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THE INTERACTION OF CONNECTIVE TISSUE WITH AQUEOUS UREA

I. REVERSIBLE AND IRREVERSIBLE EFFECTS*

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SUMMARY

The interaction of dilute aqueous urea (<2 M) with rat-tail tendons produces a reversible change in isometric force that is linearly related to concentration. Elevation of temperature (not exceeding 40°) decreases isometric force linearly and reversibly. It is proposed that alteration of collagen–water interaction is a basis for these changes.

Concentrated urea (>5 M) profoundly changes the isometric force. This can be described as Step 1, rapid force elevation that is followed by Step 2, a slow further increase in force which attains a maximum; finally, Step 3 reduces the force until the tendon ruptures. These three steps are considered to be a result of: (i) rapid diffusion of urea into flexible regions of collagen which results in their dehydration, (ii) slow melting of crystalline segments that are stabilized by hydrogen bonds, and (iii) creeping of the polypeptide chains that are liberated during Step 2.

INTRODUCTION

The purpose of connective tissue in living systems, as the name suggests, is to integrate discrete physiological structures into smoothly functioning entities. This tissue is recognized qualitatively by the presence of collagen, elastin, and reticulin; and these are surrounded by cells (fibroblasts), mucopolysaccharides, and water. The quantitative proportioning of these constituents depends on whether or not the tissue undergoes stretch, shear, or compressive deformation. Histological examination also shows that connective tissue is not sharply delineated from surrounding tissues[1,2]. In fact its ability to integrate depends primarily upon diffuseness.

Degeneration of connective tissue occurs as a result of pathology and aging. The entities called "collagen diseases" illustrate a type of pathological disintegration, whereas an opposite effect, that of aging, is slower to evolve.

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In a treatise on collagen diseases TALBOTT AND FERRANDIS[3], refer to a description of fibrinoid degeneration[4,5]; these authors point out that this is an alteration of collagen fibers and ground substance while fibroblasts concentrate and proliferate at foci of collagen degeneration. The newly synthesized elements of connective tissue as well as pre-existing ones are faulty during this disease, and they exhibit great weakness and high elasticity. Degeneration of collagen fibers proceeds so extensively that their end products accumulate in the mucopolysaccharide spaces, and the ground substance thereby is said to become gelatinized. Aging, on the other hand, produces changes in connective tissue which are contrary to depolymerization. In this situation tissue extendability is changed[6], metabolic turnover is reduced while collagen is accumulated[7], and the tissue becomes generally more refractile. From a biophysical point of view diseased connective tissue exhibiting fibrinoid degeneration shows those properties which identify the behavior of melted collagen. Aged tissue, on the other hand, resembles collagen and mucopolysaccharide macromolecules which have become crosslinked and dehydrated.

It is difficult to find a common molecular–biochemical basis for the pathology and aging of connective tissue. One approach which can be considered is to compare properties of *in vivo* degenerated tissue with those produced *in vitro* by experimentation. Such a comparison will more clearly define the physical properties of (*in vivo*) degenerated tissue because the adequately controlled (*in vitro*) alteration will be a useful guide. In support of this view, it is worthwhile to mention that physically melted collagen has certain features which closely resemble the qualitative description of diseased collagen (fibrinoid degeneration). Also, the most clearly demonstrated effects of aging in connective tissue are brought to view by measuring the isometric force[8] and isotonic length changes[9,10] of tendons that are chemically induced to melt. These two different types of connective-tissue degeneration might be more clearly described, and perhaps more readily understood, if extensive rheological data were available for diseased, aged, and "melted" connective tissue. The present studies, therefore, pertain to fundamental aspects of connective-tissue degradation, and they provide a guide for the design of later experiments using diseased and aged connective tissue.

