

DENATURATION OF COLLAGEN VIA HEATING: An Irreversible Rate Process

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■ **Abstract** Heating therapies are increasingly used in cardiology, dermatology, gynecology, neurosurgery, oncology, ophthalmology, orthopedics, and urology, among other medical specialties. This widespread use of heating is driven primarily by the availability of new technology, not by a detailed understanding of the biothermomechanics. Without basic quantification of the underlying physical and chemical processes in terms of parameters that can be controlled clinically, identification of preferred interventions will continue to be based primarily on trial and error, thus necessitating large clinical studies and years of accumulative experience. Perusal of the literature reveals that much has been learned over the past century about the response of cells, proteins, and tissues to supra-physiologic temperatures; yet, the associated findings are reported in diverse journals and the underlying basic processes remain unidentified. In this review, we seek to contrast various findings on the kinetics of the thermal denaturation of collagen and to encourage investigators to consider the many open problems in part via a synthesis of results from the diverse literatures.

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INTRODUCTION

The medical literature reveals increased clinical use of the heating of tissues to treat a multitude of diseases and injuries. Examples include the treatment of arteriovenous malformations, atherosclerotic plaques, benign prostatic hyperplasia, cardiac arrhythmias, hyperopia, joint laxity, menorrhagia, Parkinson's disease, skin lesions, and multiple malignancies. Such interventions have not been driven by a detailed understanding of the underlying biothermomechanics. Instead, they have been driven largely by technological advances in the design of the heating devices (laser, microwave, radiofrequency, etc.). Without quantifying the underlying processes, optimal heating strategies—defined as those that produce the desired change in the target tissue while minimizing changes in the surrounding healthy tissues—will remain wanting, and many devices and specific heating strategies will continue to be judged prematurely based on performance in clinical trials that explore incomplete sets of potential protocols. There is, therefore, a pressing need to quantify the coupled effects of clinically controllable parameters, such as temperature level, heating history, chemical milieu, and mechanical loads during and immediately after heating. Only with such information can one perform parametric calculations to identify candidate heating strategies that should then be evaluated in animal studies and subsequent clinical trials.

The manifold clinical effects of supraphysiological temperatures result from underlying molecular and cellular responses, including increased expression of heat-shock proteins, cell death, protein denaturation, tissue coagulation, and ablation. Associated with these heating-induced alterations can be dramatic changes in tissue structure, function, and properties. Tissues are, of course, composites of various cells and proteins, abundant water, and ions. Although the sundry cells and proteins differ in response to supraphysiologic temperatures, it appears that each exhibits some similar characteristic behaviors. Consequently, understanding well the response of a single cell type or protein can provide tremendous general insight. The focus herein is collagen, the most abundant protein in the body, and specifically, the fibrillar type I collagen. Furthermore, because many therapies treating collagenous tissues are associated with denaturation, most attention is given to heating protocols that lead to shrinkage and denaturation.

Developing predictive models of the biothermomechanical response of collagen due to a given clinical protocol requires at least three steps. First, we must quantify the rates of denaturation as a function of the temperature and load history for each biochemical state. Numerous models of the kinetics of denaturation have been developed to account for the effects of heating, but nearly all fail to account for the influence of mechanical loads. Second, we must develop stress-strain relations that account for thermal damage. The mechanical response of the tissue will change with denaturation, and in a recursive manner, heating-induced shrinkage could change the mechanical loading during treatment and thereby, the rate of damage. Moreover, mechanical properties will be different post treatment based

on the extent of the thermomechanical damage. This may well influence reparative processes that are mechano-sensitive. Third, values of thermophysical properties (i.e., specific heat, thermal conductivity, and thermal diffusivity) as functions of direction, load, and thermal damage remain wanting. These properties are needed for accurate numerical modeling of the temperature field because small changes in temperature can significantly alter the rate of denaturation. Although each aspect of the process is equally important, the focus herein is on observations and descriptions of the rate of denaturation during heating.

Much has been learned during the past century about the response of collagen to heating. Contributions to our understanding have come from many academic fields including biochemistry, clinical medicine, protein chemistry, and bioengineering. This increased understanding has been made possible by advances in technologies such as calorimetry, x-ray diffraction, NMR, and spectroscopy. Yet, some of the available data, and the interpretation thereof, appear contradictory, and many of the coupled effects of heating and mechanics remain obscure for lack of appropriate measurements and underlying theory. Uncovering these phenomena will require new insights and well-designed experiments. To help clarify the starting point for further study, this paper reviews some of what is known about the denaturation of fibrillar type I collagen due to heating and highlights some areas for future research.

BACKGROUND

Type I collagen molecules are composed of three polypeptide chains, each consisting of 1300–1700 amino acid residues, the majority of which (~1000) are organized into a central triple helix configuration (1). This triple helix is on the order of 285-nm long and 1.4 nm in diameter, and consists of a repeating triplet, (G-X-Y)_n, where G is glycine and X and Y are often proline or hydroxyproline. The collagen molecule is stabilized by extensive hydrogen bonds, many associated with the hydroxyproline. Type I collagen exhibits a characteristic 67-nm periodicity, which results from quarter-staggered assemblies of 4–5 molecules into microfibrils. These structures are the building blocks for collagen fibers, which are stabilized further by intra- and intermolecular cross-links (many involving lysine or hydroxylysine; see Figure 1). The effects of heating collagen can be “reversible” or irreversible. Moderate heating can result in a local unfolding within the protein, which appears to regain its native structure upon the restoration of normal temperatures. This unfolding may be due to the breaking of a small number of consecutive hydrogen bonds. Severe heating results in a time-dependent irreversible transformation of the native triple helical structure into a more random (coiled) structure (2,3). It is thought that the latter transformation occurs primarily via the breaking of longer sequences of hydrogen bonds that stabilize the triple helix; particular subdomains along the molecule [i.e., between residues 877 and 936; Miles et al. (4)] may be more susceptible to the breaking of consecutive hydrogen bonds. The thermal

