[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Melting Equilibrium for Collagen Fibers under Stress. Elasticity in the Amorphous State[1]

BY P. J. FLORY AND O. K. SPURR, JR.

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The relationship between the tensile force f and the melting temperature T_m for tendon collagen (rat tail) cross linked with *p*-benzoquinone was investigated in pure water, in 1 *M* KCNS and in 3 *M* KCNS. The latent change in length $\Delta\bar{\bar{L}}$ for the transformation was established as a function of stress and temperature through investigation of the stress–strain–temperature relationships for amorphous collagen in the same media. The heats of transformation $\Delta\bar{\bar{H}}$ under these conditions have been deduced through use of the thermodynamic relationship $[\partial(f/T)/\partial(1/T)]_{p,eq} = \Delta\bar{\bar{H}}/\Delta\bar{\bar{L}}$. The heats of fusion obtained from $\Delta\bar{\bar{H}}$ by correction for the integral heats of dilution associated with swelling of amorphous collagen are 1.35, 1.0 and 0.45 kcal. per mole of peptide unit in the respective media. The result for pure water is supported by early calorimetric measurements of Wöhlisch and de Rochemont and of Küntzel and Doehner. The corresponding entropy of fusion accords with values found for the melting of other polymers. At temperatures well above T_m, the stress for amorphous (shrunken), cross-linked collagen maintained at fixed elongation is approximately proportional to the absolute temperature. The stress is therefore predominantly entropic in origin; the elasticity under these conditions is rubber-like.

Introduction

Characteristic features of the shrinkage of collagen include: contraction to about one-fifth of the length in the native state; disappearance of X-ray crystalline reflections, these being replaced by amorphous halos[2,3]; disappearance of the large optical birefringence of native collagen; a shift in the infrared N–H frequency[4] by 30 cm.$^{-1}$; a distinct, though small, increase in volume[5,6]; an appreciable absorption of heat[7,8]; and, if in contact with water or another swelling medium, an increase in swelling following contraction. Shrinkage occurs abruptly on warming; it may be confined to a range of temperature not exceeding 2 or 3°. It occurs in the vicinity of 60° for virtually all mammalian collagens, irrespective of origin, when the undispersed native fibers are immersed in water. The shrinkage temperature depends however upon the liquid medium. It may be raised by incorporation of cross linkages into the structure[9] or by application of a tensile stress.[7,10–14]

Dilute solutions of native collagen display correlated changes manifested by a sharp decrease in viscosity and, simultaneously, a large decrease in the (negative) optical rotation.[15–17] These changes take place from 35 to 40° in pure water and from 45 to 48° in ethylene glycol.[5] The equilibrium transformation temperatures for concentrated collagen (or gelatin)–diluent mixtures, when plotted against composition and extrapolated to infinite dilution, yield values which fall within these ranges.[5] The transformation observed in dilute solutions of molecularly dispersed collagen and the shrinkage of coherent fibers comprising collagen at much higher concentrations are therefore manifestations of the same process at the molecular level.

The shrinkage of collagen has been shown beyond question to occur directly as the result of melting of the ordered crystalline structure of the native form.[5,12] Well defined latent changes in volume, enthalpy and length, these being confined to temperature ranges of several degrees at most, furnish solid justification for treating the process as a phase change of the first order. The suggestion that it should be so regarded was made long ago,[7,8,18] but contrary opinions prevailed until recently. Partial reversion to the native form has been demonstrated both in dilute solutions[15–17,19] and in mixtures of intermediate composition[5]; the native crystalline form can be regenerated either by cooling transformed material, or, in the case of fibers, by stretching. That the structure thus regenerated is equivalent to that of the native form is demonstrated by coincidence of the temperature of its disappearance upon re-warming with the melting temperature T_m characteristic of the native protein under the same conditions.[5,17] Further evidence for this equivalence is furnished by recent electron microscopic observations[20] on the structure of fibrils

(1) From a thesis submitted by O. K. Spurr, Jr., to the Graduate School of Cornell University, 1958, in partial fulfillment of the requirements for the Ph.D. degree.

(2) W. T. Astbury, *Trans. Faraday Soc.*, **34**, 378 (1938).

(3) R. S. Bear, "Advances in Protein Chemistry," Vol. VII, Academic Press, Inc., New York, N. Y., 1952, p. 69.

(4) J. T. Randall, H. D. B. Fraser, S. Jackson, A. V. W. Martin and A. C. T. Horth, *Nature*, **169**, 1029 (1952).