Early work done on the melting of collagen shows that the transformation is easily discerned by changes in dimensions, *i.e.*, longitudinal shortening and diametrical swelling[11,12]. Equilibrium thermodynamic analysis of the melting phenomenon shows that it is a first-order phase transition[13], and it can be reversed provided that the thermal treatment is not drastic[14]. Previous theoretical work by FLORY[15] showed that a relationship exists between the degree of solvation of a polymeric network and the melting temperature. Experiments by WITNAUER AND FEE[16] demonstrated the applicability of this relationship to equilibrium melting of hide collagen, and also showed that solvation of connective tissue favors melting of collagen by lowering its phase-transition temperature. Collagen–water interaction, therefore, is an important factor (in addition to crystallinity and crosslinking) that influences the ease of depolymerization of connective tissue.

The studies reported in these papers (I and II) deal with chemical degradation of connective tissue (rat-tail tendons) using dilute and conc. aq. urea. They provide new measurements of isometric force *vs.* time of reaction which can be compared with isotonic length–time data obtained in earlier studies[8-10,17].

METHODS

Preparations of tendons

Tendons were removed from tails of adult rats (Sprague–Dawley). They were washed in normal saline to remove blood and solubles, rinsed in distilled water, and dried (70% relative humidity at 25°). This procedure required less than 60 min to complete. Next, they were weighed to an accuracy of 0.01 mg, and measured to an accuracy of 0.5 mm for length. The ratio, weight: length, was computed and expressed as g/12 cm (W_0); this represents a measurement of tendon size (W_0 = area × density). Allowing tendons to dry to equilibrium with room air, but no further, was a safeguard against producing irreversible changes which develop by exhaustive dehydration[18].

Apparatus

All measurements of force as a function of time were made with the Instron testing instrument (Instron Engineering Corporation, Canton, Mass.). This apparatus consists of a load-weighing system, an adjustable (vertical movement) crosshead, a recorder, and appropriate controls. Gear-ratio adjustments provide controls for the vertical extension of the sample and the chart recorder (operation between 0.2–50 in/min). Controls also are provided for automatic cycling of load or extension, rapid return, direct reading of sample length to 0.001 in, and selection of various tensile loads. Two cells that were found convenient are A (10, 20, and 50 g with 1 g extra-sensitive) and B (100, 200, 500, 1000, and 2000 g) for full scale of 10 in chart width. Rubber-coated fiber clamps (standard accessories) were found satisfactory for attaching the soft and easily damaged tendons to the crosshead and load-weighing cell. Calibration of the load-weighing circuit is easily accomplished, and the drift of settings was insignificant.

Cell assembly

A rat-tail tendon was folded, inverted, and attached to a fiber clamp. This in turn was held by a fitting on the crosshead of the instrument. A wire suspended from the load-weighing cell (overhead) was used to establish and register loads generated in the tendon. The assembly was so constructed (Fig. 1) that the tendon could be bathed in aqueous solutions with suitable temperature regulation. Water was pumped from a Sargent constant-temperature water bath through a stainless-steel heat exchanger (essentially a U-tube). A small stream of bubbles agitated the solution around the heat exchanger, and temperature within the cell was indicated by a suitably placed thermometer or thermistor.

Details of the cell assembly are as follows: The cell (Fig. 1) was constructed from commercially available lucite. A 2-in hole was drilled into the center of a 4 × 4 in square base 0.5 in thick. Surrounding the hole were a 2-in ID round tube 6 in high and a 6-in ID tube 6.25 in high. The annular space acts somewhat as a heat insulator and also as a reservoir for any solution spilled out of the inner chamber. This protects the Instron apparatus from overflow of liquids (should it occur). These pieces were cemented to the base with a chloroform solution of lucite chips. Fig. 1 shows a rubber stopper which fits over a standard Instron fiber-clamp assembly to form a seal for attaching the cell to the crossarm of the Instron apparatus. Two plastic trimmers, 2-in dia. tubing with one end cut at 90° and the other end cut at 30° angles, were used to support the cell after it was forced down the rubber stopper. Folded emery cloth

was used at places of plastic–plastic and plastic–metal contact to reduce slippage.