COLLAGEN STRUCTURE

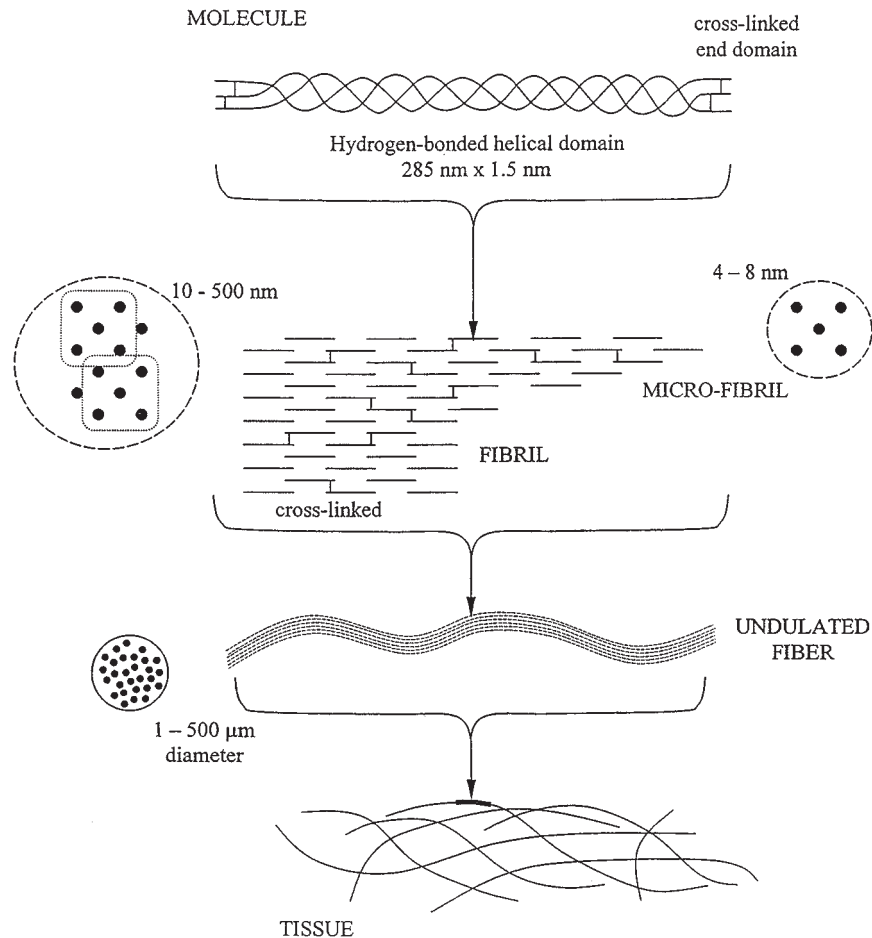


Figure 1 Molecular/fibrillar configuration of Type I collagen (drawing by J. Harris).

stability of collagen increases as the concentration of hydroxyproline increases in an α -helix (5) and as the percentage of reducible cross-links decreases (6). Regardless of the underlying molecular mechanisms, denaturation causes collagen to shrink, which is a convenient gross metric. Indeed, Hörmann & Schlebusch (7) showed that gross shrinkage provides much of the same information on the kinetics of denaturation as many biochemical assays, although not the same detail on underlying mechanisms.

Soft collagenous tissues consist primarily of water, which plays a significant role in governing their gross properties [e.g., see (8, 9)]. Weir (10), for example, demonstrated that the rate of shrinkage, and hence denaturation, increases with increased hydration at a given temperature. The level of hydration may vary, in turn, due to heating. Luescher et al. (11) suggested that "... primary hydration water is set free during the process of denaturation ... it is slowly rebound to the amorphous protein, which thereby undergoes a partial renaturation." Hörmann & Schlebusch (7) further suggest that this partial renaturation is due, in part, to the formation of additional water bridges within the more disorganized molecule. At the tissue level, this imbibing of water likely occurs with characteristic times on the order of hours following heating and the restoration to body temperature. Hörmann & Schlebusch also suggest that renaturation is greater if the tissue is maintained under load because increased load tends to organize the structure and thereby promote reformation of some of the triple-helix structures. This possibility emphasizes the importance of thermomechanical coupling. There is a pressing need to quantify fluid transport due to thermal damage.

EMPIRICAL FINDINGS

Shrinkage has long been used as a measure of heating-induced damage to collagen and collagenous tissues (5, 10, 12); other metrics include changes in enthalpy (13) and loss of birefringence (14). For collagen in solution, changes in viscosity have also been correlated with the degree of denaturation (15, 16). Regardless of the metric, most experiments employ one of two heating protocols: temperature-jump or controlled heating-rate. In a temperature-jump test, the specimen is heated quickly to a specified supraphysiologic temperature and maintained isothermally during the test period. The chosen metric is measured either throughout the isothermal period, such as with video-based measurement of shrinkage, or after the specimen has been cooled quickly to a temperature at which the reaction (i.e., denaturation) rate is imperceptible. Kinetic parameters are evaluated using the results of several tests conducted with different temperature jumps, and in the case of endpoint measurements, with different heating durations at each temperature. Conversely, controlled heating-rate experiments raise the temperature of the specimen over time, usually at a constant rate, and record changes in the metric, typically enthalpy or the force required to maintain the specimen at constant length, during the heating. Kinetic or thermodynamic parameters are then calculated based on an assumed reaction model, e.g., first-order kinetics or equilibrium thermodynamics. An advantage of the temperature-jump experiments is that time and temperature are left explicitly as independent variables, thus easing recognition of their individual roles. A disadvantage is that many tests and specimens may be required to reveal fully the material response. The advantage of controlled heating-rate tests is the comparatively reduced number of specimens, with the disadvantage of increased difficulty in the deconvolution of the reaction process.