(5) P. J. Flory and R. R. Garrett, THIS JOURNAL, **80**, 4836 (1958); R. R. Garrett and P. J. Flory, *Nature*, **177**, 176 (1956).

(6) C. E. Weir, *J. Research Natl. Bur. Standards*, **41**, 279 (1948); **42**, 17 (1949).

(7) E. Wöhlisch and R. de Rochemont, *Z. Biol.*, **85**, 406 (1927); E. Wöhlisch, *Biochem. Z.*, **247**, 329 (1932); *Kolloid Z.*, **89**, 239 (1939); *Naturw.*, **28**, 305 (1940).

(8) A. Küntzel and K. Doehner, *Angew. Chem.*, **52**, 175 (1939); A. Küntzel, in "Stiasny Festschrift," Roether, Darmstadt, 1937, p. 191.

(9) K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic Press, Inc., New York, N. Y., 1956.

(10) F. G. Lennox, *Biochim. Biophys. Acta*, **3**, 170 (1949).

(11) E. R. Thies and R. G. Steinhardt, *J. Am. Leather Chemists' Assoc.*, **45**, 591 (1950).

(12) B. A. Wright and N. M. Wiederhorn, *J. Polymer Sci.*, **7**, 105 (1951).

(13) A. V. Tobolsky, R. Haselkorn and E. Catsiff, THIS JOURNAL, **78**, 5957 (1956).

(14) J. F. M. Oth, E. T. Dumitru, O. K. Spurr, Jr., and P. J. Flory, *ibid.*, **79**, 3288 (1957).

(15) J. D. Ferry and J. E. Eldridge, *J. Phys. Chem.*, **53**, 184 (1949). C. Robinson, in "The Nature and Structure of Collagen," Ed. by J. T. Randall, Academic Press, Inc., New York, N. Y., 1953, p. 96.

(16) H. Boedtker and P. Doty, THIS JOURNAL, **77**, 248 (1955), **78**, 4267 (1956). P. Doty and T. Nishihara, in "Recent Advances in Gelatin and Glue Research," Ed. by G. Stainsby, Pergamon Press, New York and London, 1958, p. 92.

(17) P. J. Flory and E. S. Weaver, THIS JOURNAL, **82**, 4518 (1960).

(18) A. Van Hook, *Biodynamica*, **6**, 81 (1947).

(19) P. H. von Hippel and W. F. Harrington, *Biochim. Biophys. Acta*, **36**, 427 (1959).

(20) R. V. Rice, *Proc. Natl. Acad. Sci.*, **46**, 1186 (1960); A. Veis and J. Cohen, *Nature*, **186**, 720 (1960).

regenerated from transformed collagen. The transition is therefore a reversible one.

The heat of fusion of collagen in ethylene glycol has been determined from the dependence of the melting temperature on the composition of the mixture.[5] The heat of fusion may be deduced also from the effect of a tension f on the equilibrium melting temperature through use of the unidimensional form of the Clapeyron equation, which may be written[21]

$$[\partial(f/T)/\partial(1/T)]_{p,eq} = \Delta\bar{\bar{H}}/\Delta\bar{\bar{L}} \qquad (1)$$

where the double-barred quantities represent total changes of enthalpy and length for the process as it occurs when swelling equilibrium is maintained with surrounding liquid. Thus, $\Delta\bar{\bar{H}}$ and $\Delta\bar{\bar{L}}$ include, in addition to the latent changes in enthalpy and in length for fixed composition, the integral changes associated with dilution of the amorphous phase formed by melting.

As we have pointed out previously,[14] the shrinkage temperature T_s, *i.e.*, the temperature at which transformation sets in upon warming native collagen, differs appreciably from the equilibrium melting temperature T_m. Determination of the latter requires coexistence of amorphous and crystalline phases in juxtaposition along the length of the fiber. Initiation of melting in absence of a previously formed amorphous zone along the fiber may require superheating by 10° or more above T_m. Hence, serious errors may arise from failure to discriminate between T_m and T_s. It is the former which must be used in eq. 1.

The present investigation has been concerned primarily with establishing the relationship of the equilibrium melting temperature to the tension applied to rat-tail tendon collagen (RTT) in pure water, in 1 *M* KCNS and in 3 *M* KCNS. Networks of adequate stability have been prepared by cross linking the native tendons with *p*-benzoquinone.[9] These represent a considerable improvement over specimens cross linked with formaldehyde, which were used in the investigation[14] briefly reported several years ago. As a necessary adjunct to the analysis of the stress-temperature relationship for phase equilibrium, we have investigated also the equation of state for amorphous, cross-linked collagen in aqueous media. The observed stress–length–temperature relationships are compared with the theory of rubber elasticity.