The load-weighing cell (overhead) was easily attached to the inverted U-shaped tendon that was held by the bottom clamp. A standard Instron clamp with rubber faces was attached to the load-weighing cell. The end of a piece of 0.06 in brass wire

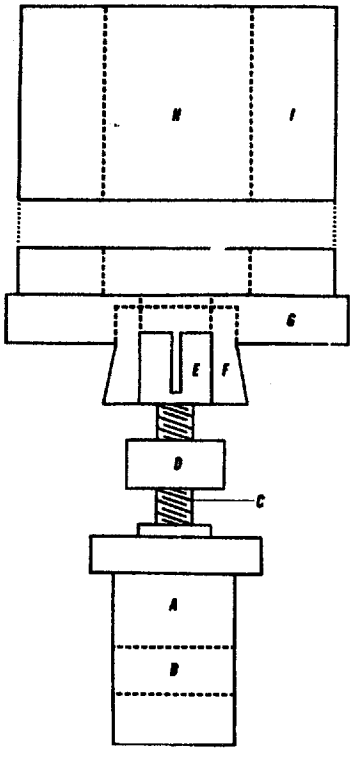

Fig. 1. Details of cell assembly. A is a cylindrical plug which fits into the movable crosshead of the Instron tensile-testing apparatus. B is a hole through which a retaining pin is inserted. C and D are thread and locking nut that attach the fitting E to the cylinder. A fiber clamp is inserted into the slot of E, and F is a rubber stopper which attaches the plastic assembly to the metal fittings. G is the square base of the lucite chamber, H is the hollow cylinder in which solutions are poured, and I is an empty circular space.

was folded into a hook and the other end was held by the upper clamp; the hook was slipped in turn under the fold of the inverted tendon. The heat exchanger, thermometer, and air bubbler (1-mm dia. tubing) were installed, and the apparatus was ready for use.

Measurement of physical properties

Each tendon was temperature equilibrated in phosphate buffer (pH 7.0, I 0.02) at 40° for 15 min. The folded tendon was then stretched at a rate of 0.2 in/min from a position of no load to a point where the load was 50 g. Extension was stopped and gage length was read off the indicator. The true gage length at zero load was calculated from the inscribed load–length curve. Following this the tendon was worked, or conditioned, by automatically cycling the length 10 times so that the load was changed from 0 to 50 to 0 g. At the end of the last extension, the sample was allowed to stress-relax for 30–45 min in buffer at 40°. The next steps were individually determined for the particular studies to be undertaken. They were essentially the same type of manipulations as the above steps, but the exact procedures are more easily presented with the results of the studies.

RESULTS

Effect of temperature

After a standardized period of work conditioning, force was measured as the temperature of buffer solution was changed from 40° to 15°. Heating and cooling were conducted at a rate not in excess of 2°/min, and it was established that thermal data could be reproduced with coincidence. Fig. 2 shows a typical plot of force on the y-axis as a function of solution temperature on the x-axis. Application of heat, therefore, lowers the net balance of forces which results in tendency of a tendon to retract when held at constant extension.

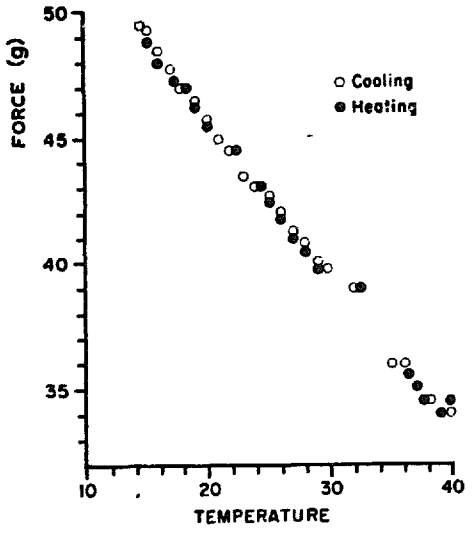

Fig. 2. Reversible influence of heating on force produced in a tendon exposed to 2 M urea. ●, heating; ○, cooling.