Temperature-Jump Tests

In 1949, Weir (10) clearly demonstrated the kinetic nature of denaturation by measuring the shrinkage of kangaroo tail tendon as it was heated in isothermal baths. Shrinkage was hastened by increased hydration and slowed by tanning agents or increased tensile loads. Shrinkage, defined as $\xi_W = (l - l_\infty)/(l_0 - l_\infty)$ where l , l_0 , and l_∞ are the current, original, and “ultimate” lengths of the tendon, respectively, occurred more quickly at higher temperatures ($62.4 \leq T \leq 67.0^\circ\text{C}$). The shrinkage was well correlated with first-order chemical kinetics, with the specific reaction rates related to temperature via the Arrhenius relation or absolute reaction rate theory. The observation that increased uniaxial loading slows the rate of shrinkage is important clinically because tissues are often loaded in vivo and many heating therapies may further stress target tissues. These important findings apparently went largely unnoticed for nearly 50 years. Aware of Weir’s paper only recently, we had independently confirmed and extended his findings (17). That is, we measured shrinkage of bovine chordae tendineae over a wider range of temperatures ($65 \leq T \leq 90^\circ\text{C}$) and loads ($0 \leq P \leq 650$ kPa, where P is the uniaxial first Piola-Kirchhoff stress or actual load per undeformed cross-sectional area). Shrinkage, $\xi_C = (l_0 - l)/l_0$, was defined independent of a “final” state. Figure 2 shows the time course of ξ_C during different isothermal conditions for $P = 500$ and $P = 650$ kPa. Compare the shrinkage history at 80°C , which is shown by “+” symbols in both panels. The behavior is similar, but delayed at $P = 650$ kPa as compared with $P = 500$ kPa. Indeed, all behaviors were similar: There was an initially slow, small-shrinkage regime, then a rapid, large-shrinkage regime, and finally a slow continuing-shrinkage regime. This sigmoidal response suggests three natural characteristic times: τ_1 the time at which rapid shrinkage begins, τ_2 the time at which rapid shrinkage ends, and $\tau_{1/2}$ the half-time of the rapid shrinkage process. The most remarkable finding of these studies is that when the heating time τ is scaled by a characteristic time, curves of measured ξ_C from the 17 different thermomechanical loading protocols collapse to a single “master” curve (Figure 3).¹ This reveals a time-temperature-load equivalency: A specific level of heating damage can be achieved via a multitude of combinations of temperature level, applied mechanical stress, and duration of heating as long as a scaled duration of heating (e.g., τ/τ_2) remains the same. A potentially important clinical consequence of this equivalency is that it allows one to pick from multiple strategies that would achieve the same outcome by different paths. A clinician, of course, must confine the thermal damage to a region of interest (e.g., a tumor). By performing associated bioheat transfer analyses for each candidate temperature-load-duration protocol, one may be able to identify the protocol that achieves a desired result while minimizing damage to surrounding tissue. An analysis of hypothetical heating in an artery (18) shows that preferred strategies are not always

¹We used τ_2 to scale τ since it was measured most reliably, but τ_1 or $\tau_{1/2}$ can be used similarly (22).

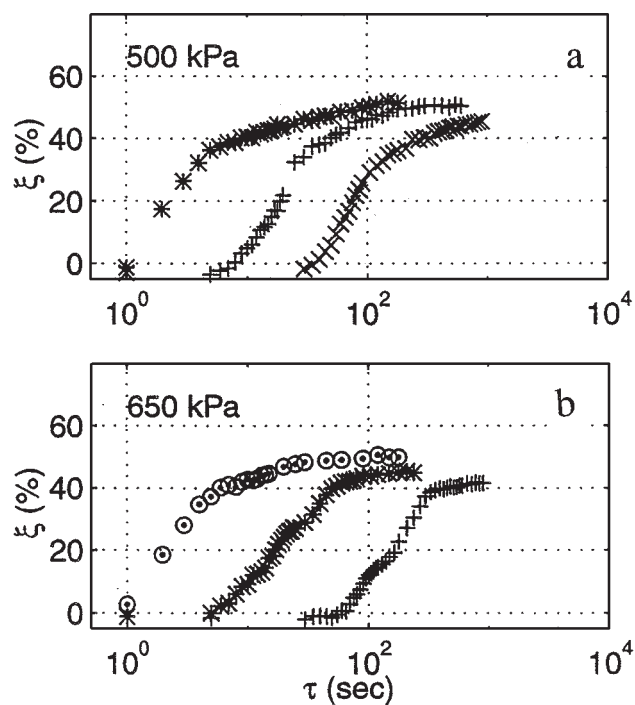


Figure 2 Isotonic shrinkage history for (a) $P = 500$ kPa, and (b) $P = 650$ kPa (17). Symbols represent different constant temperatures. See Figure 4a.

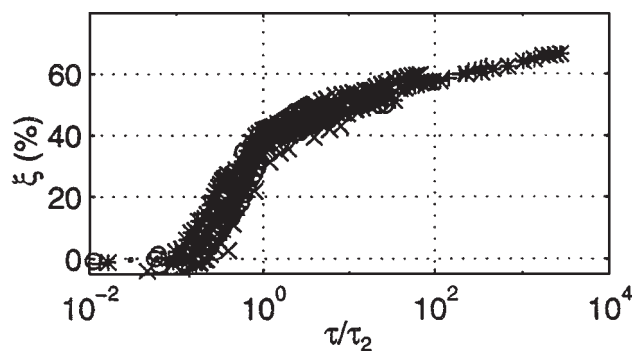


Figure 3 Shrinkage master curve for $65 \leq T \leq 90^\circ\text{C}$ and $0 \leq P \leq 650$ kPa (17).

intuitive. Thus, there is clear motivation to understand and then to exploit this equivalency. Much remains to be uncovered in this regard.

