° showed a progressive increase in relaxation rate, and relaxation was also induced by reheating to 40° those tendons which failed to yield Step 3 at 12°. Rate analysis of force produced in Stages 2 and 3 obtained by subsequently heating the cooled tendons to 40° showed that the empirical laws of Fig. 5 were obeyed.

Data shown in Fig. 10 reveals a most prominent effect of solution pH on rate of tendon degradation. Acid pH allows the tendon to be more readily attacked by urea than does neutral or alkaline pH. The tendon, therefore, is more inert to conc. urea at pH 8.0 than at lower pH, while a smooth increase in relaxation rate continuously bridges the behavior from pH 8.0 to pH 5.7. Acid and base buffers alone produced a real, but comparatively small, change in force. When 5 M urea at pH 7.0 was added to these pretreated tendons, the time required for rupture decreased in proportion to the time that a tendon was exposed to buffer (pH 5.7). Furthermore, the

force developed was lessened and the rate of force relaxation was hastened as a result of exposure to acid pH. It is evident that collagen alteration does occur at these acid pH, but this does not result in pronounced change of isometric force at the time of exposure to low pH.

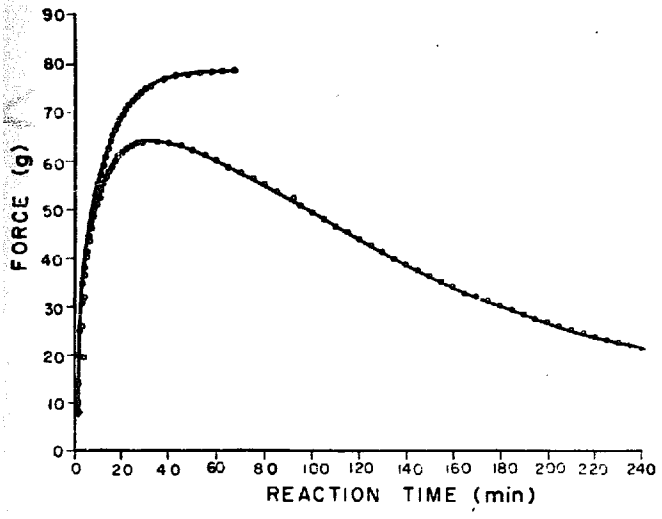

Fig. 9. Influence of temperature on isometric force produced by melting tendons, in 5 M urea, at 12° (●) and 40° (○). Relaxation was completely inhibited at 12°.

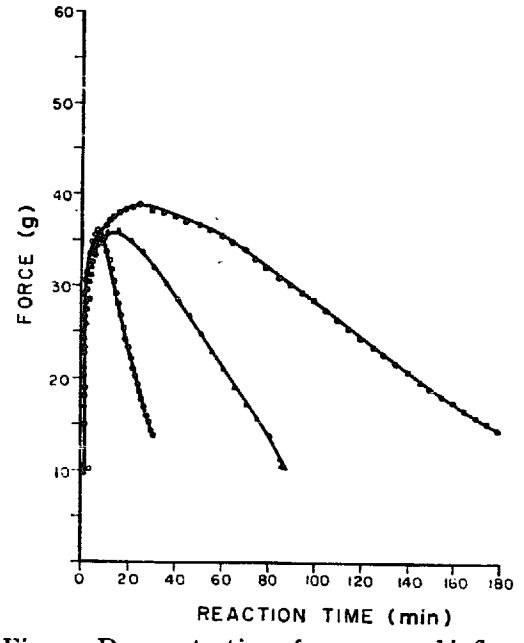

Fig. 10. Demonstration of pronounced influence of pH on rate of relaxation in Step 3. Notice that Step 1 and Step 2 are not influenced significantly. In 5 M urea at 40° and pH 5.7 (○), 7.0 (▲), 8.0 (●), respectively.

DISCUSSION

Numerous experiments concerning the effects of aging on connective tissue (tendons) have been done by measuring water uptake and rate of isotonic and isometric melting. It is generally believed, as a result of these and other measurements, that crosslinks become incorporated into or between macromolecules of collagen. The position and identity of these tertiary polypeptide bonds are not known, but their identification is being pursued by VEIS AND COHEN[2], GALLOP *et al.*[15], and GRASSMAN *et al.*[16]. The proposal of using isometric and isotonic mechanical properties of melting tendons, therefore, is an attempt to expediently determine the response of connective tissue to age-induced cross-linking reactions.

Collagen interactions with water and urea

The sorption of water by collagen was reviewed by GUSTAVSON[17], and he points out that side-chain acid and base groups cannot account for the observed hydration. Strong association of water with peptide groups, however, does account for a major fraction of collagen water. Furthermore, BEAR AND MORGAN[18], and TOMLIN AND WORTHINGTON[19] suggest that a hydrophilic flexible segment is present in collagen. Also, VON HIPPLE *et al.*[20,21] state that a water–carbonyl oxygen bridge assists in the stabilization of the collagen-fold, but NMR measurements by BERENDSEN[22] fail

to show the presence of water bonded to peptide groups. They instead demonstrate the presence of longitudinal chains of hydrogen-bonded water molecules along the longitudinal axis of collagen chains. The possibility was admitted, however, that intermolecular stabilization of collagen might depend upon side–side interaction of these water and collagen chains.

The findings illustrated by Fig. 2 show that interaction of mature tendons with dilute urea does not lead to depolymerization. Since force reached a constant value after addition of aq. urea and remained there for a considerable period in some experiments, it is accepted that further reaction beyond Step 1 is not achieved by 2 M urea. The influence of initial force (5, 15, and 32 g) on Step 1 can now be interpreted if the presence of hydrophilic flexible segments is accepted.