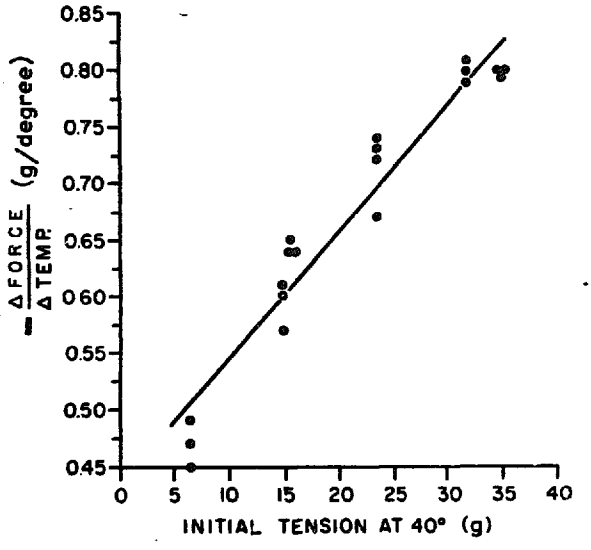

Fig. 3. Dependence of force-thermal coefficient on initial tension of tendon in water.

It was possible to program the change in temperature of the cell by using 2 water baths in parallel. One was set at 40° while the other was held at 20°; a valve system permitted either one to be switched to the heat exchanger in the cell. This assembly was used to produce a change of about 10° in the temperature of the solutions surrounding the tendon. The simultaneous change in tension was enscribed on the chart and used to calculate a thermal-tension coefficient ($-\Delta$ force/Δ temperature, g/°C).

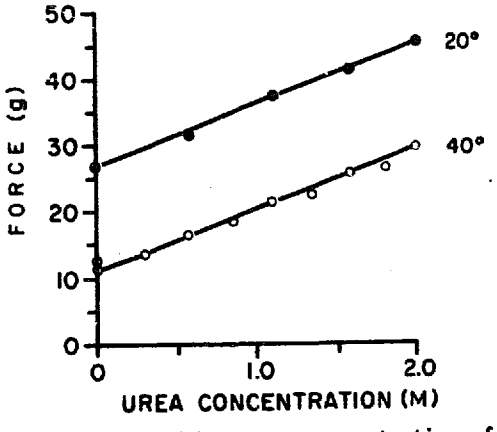

Fig. 4. Reversible dependence of force on concentration of urea at 20° and 40°.

Fig. 3 shows a plot of these coefficients for tendons in buffer as a function of different initial tension.

Effect of dilute (0–2 M) urea

Concentration of urea was raised within the cell by titrating a 10 M stock from a burette into the buffer solution. The force produced in the tendon is shown in Fig. 4 as a function of urea concentration. It was noticed that force equilibration was not instantaneous, but instead showed a transitory response lasting upwards to 5 min (see Part II of these reports). Data plotted here are those coordinates of force–molarity corresponding to a steady state. Dilution of urea in the cell by titrating with phosphate buffer reversed the former increase in force. Furthermore, the steady-state force–molarity relationship obtained for urea addition coincided with that resulting when urea was diluted with buffer. In this respect the system was reversible.

It is evident that force increased linearly and reversibly with addition of urea. Curves obtained at 20° and 40° were parallel, but the latter were lower. Urea concentration was not raised higher than 2 M during these particular measurements, and solutions within the range 0–2 M are considered here to be dilute.

Effect of conc. urea

Addition of 5–10 M urea profoundly altered the force of tendons previously saturated with pH-7.0 buffer at 40°. This irreversible phenomenon was measured by