Pipkin (19) suggests that time-temperature “superposition” in polymers—wherein the same viscoelastic response occurs faster at a higher temperature—suggests the existence of an internal material clock. That is, a given process always occurs at the same rate relative to the material’s clock, which speeds or slows with changes in temperature. The advantage of clock models is that constitutive equations need not depend explicitly on T . Moreover, time-temperature equivalency suggests that slow is a relative, not absolute, term because the same process that occurs slowly at “low” temperatures occurs quickly at “high” temperatures. Indeed, consistent with studies of hyperthermia and cell death related to oncology (20), similar outcomes can often be achieved via an order of magnitude decrease in heating time for each increase of 5°C , at least over the range of $55 \leq T \leq 90^\circ\text{C}$ (21, 22).

Figure 4 shows how the characteristic time τ_2 varied with stress P and temperature T during isotonic, isothermal measurements of shrinkage of chordae tendineae. Note the Arrhenius-type behavior. The families of results in Figure 4 suggest that

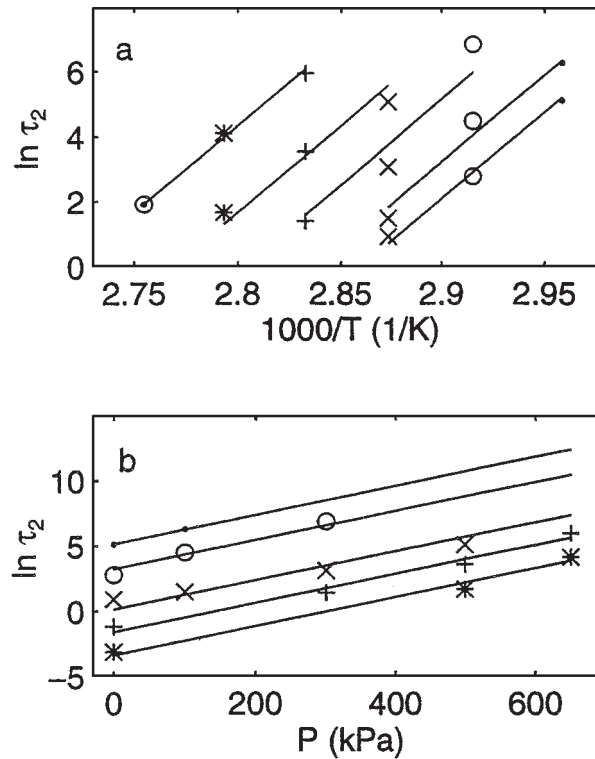


Figure 4 Characteristic times τ_2 for chordae tendineae for $65 \leq T \leq 90^\circ\text{C}$ and $0 \leq P \leq 650$ kPa (17).

$$\tau_2(P, T) = e^{(\alpha + \beta P)} e^{m/T} \equiv A e^{\beta P} e^{m/T}, \quad (1)$$

where α , β , and m are material parameters. This equation reveals that the effect of applied load on the denaturation kinetics is functionally the same as that for the effect of temperature level—both are exponential. It is common to interpret Arrhenius-type behavior (with the characteristic time $\tau_{1/2}$ associated with the maximum rate of the process) in terms of an activation energy E_a . Although this must be done cautiously for the denaturation of a complex (triple helix) protein such as collagen, m would then be interpreted as $-E_a/R$, where R is the universal gas constant. Doing so yields a value of $E_a = 458 \text{ kJ mol}^{-1}$ for our chordae data, which is similar to the 430 kJ mol^{-1} reported for vascular collagen (21). Of course, the nearly constant value of m (i.e., parallel lines in Figure 4) suggests that the energy barrier to denaturation is independent of applied load within the range studied. This is reasonable given that tissue elasticity is mostly due to changes in configurational entropy, not internal energy (23). That is, applied loads increase the “order” of the collagen, thereby decreasing the configurational entropy rather than changing heat-labile bonds and thus the activation energy. This possibility is consistent with the interpretation that the intercept in the Arrhenius plot relates to an activation entropy (4, 24). That the activation entropy S_a may change with the application of load during heating emphasizes again the importance of thermomechanical coupling, which has often been ignored in the past.

Recently, others have measured shrinkage of collagenous tissues. Wall et al. (25) measured the uniaxial isotonic shrinkage of bovine flexor tendon in temperature-jump experiments from 59.5 to 65.5°C. Similar to the finding of Chen et al. (26), the compliance increased with increasing final shrinkage. However, Wall et al. neglected time-temperature-load equivalence and the partial recovery following heating. Likewise, Moran et al. (27) measured the shrinkage of small cylindrical specimens cut from bovine joint capsules. Changes in area were measured optically for temperature levels ranging from 45 to 70°C and heating times up to 60 minutes. The temperature dependence of the specific reaction rate was measured to be significantly greater for temperatures in excess of 63°C.

Loss of birefringence (14, 28) and altered scattering and absorption (29) are optical measures of the response of collagen and collagenous tissues to heating. The rate of change of these optical properties resembles those of the burn response (30). Boedtker & Doty (15) and Burge & Hynes (16) measured the change in optical rotation and viscosity of soluble collagen extracted from carp swim bladders after isothermal heating for 30 minutes at various temperatures from 5 to 45°C. After heating, the collagen solutions were rapidly cooled to arrest denaturation, at which point the viscosity was measured again. Fixing the duration of heating to 30 minutes defined a range of temperatures for which denaturation would occur; at the lowest T , no perceptible change occurred, whereas at the highest T , the change in viscosity appears to have reached an asymptote. Assuming the collagen exists only in native and denatured states, the measured viscosity reflected the concentration of each component. From these data, E_a was calculated using the Arrhenius equation and

S_a was calculated using transition state theory. Boedtker & Doty suggested that the narrow range of temperatures over which shrinkage occurs is approximately E_a/S_a , and that this decreases to approximately 30°C when collagen is dissolved into solution. Burge & Hynes (16) credit this with being the first definition of a “denaturation temperature” T_d .