Considering that such units are in a contiguous series with hydrophobic crystalline segments, application of longitudinal force more readily extends the flexible segments. When dilute urea is added the flexible hydrophilic segments dehydrate; but the strain imposed by high initial force opposes that disruption which would be produced had the reaction taken place at lower initial force. Therefore, at a certain point there is an initial force which balances the constrictive force produced by interaction of dilute urea with tendon water of hydration. No change in force occurs at this initial force.

The kinetic analysis of Step 1, based upon cylindrical-diffusion theory, strongly suggests that rate of entry of urea into tendons is the limiting step for elevation of force. In solutions of urea above 2 M concentration, SCHELLMAN[23] presented an analysis showing the association of monomeric urea to form dimers, trimers, etc. At 2 M concentration, there are appreciable numbers of monomers which have polymerized. When these are present near a polypeptide group, one should expect to find the urea molecule associating with the peptide bond. X-ray-diffraction experiments by SHAW[24] reveal that urea does crystallize on the collagen polypeptide, and they also become oriented with respect to the longitudinal axis of collagen. Disruption of collagen-bound water by urea, therefore, would arise by a competitive association of urea and water with collagen.

Degradation of collagen by urea

When urea concentration is raised to 5 M at 40° and pH 7.0, the data of Fig. 3 shows that a slower chemical reaction follows Step 1, and this is believed to represent degradation of crystalline segments in collagen (Step 2). Lowering the temperature to 20° did not significantly alter the velocity of this reaction. Comparison of the slopes of data plotted in Figs. 5a and 4 shows the nearly 1 0-fold difference in time over which these events take place. The different velocities of reaction and the pronounced limitation of Step 2 to concentrations above 2 M urea are used as evidence for delineating these two parts of the overall F–t curve of melting tendons.

When conc. urea is allowed to react with tendons at 40°, Step 2 (slow force-elevation) is followed by even slower Step 3 (force-relaxation). The fundamental velocity equation which governs the F–t relationship for Step 2 and 3 was found to be Eqn. 4, and there appear to be two distinct reactions. Disruption of crystalline segments in collagen yields an elevation of force because the liberated chains tend to shorten. Progressive degradation, however, frees these from secondary restraint,

and they slip under the generated load; however, when all of the crystallites become degraded, the reaction becomes principally one of slippage of liberated polypeptide chains.

Data plotted in Fig. 5a show that Step 2 follows a simple rate expression, *viz.*, log. $\Delta F/\Delta t = A - Bt$, for most of the time interval of Step 2. If the rate of relaxation $(\Delta F/\Delta t)_r$ in Eqn. 4 is set equal to zero, then the resulting equation agrees with the data of Fig. 5a. Thus, it appears that slippage does not become significant until the terminal stages of crystalline degradation.

When initial force is raised sufficiently high, so as to oppose the elevation of force normally expected by crystalline degradation of Step 2, then conc. urea allows relaxation to develop immediately upon its addition to the tendon. This point is substantiated by the data shown in Fig. 7. It is not possible, however, to arrive at the observed relaxation rate of Fig. 8, *viz.*, $1/F(\Delta F/\Delta t)$ *vs.* time, by making $(\Delta F/\Delta t)_c$ equal to zero in Eqn. 4. Consequently, there must be some dependence of $(\Delta F/\Delta t)_r$ on $(\Delta F/\Delta t)_c$, but probably not *vice versa*. Furthermore, the data of Fig. 8 demonstrate that relaxation rate is dependent upon urea concentration. This shows that the slippage mechanism indeed is dependent upon the interaction of urea with collagen.

The striking influence of pH on relaxation rate perhaps is the most interesting finding of this study. Rapid disintegration at pH 5.7 and moderate resistance to depolymerization at pH 8.0 closely parallels changes in acidity. Low pH shifts hydrogen ions toward collagen so that its carboxyl and amine groups carry protons, *viz.*, –COOH and $-NH_3^+$. The net positive charge of the ammonium ion results in electrostatic repulsion of these units along the polypeptide chains and in between the chains—rapid dissociation of the structure is favored because of this electrical instability. Alkaline pH, on the other hand, favors dissociation of carboxyl groups to give the carboxylate ion ($-COO^-$) but it probably cannot completely dissociate the ammonium ion ($-NH_3^+$). The alteration of positive and negative charges provides local stability, and a rapid depolymerization conceivably would not be expected.

The dependence of relaxation rate on temperature also is an important observation. Since low temperature favors collagen–water interaction, it is likely that larger kinetically effective units are formed at 12° than at 40°. Retardation of force-relaxation (Step 3) would be expected to develop because these larger units would have greater frictional resistance to flow under an applied load, and also because their thermal motion would be reduced at the lower temperature. The comparatively insignificant change in rate of Step 2 with temperature (away from the transition temperature) points out that isometric melting, degradation of crystalline segments, is primarily a local reaction which does not depend greatly upon influence of temperature for large-scale movement of polypeptide chains.

Degradation of collagen molecules in solution was studied by ENGEL[25], and he observed a two-step mechanism. ENGEL considered that the second stage depended upon rupture of interpeptide bonds, present in the originally intact molecule of collagen, which now restricted the separation of collagen chains melted during Stage 1. Isometric force produced in tendons by heating[26], and degradation of soluble collagen exposed to conc. urea[27] show transient behavior which suggest a two-step degradation. Experiments by WEIR[28], CHVAPIL *et al.*[29,30], and CREWTHER AND DOWLING[31] also are interpreted to show a two-step degradation. These findings, therefore, agree with the interpretation proposed here for urea-induced degradation of tendons.

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