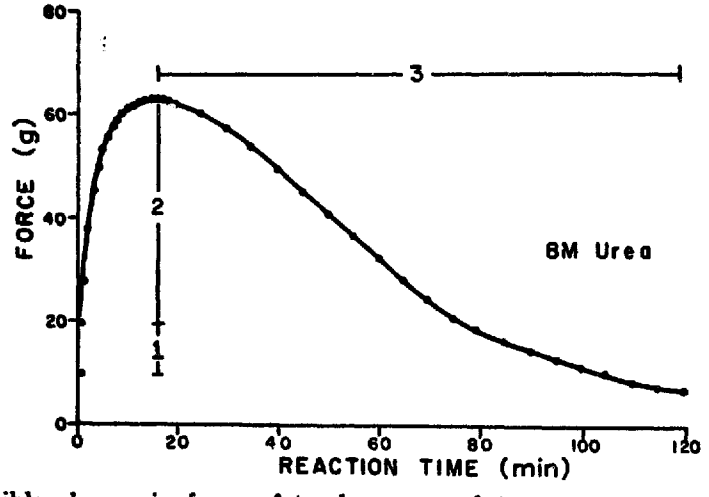

Fig. 5. Irreversible change in force of tendon exposed to conc. aq. urea at 40° and pH 7.0.

continuously recording force–time curves for tendons exposed to conc. urea, and by periodically determining the thermal-force coefficient and the transitory stress–strain curve. Fig. 5 illustrates the characteristic dependence of force on reaction time for tendons exposed to conc. urea. There is a rapid increase in force (Step 1) which requires about 1–2 min. The force then continues to increase (Step 2) until it attains a maximum, where it decreases more slowly (Step 3) until finally the tendon ruptures.

Fig. 6 shows a plot of the thermal-force coefficient as a function of force. The line marked "water" refers to 0 M urea, and is data of Fig. 4 which is redrawn. Elevation of urea concentration to 2 M did not change significantly the slope of this line. Where urea concentration was raised to 4 and 6 M, the force increased rapidly, reached a maximum, and decreased slowly until the tendon ruptured. Measurements

of thermal-force coefficient now became more difficult because the force of the tendon held at 40° was changing. The ordinates of the graph for 4 and 6 M were obtained, therefore, over an average value of force. These are shown in Fig. 6, however, as a

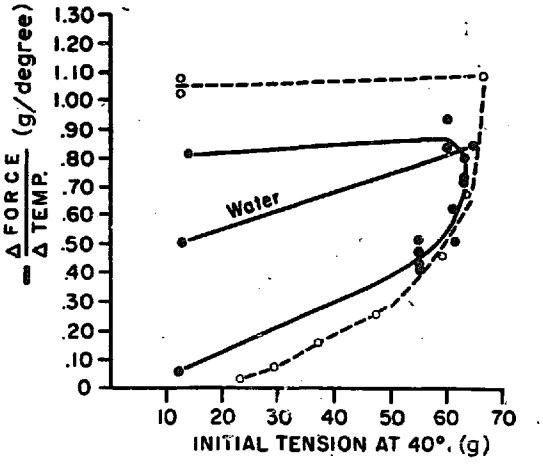

Fig. 6. Change in force-thermal coefficient during course of reaction with dilute and conc. urea. O—O, 6 M urea; ●—●, 4 M urea.

function of the force prevailing at the beginning time of measurement. The chart indicates that the thermal-force coefficient increases during force production, but after reaching its maximum value it decreases well below the previous level. 6 M urea resulted in ordinate values that were below those of 4 M urea. The latter obviously were below those obtained in water as a function of initial force produced simply by stretching the tendon.

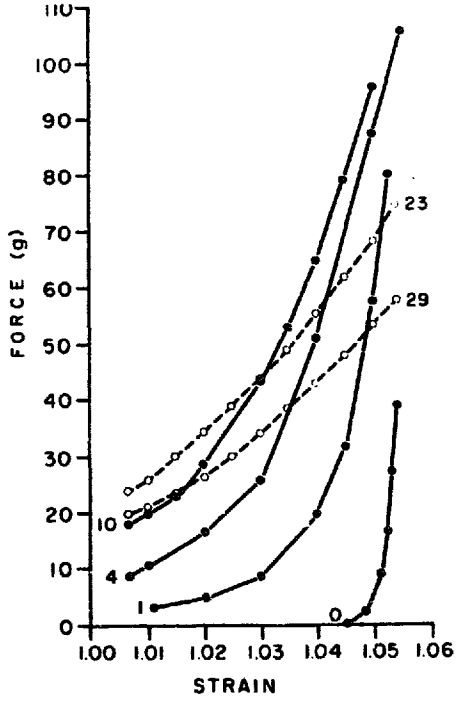

Fig. 7. Dependence of force produced in a melting tendon on strain and reaction time.