Controlled Heating Rate Tests

In both differential scanning calorimetry (DSC) and hydrothermal isometric tension (HIT) testing, the temperature of a specimen is raised, often at a constant rate: $r \equiv dT/d\tau \sim 1$ to 10°C/min for $15 \leq T \leq 100^\circ\text{C}$. Changes in the enthalpy of a sample are monitored during DSC tests as the temperature of the sample is increased; denaturation is assumed to occur at a peak in the enthalpy trace. During HIT, one monitors the force needed to maintain a tissue at a fixed dimension; denaturation is assumed to occur when there is a sudden increase in the force. HIT and DSC yield results that are nearly equal for similar scan rates (31). Analyses of DSC and HIT results usually omit explicit time dependence by transforming time to temperature via the scanning rate using

$$T = T_0 + r\tau, \quad (2)$$

where T_0 is the temperature at the start of the test and r the temperature-scanning rate. Thus, changes with respect to time are written

$$\frac{d\Phi}{d\tau} = \frac{d\Phi}{dT} \frac{dT}{d\tau} = r \frac{d\Phi}{dT}, \quad (3)$$

where Φ represents a dependent variable of interest. Many studies then neglect time as an independent variable and cite only temperature because the changes in enthalpy or force have been plotted with respect to temperature.

Calculating the activation enthalpy or entropy (H_a and S_a) from DSC measurements can be difficult for proteins in general (32, 33) and for collagen specifically (4, 13). In addition to the transformation by Equation 3 obscuring the time dependence of denaturation, the high activation energy of the shrinkage of collagen may contribute to the failure by some to recognize denaturation as a rate process (10). Lepock et al. (33) showed that the apparent transition temperature depends on scan rate and that even if it were independent, such a condition is insufficient to justify an equilibrium thermodynamic behavior. Controlled heating rate tests have measured large changes of the metric (e.g., enthalpy, volume changes) on the first scan, with subsequent scans showing little change. For example, the volume changes measured by Flory & Garrett (34), which led, in part, to their conclusion that denaturation results from the reversible melting of a crystalline structure, showed such a trend. Miles & Ghelashvili (35) have since demonstrated that the same functional dependence of volume change on temperature level results when the time dependence and irreversibility of denaturation are included.

Notwithstanding certain limitations, DSC and HIT testing have increased our understanding; they have shown for the first time or confirmed independently that the thermal stability of collagen decreases with increased tissue hydration or increased ionic composition in the bathing solution, but it increases with age (i.e., increased cross-linking) or mechanical load (4, 6, 31, 36–38). Although DSC and HIT tests may continue to yield important information, other limitations must be recognized. Particularly noted here is that DSC tests are typically performed in the absence of applied tractions; hence, they cannot supply information on thermomechanical coupling. Conversely, traditional HIT tests induce more complex deformations than implied; albeit isometric along the axis of interest, the lateral sides are traction-free (i.e., isotonic with an applied load of zero) and analysis of data universally omits the time-dependent shrinkage in the lateral direction. A complete understanding of the thermomechanics of the denaturation process will require various isotonic, isometric, and combined isometric-isotonic tests, each under many different well-controlled multiaxial conditions. Hence, neither traditional DSC nor HIT can be used to evaluate the known, yet poorly understood, coupling between denaturation and the multiaxial mechanical loading that often exists *in vivo*.

MODELS OF HEATING-INDUCED CHANGES

Weir (10) modeled the shrinkage of collagen using first-order reaction kinetics, that is

$$\xi_w = e^{-k(T)\tau}, \quad (4)$$

where $k(T)$ is the specific reaction rate for the shrinkage. Weir noted that multiple reactions likely occur during shrinkage, yet the overall trend is represented well by Equation 4. His analysis considered only one set of isotonic (14-g load) shrinkage data even though he observed a delay in the denaturation due to increased uniaxial load, the use of tanning agents, and, as mentioned above, decreased hydration. Hence, a more general reaction rate would be $k = k(T, \text{load, hydration, pH, } \dots)$. This needs further study.

Closely preceding Weir's study of collagen, Henriques (39) studied the evolution of burns to porcine and human skin (30). A damage parameter Ω was defined by integration with respect to time of an Arrhenius relation, that is

$$\Omega_H(\tau) = \int_{t=0}^{\tau} A e^{-E_a/RT} dt, \quad (5)$$

where A is the pre-exponential or frequency factor, E_a the energy barrier to the reaction, T the absolute temperature, and R the universal gas constant. The integral was assigned a value of $\Omega = 1$ for complete epidermal necrosis; reported values of the parameters were $A = 3.10 \times 10^{98} \text{ s}^{-1}$ and $E_a = 627 \text{ kJ mol}^{-1}$. Clearly, for

isothermal heating, Equation 5 suggests that Ω increases linearly with heating time, and thus the damage integral represents zeroth-order kinetics. Henriques further analyzed these data, noting that damage to tissue likely involves more than a single protein, in terms of transition state theory:

$$\frac{d\Omega}{dt} = \ln\left(\frac{[N]_0}{[N]}\right) = \frac{k_B T}{h} e^{S_a/R} e^{-E_a/RT}, \quad (6)$$

where $[N]_0$ is the initial concentration of undamaged (native) protein, $[N]$ the current concentration, k_B the Boltzmann constant, and h the Planck constant. Equation 6 is equivalent to a first-order kinetics description of the change in concentration of native molecules. Pearce et al. (40) demonstrated that the intensity of light I transmitted through rat skin specimens can be related to Ω using

$$\Omega_P(\tau) = \ln\left(\frac{I_0}{I(\tau)}\right) \quad (7)$$

in studies of changes in birefringence of thermally coagulated collagen. Others have used this concept of the damage integral to correlate heating induced changes in collagen and collagenous tissues (e.g., 41, 42).

Xu & Quan (43) developed a curve fit of the Moritz & Henriques (30) burn data by assuming that the rate of burn injury is equal to the rate of inactivation of an unidentified enzyme. Specifically, based on enzyme deactivation kinetics, the equation developed is

$$\Omega_X(\tau) = \int_{t=0}^{\tau} \frac{B e^{-\alpha z}}{1 + C e^{-\beta z}} dt, \quad (8)$$

where B is a constant, $z = 1 - T_0/T$, $C = S/K_{m0}$, $\alpha = G_d/RT_0$, $\beta = G/RT_0$, where G is the difference in Gibbs energy functions for the formation and decomposition of the substrate-enzyme complex, and G_d refers to the activation energy of enzyme deactivation. The parameters B , C , α , and β are found by regression fits of the data. With the “biological reference temperature of tissue,” T_0 , chosen to be 32.5°C, the curve fit matches well the data of Moritz & Henriques (30) except at the highest temperatures.

Lumry & Eyring (44) proposed a three-state model for the thermal denaturation of globular proteins, with an intermediate state that could either refold to the native state or transform irreversibly to the denatured state. Such a reaction may be written as



where U is the unfolded state, D the denatured state, and $k_i(T)$ the temperature dependent specific reaction rates. This reaction can be modeled by a system of

first-order rate equations:

$$\frac{d[N]}{dt} = -k_1(T)[N] + k_2(T)[U], \quad (10)$$

$$\frac{d[U]}{dt} = k_1(T)[N] - k_2(T)[U] - k_3(T)[U], \quad (11)$$

and

$$\frac{d[D]}{dt} = k_3(T)[U], \quad (12)$$

where $[U]$ and $[D]$ are the concentrations of the reversibly unfolded and denatured states, respectively. The advantage of such an approach is that the relationship between the forward and backward rates of unfolding and the rate of irreversible denaturation may be examined. Lepock et al. (33) examined the three-state Lumry-Eyring model for temperature scan rates covering five orders of magnitude; they omitted the simplifying assumption of thermodynamic equilibrium between the native and unfolded states (32). The analysis included the asymptotically small and large values of the ratio of k_1/k_3 , which reduce to the reversible unfolding and two-state (native and denatured) irreversible denaturation cases, respectively.

Temperatures

Transition temperatures represent a common model of the denaturation process because they contain a single parameter and, as mentioned above, controlled heating rate tests mask time as an independent variable. Sometimes temperatures are assumed to denote a phase transition (e.g., the melting temperature of the crystalline regions T_m), whereas often they are recognized as apparent temperatures that result from the kinetic reaction [e.g., the measured transition temperature T_{app} , Lepock et al. (33)]. Unfortunately, citing these temperatures may lead some to conclude that there is a specific thermodynamically defined temperature for the denaturation of collagen. Thus, although often recorded, T_d is neither a property of the material nor is it uniquely defined for a given sample. Indeed, Privalov (3) suggested that "... only the value [of T_d] obtained by extrapolation to zero heating rate can be used as a characteristic of this process." Yet, whereas $r = 0$ implies the need for isothermal tests, Miles et al. (4) wrote that because T_d "varied logarithmically with scan rate ... it is therefore not possible to quote a value [of T_d] at zero scan rate." Together these two observations correctly imply that different constant temperature levels have different characteristic durations of heating that can yield equivalent denaturation. This is consistent with the aforementioned concept of time-temperature equivalence.

Rule of Mixtures

Danielsen (45) suggests that denaturation kinetics in constant heating rate tests can be described using a simple rule-of-mixtures model. Although such a model can be interpreted as a "two-state model," which is to say that only two molecular

conformations exist in the rapid transition regime, it need not be considered in this way. Given the complexity and incomplete understanding of the biochemical reactions that take place when collagen denatures, it may prove more expedient to adopt a phenomenological approach to modeling (46), one that provides predictive capability without proposing a molecular model based on a host of simplifying assumptions. Hence, consider a mixture model (47) for the master curve in Figure 3:

$$\xi_C(\nu) = [1 - f(\nu)](A_0 + A_1\nu) + [f(\nu)](a_0 + a_1\nu), \quad (13)$$

where A_0 , A_1 , a_0 , and a_1 are material parameters; $\nu \equiv \ln(\tau/\tau_2)$; and $f(\nu) = 0$ for all $\tau < \tau_a$ and $f(\nu) = 1$ for all $\tau > \tau_b$ [τ_a and τ_b are similar to τ_1 and τ_2 ; see Chen et al. (47)]. Danielsen (45) shows that the values of f for $\tau_a < \tau < \tau_b$ can be computed directly from data via $f = \xi_i/(\xi_i + \xi_f)$, where ξ_i (initial) and ξ_f (final) are absolute values of the difference between values of measured ξ and those extrapolated linearly from the initial and final regimes wherein the shrinkage progresses linearly in time. If these values are plotted as $\ln(f/[1 - f])$ versus ν for shrinkage of collagen (17), one obtains a linear relationship, which immediately suggests a specific functional form for $f(\nu)$, namely,

$$\ln\left(\frac{f}{1-f}\right) = a(\nu - \nu_m) \rightarrow f(\nu) = \frac{e^{a(\nu - \nu_m)}}{1 + e^{a(\nu - \nu_m)}} \forall \nu \in [\ln(\tau_a/\tau_2), \ln(\tau_b/\tau_2)], \quad (14)$$

where a is a material parameter and ν_m is the value of ν at $f = 0.5$. Figure 5 shows