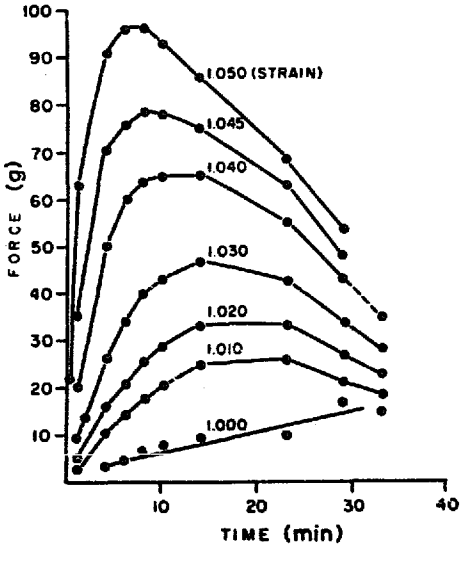

Fig. 8. Dependence of maximum force produced in a tendon on strain and reaction time.

Stress–strain curves were obtained periodically in conc. urea by using the automatic cycling features of the Instron testor. After work-conditioning the sample, its gage length was slacked off about 5 % of zero-load length. The automatic cycling control was set so that one extension and shortening cycle could be executed by momentarily closing the appropriate switch that initiates the cycle. In this manner the tendon remained in a zero-load condition for a short period except when the cycle was in operation. Conc. urea was added at 40° to the tendon, and a stopwatch was started when the cell was half full of solution. At a certain time (shown on each curve in Fig. 7) the cycle was started and a load–elongation curve obtained. In this particular experiment the rest length was not short enough to render the tendon completely free of tension except during no measurement. The "strain" is not, therefore, relative extension from point of maximum length for zero load.

The curves shown in Fig. 8 reveal that the load–strain relationship changes as a result of the action of conc. urea on tendons. First, there is a rapid shortening of the tendon. 1 min after addition of aq. urea the force at maximum zero-load length (our strain = 1.045) had increased to over 30 g. Meanwhile, the load at strain = 1.01 had increased to about 2 g. Continued examination of the data shows that the load obtained at high extension (1.050) increased faster and reached a higher value than that obtained at low extension (1.010). At 23 min the load at 1.050 extension had passed its maximum and was decreasing, while that at 1.010 extension was just reaching maximum. These findings may be more easily apprehended by examining the replotted data shown in Fig. 8. Here, the load is plotted on the ordinate for a particular extension as a function of time following addition of urea. One can see that the time required to reach maximum force decreases linearly with increasing strain. The tension at strain = 1.00 increases linearly with time and shows no maximum.

DISCUSSION

Hydration of collagen

The interaction of collagen with water causes an alteration in molecular configuration of tendons[19], and ROUGVIE AND BEAR[20] demonstrated that an X-ray-diffraction spacing in tendon elongates from 600–680 Å when they are hydrated. Also, during the elongation the equatorial spacing increases from 10.4 Å to 11.5 Å. Furthermore, water appears to react with electron microscopic "bands", but does not interact with "interband" crystalline regions[21,22]. Groupings of electron-dense amino acid side chains which form the "bands", therefore, are hydrophilic and presumably impart flexibility to the macromolecule.

Studies on hydration of collagen (cited by RICH AND CRICK[23]) point to a specific role for water in maintaining the structural integrity of this macromolecule. Although there is no agreement on the location of sites for collagen–water interaction, it is generally accepted that dehydration forces certain segments of the molecule to contract and separate, whereas hydration allows them to elongate and weld together. It is clear, however, that water does not interact with the crystalline region, because wide-angle X-ray-diffraction patterns originating from these points do not change with hydration.

Consider a molecular model of rat-tail tendon which has the following structural features. Collagen macromolecules (tropocollagen) are arranged with their longitudinal

axes closely parallel to the tendon longitudina; these macromolecules are aggregated end to end with considerable strength in the axial direction, but they are weaker in the lateral direction; certain tertiary crosslinks supplement the lateral forces at distant points; and, hydrophobic crystalline and hydrophilic flexible (when wet) regions are in series and contiguous contact. In native tendon subjected only to hydration and dehydration, the elastic moduli and relative number of crystalline and flexible units determine the elasticity of the tissue. When the tendon is melted, both flexible and crystalline units depolymerize and polypeptide chains separate. The crosslinks of tertiary structure now greatly influence tissue rheological properties, because they alone tie together the polypeptide chains just liberated. This model is a useful guide for interpreting the findings of this study.

Thermoelasticity of tendons

Reduction in tension as a result of heating to 40° suggests an alteration in collagen–water interaction, or that a shift has occurred in the crystalline–amorphous content. Similar behavior was shown by FEE *et al.*[24] for strips of chrome-tanned hide which elongated when they were heated in water below the shrinkage temperature. WEIR[25] showed by measuring the volume expansivity that heating below the shrinkage temperature caused a reversible increase in volume of $540 \cdot 10^{-6}$ per °C which is typical of liquids. MITTON[26], on the other hand, showed that longitudinal thermal expansion was considerably less ($22 \cdot 10^{-6}$ per °C) than expected from WEIR's data which assumed isotropic dilatation. Since collagen is not isotropic, the low axial expansion compared to radial and volume expansion should be expected.

The interaction of collagen with water is temperature dependent, and KANAGY[27], showed that the adsorption of water vapor decreased with elevation of temperature from 28° to 70°. Adsorption isotherms otherwise were parallel at 28°, 50°, and 70°. Reduction in isometric force with elevation of temperature, therefore, presumably involves favored collagen–collagen interaction which causes elongation.

Collagen–urea

The tendon model proposed above allows certain qualitative deduction to be made based on the data shown in Figs. 3–8. It seems quite likely that urea molecules should diffuse into the open (flexible) regions more readily than into the crystalline segments. These flexible regions also would strain more readily (stretch) when stress is applied because they have fewer secondary bonds for stabilization. On the basis of these considerations, Step 1 of Fig. 5 is considered to represent the rapid interaction of conc. urea with the flexible units.

Following this, Step 2 would then depend upon penetration of urea into the crystalline regions adjacent to the flexible segments. Degradation of the crystalline regions would produce an additional increment of force due to the increase in entropy of the liberated chains. Concurrent slipping of the freed polypeptide chains allows the force to relax from the maximum generated by Step 2. The main restraining factors then become the tertiary crosslinks and bulk size or shape of the liberated (melted) polypeptide held together by these links. Step 3, therefore, represents the slow creeping of polypeptide chains and liberation of the recently described multiple components of collagen[28].

Transient stress–strain curves shown in Fig. 7 are consistent with the model.

They show a rapid increase in stress required to give a certain strain which is what should occur as a result of dehydration in Step 1 of Fig. 5. Subsequent increase in stress at low strain reflects the additional force generated by melting of the crystalline region of collagen. Simultaneous diminution of stress at high strain is based on creeping of the liberated polypeptide chains. Reconsidered on a time basis in Fig. 8, one sees that maximum force is obtained early for high strains where onset of creeping is sooner, and maximum force occurs later for low strains, where creeping should be less pronounced.

The data of Fig. 3 showing an increase in the energy term with stretching might suggest a reduction in the number of flexible segments. Naturally, this would be expected if these segments become inflexible when the tendon is stretched. When urea is added the water of hydration in these strained flexible units diffuses out and the segments become more rigidly fixed. Data plotted in Fig. 6 shows the anticipated elevations of $-\Delta F/\Delta T$ as a positive slope for the linear portion of data. When Steps 2, 3 ensue, the negative coefficient decreases because both fixed and free flexible segments as well as the crystalline segments have been disrupted. The whole structure is now composed of coiling and slipping polypeptide chains which are attached only at the point of original tertiary crosslinks.