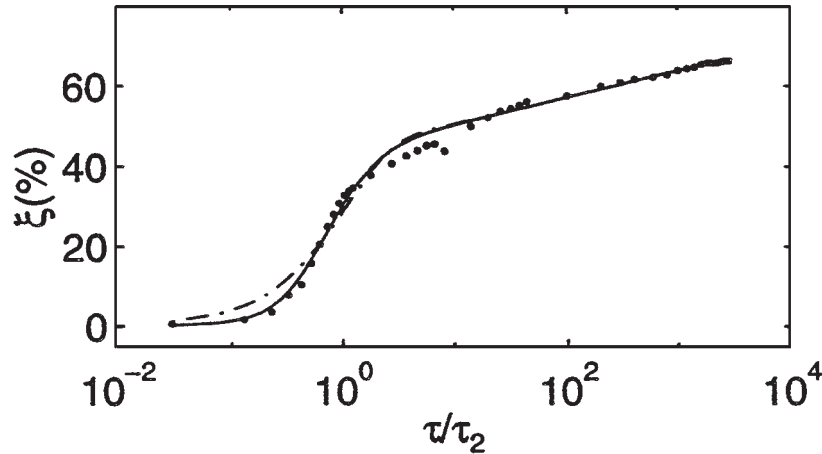


Figure 5 Fit of the mean free-shrinkage response during heating (*symbols*) by the mixture model using two different forms of $f(\nu)$: first-order kinetics (*dot-dash line*) and phenomenological model (*solid line*) (47).

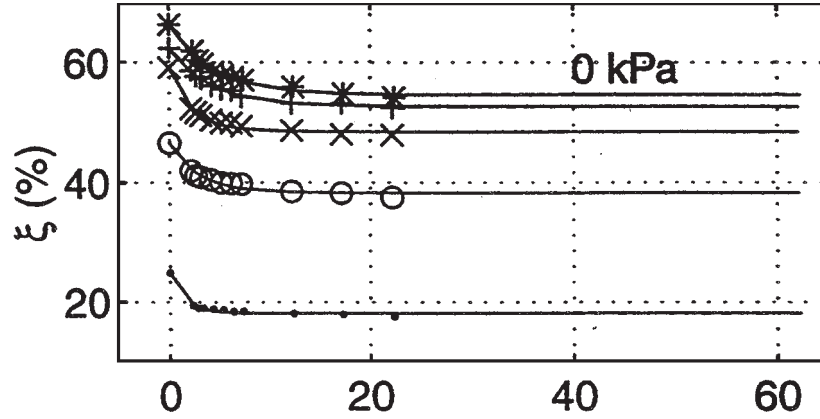


Figure 6 Partial recovery following heating in the absence of load and at 37°C. The solid lines show the fit of Equation 15 (17).

that this model (Equations 13 and 14) fits the master curve well (i.e., shrinkage measurements scaled using time-temperature-load equivalency). Recalling from Equation 1 the scaling that yielded time-temperature-load equivalency, Equations 13 and 14 are the only available descriptors of shrinkage kinetics that include the effect of temperature to hasten the process and load to delay it.

Because of the clinical importance of the immediate post-heating response, Chen et al. (17) unloaded the chordae specimens and returned them to 37°C following heating. This resulted in a small elongation over time t towards an asymptotic shrinkage ξ_e (Figure 6). This partial “recovery” may result from a partial renaturation, which, as noted above, likely relates to the imbibing of fluid to form water bridges in the more random molecular structure. Regardless of the exact mechanisms, this process was well modeled by

$$\xi(T = 37^\circ\text{C}, P = 0 \text{ kPa}, t) = B_0 - B_1(1 - e^{-t/t_c}), \quad (15)$$

where $B_0 - B_1 \equiv \xi_e$, where B_0 is the maximum shrinkage and B_1 and t_c are material parameters.

Chen et al. (47) further demonstrated the utility of the phenomenological model by predicting the response of chordae that were subjected to a series of two-minute heating periods at successively higher temperatures, each followed by a recovery period of 20 minutes at 37°C (see Figure 7). Hence, each specimen would shrink, recover partially, shrink more, and so on. Figure 7 shows the mean data (*symbols*), and the solid line shows the prediction of Equations 13–15 with parameters based on prior data, for which individual specimens experienced only one elevated temperature. The data were predicted well, except that recovery was underestimated following heating at 70°C. Because ξ is a metric of

underlying denaturation, it is easy to see the potential power of such models. One can couple these results with bioheat transfer analyses and compare multiple candidate heating protocols (i.e., vary the temperature, duration of heating, uniaxial load during heating, and intermittent recovery periods) to identify preferred methods.