If melting of collagen converts crystalline regions into amorphous coils, then the tendon must become rubbery. Ideal rubbers have a zero energy term for thermoelastic stretching, and force is based entirely on entropy of deformation. The change in force with elevations of temperature should be positive, but real rubbery materials have some residual energy contribution. Data shown in Fig. 6 for melting tendons demonstrates that the energy term decreases, but it was difficult here to show a predominance of entropic effect over residual energy contributions due to slippage of melted polypeptides.

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REFERENCES

1 A. MAXIMOW AND W. BLOOM, *A Textbook of Histology*, W. B. Saunders, Philadelphia, 1930, p. 72.
2 W. BONDAREFF, *Gerontologia*, 1 (1957) 222.
3 J. H. TALBOTT AND R. M. FERRANDIS, *Collagen Diseases*, Grune and Stratton, New York, 1956.
4 F. KLINGE, *Ergeb. Allgem. Pathol. Pathol. Anat.*, 27 (1933) 1.
5 E. NEUMANN, *Arch. Mikrobiol. Anat.*, 18 (1880) 130.
6 J. D. SPIKES, *Federation Proc.*, 19 (1960) 142.
7 H. R. ELDEN, N. L. NOBLE AND R. J. BOUCEK, *Gerontologia*, 3 (1959) 204.
8 J. BROCOS, AND F. VERZAR, *Gerontologia*, 5 (1961) 223.
9 M. CHVAPIL AND Z. HRUZA, *Gerontologia*, 3 (1959) 241.
10 I. BANGA, J. BALO AND D. SZABO, *Experientia, Suppl.*, 4 (1956) 28.
11 C. F. WEIR, *J. Res. Natl. Bur. Std.*, 42 (1949) 17.

[12] H. R. ELDEN AND B. CASSAC, *J. Polymer Sci.*, 59 (1962) 283.
[13] P. J. FLORY AND R. R. GARRETT, *J. Am. Chem. Soc.*, 80 (1958) 4836.
[14] P. J. FLORY AND E. S. WEAVER, *J. Am. Chem. Soc.*, 82 (1960) 4518.
[15] P. J. FLORY, *J. Chem. Phys.*, 17 (1949) 223; 15 (1947) 684.
[16] L. P. WITNAUER AND J. G. FEE, *J. Polymer Sci.*, 26 (1957) 141.
[17] H. R. ELDEN AND G. WEBB, *Nature*, 192 (1961) 742.
[18] D. JORDAN-LLOYD AND R. MARRIOTT, *Proc. Roy. Soc. London Ser. B*, 118 (1935) 439.
[19] K. PANKHURST, *Nature*, 159 (1947) 538.
[20] M. A. ROUGVIE AND R. S. BEAR, *J. Am. Leather Chemists' Assoc.*, 48 (1953) 735.
[21] R. S. BEAR AND R. S. MORGAN, *Symposium on Connective Tissue*, Blackwell, 1957, p. 321.
[22] S. G. TOMLIN AND C. R. WORTHINGTON, *Proc. Roy. Soc. London Ser. A*, 235 (1956) 189.
[23] A. RICH AND F. H. C. CRICK, *J. Mol. Biol.*, 3 (1961) 483.
[24] J. G. FEE, R. R. CALHOUN AND L. P. WITNAUER, *J. Am. Leather Chemists' Assoc.*, 51 (1956) 530.
[25] C. E. WEIR, *J. Am. Leather Chemists' Assoc.*, 44 (1949) 79.
[26] R. G. MITTON, *J. Soc. Leather Trades' Chemists*, 29 (1945) 169.
[27] J. R. KANAGY, *J. Am. Leather Chemists' Assoc.*, 42 (1947) 98.
[28] W. GRASSMAN, K. HANNIG AND J. ENGEL, *Z. Physiol. Chem.*, 324 (1961) 284.