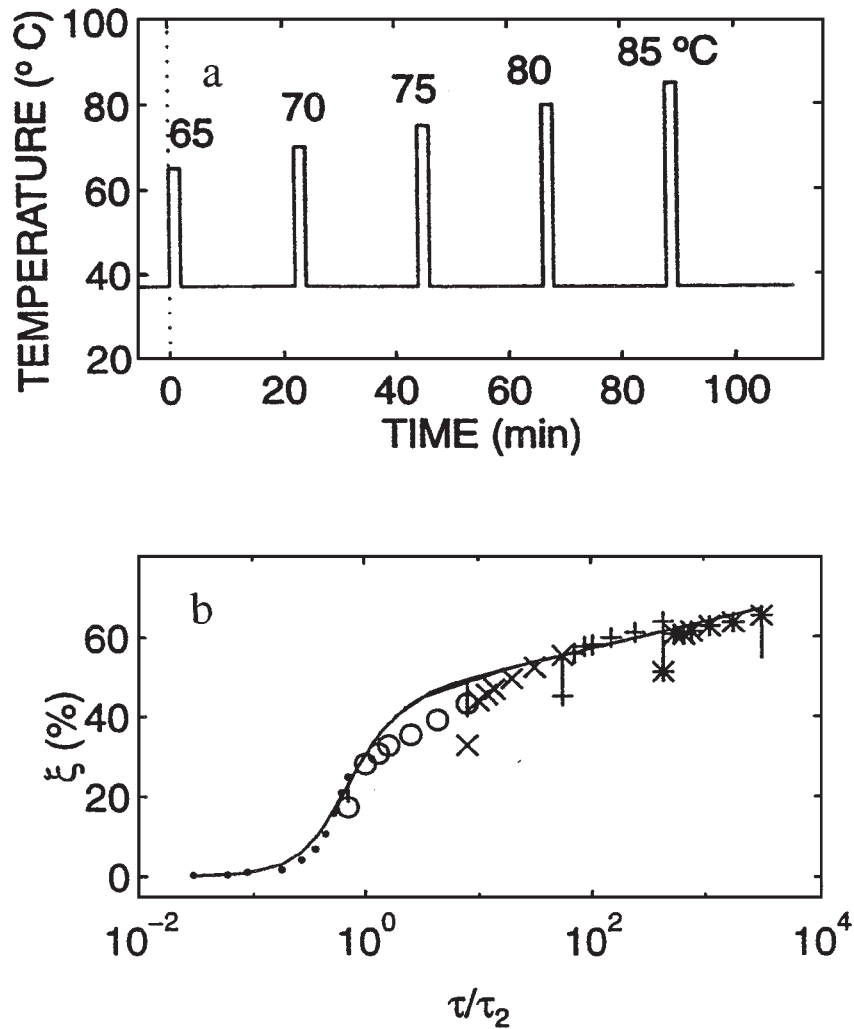


Figure 7 Shrinkage due to successive heating and cooling periods. (a) Saline bath temperatures for multiple heating protocol. (b) Shrinkage predicted using the model of Equations 13–15 with the model parameters developed from results of previous experiments (47).

CLOSURE

As the most abundant structural protein, collagen commands considerable study by researchers in a variety of disciplines. The array of available tests, experiments, and assays should speed discovery of the behavior of collagen under many conditions, and specifically, its denaturation upon heating. Although this advancement continues to come from different communities, the lack of communication across disciplines hinders researchers from availing themselves of the broadest cross-disciplinary understanding. Engineering, with its emphasis on mechanics and transport phenomena, is particularly well suited to uncover the role of mechanical loading and heat transfer in the denaturation of collagen. Though not exhaustive, this review has examined results from biologists, biochemists, biophysicists, physicians, and engineers to illustrate the range of results on the denaturation of type I collagen.

The denaturation of collagen is an irreversible kinetic process that is governed by the temperature history, mechanical load, specimen hydration, chemical milieu, and cross-linking, amongst other variables. The kinetic nature of collagen shrinkage and denaturation has been demonstrated through many empirical findings and, for example, the applicability of time-temperature-load equivalence over wide ranges of temperatures. Conversely, such scaling would not apply if denaturation were a simple phase transition with time dependence being heat transfer limited. The transformation of time to temperature as the independent variable, via the temperature scanning rate, has sometimes obscured the kinetic nature of denaturation. This has then been used to justify the concept of T_s and T_d as material properties.

Although the precise mechanism of denaturation remains unknown, models based on first-order kinetics approximate the denaturation response of collagen and collagenous tissues over limited ranges of independent parameters. It is likely, however, that reactions of other orders occur during denaturation from the native state to gelatin (15, 16). Such reactions may be further evident for collagenous tissues *in situ*, as these contain other constituents, including other proteins such as elastin, cells such as fibroblasts, and proteoglycans. Phenomenological models may better model denaturation than those based on first order chemical kinetics—for example, for the shrinkage of chordae tendineae, which consists predominantly of type I collagen and water with a smaller component of type IV collagen (47). Such phenomenological models have the advantage of applicability over the range of parameters for which they are developed, regardless of the changes in the underlying mechanisms during the process. Nevertheless, significant advances are being made to describe denaturation in terms of the molecular mechanisms (35, 48).

As might have been anticipated from time-temperature equivalence in polymer mechanics, the temperature dependent characteristic times for collagen shrinkage are well described by an Arrhenius relation. Moreover, Chen et al. (17) found that the functional form by which uniaxial tension delays shrinkage during heating is

exponential, suggesting that mechanical loading is as important functionally as is the temperature level during shrinkage. The entropic origin of the elasticity of collagen and the functional form that uniaxial load plays in delaying the rate of denaturation (Equation 1) invites comparison with the role of entropy in the absolute rate theory description (Equation 6) of the specific reaction rate of collagen denaturation. This potential source of the thermomechanical coupling requires careful design of experiments to uncover its true nature and its applicability to multiaxial loading. Furthermore, experiments with multiaxial loading are required to uncover better the thermomechanical response to heating during likely clinical protocols. In this regard, experiments for which both the temperature and mechanical load are changing require careful design and analysis.

Although there has been clear improvement in understanding the molecular mechanisms of damage [e.g., the polymer-in-a-box model; (35)] and the tissue level manifestations of damage, much remains unknown. Questions remain, particularly with regard to the combined effects of heating, multiaxial mechanical loads, and biochemical chaperones as well as the response of collagen to heating via absorption of electromagnetic radiation, which is of clinical importance. Further study is also required of the load-dependent aggregation that collagen experiences following heating, wherein some of its mechanical properties are regained (15). Clearly, the results from the literature on cell death and denaturation of other proteins due to heating aid in the understanding of time-temperature-load equivalency in collagen. Likewise, the similarities of some of the basic mechanisms of the denaturation of collagen may have broader implications to the heating of other proteins and tissues.

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