Glossary of Principal Symbols[22]

T_s Shrinkage temperature.
T_m Melting temperature, *i.e.*, temperature for phase equilibrium at pressure p, stress τ_{eq}^*, etc.
T^i_m Value of T_m at $\tau_{eq}^* = 0$, other conditions being the same.
V; V^* Volume of swollen polymer; volume unswollen.
L^0 Length of sample when entirely crystalline (native form) and immersed in excess diluent.
L^s Length of sample, totally amorphous, swollen, and at tension f, temperature T, etc.
L_i Length undistorted (isotropic, amorphous state) at the prevailing (swollen) volume V.
$L_i^* = L_i v_2^{1/3}$ Isotropic amorphous length unswollen.
$v_2 = V^*/V$ Volume fraction of polymer in swollen network.
$\alpha = L^s/L_i$ Extension ratio.
f; f_{eq} Tension; tension for equilibrium between crystalline and amorphous phases.
τ^*; $\tau_{eq.}^*$ "Stress" referred to unswollen, undistorted cross section in the amorphous state; "stress" for phase equilibrium.
$\langle r^2\rangle_0$ Mean-square end-to-end distance for free chains.
$\langle r^2\rangle_i^*$ Mean-square end-to-end distance for chains of the unswollen, undistorted network.

Experimental

Collagen Fibers.—The longer tendons were removed from fresh rat tails by the method of Dumitru and Garrett.[23] They were immediately immersed in distilled water at room temperature for a period of about 20 min., the water being replaced several times during this interval. A bundle of about 30 washed tendons was secured by clamps at either end and suspended in a large excess of freshly prepared solution of *p*-benzoquinone in distilled water (*p*H *ca.* 7, unbuffered). Gentle agitation averted depletion of quinone from the solution adjacent to fiber surfaces. At the conclusion of the treatment the bundle of fibers was rinsed with water, then stored in distilled water at room temperature. There was no evidence of deterioration of the samples under these conditions for periods up to one month. Samples were not stored for longer periods prior to use.

The effects of quinone concentration, of temperature and of time of treatment were explored in preliminary experiments. The shrinkage temperature T_s and the melting temperature T_m^i under null stress (see below) were adopted as criteria for the degree of cross linking attained. Results at 25° are shown in Fig. 1. Cessation of further increases in T_s and T_m^i after about 10 hr. suggests that the process is limited by the number of functional groups available for reaction with quinone. The comparatively small effect of quinone concentration is in harmony with this view.

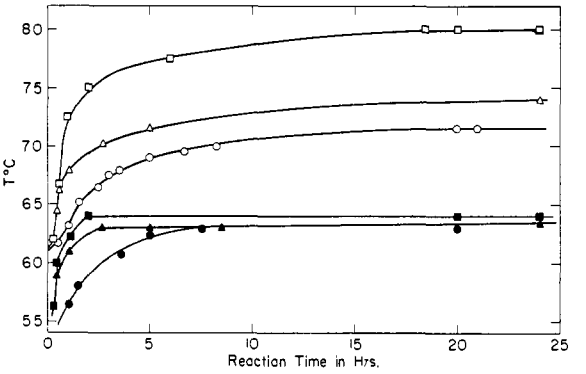

Fig. 1.—Shrinkage temperatures T_s for RTT after treatment at 25° with aqueous benzoquinone solutions at concentrations of 0.01% (○), 0.1% (△), and 1.0% (□) for the periods of time on the abscissa axis. Equilibrium melting temperatures T_m^i for the same preparations are plotted using symbols ●, ▲ and ■, respectively.

Preparations used in principal experiments of this investigation are listed in Table I.

Cross linkages established by treatment with quinone in the foregoing manner withstand heating in water at temperatures up to 80° for periods of 1 to 4 hr. without appreciable change, as evidenced by reproducibility of the stress-strain curve in the amorphous (shrunken) state and by the stress τ_{eq}^* required to maintain equilibrium between crystalline

(21) P. J. Flory, THIS JOURNAL, **78**, 5222 (1956); G. Gee, *Quart. Rev.*, **1**, 265 (1947).

(22) The present notation corresponds with that introduced previously[21] (1956) except for introduction of the asterisk superscript to denote the unswollen state instead of a subscript zero, *e.g.*, L_i^* is used instead of L_{i0}. We thus preserve the subscript zero for the reference state of volume V_0 such that $\langle r^2\rangle = \langle r^2\rangle_0$ when undistorted.

(23) E. T. Dumitru and R. R. Garrett, *Arch. Biochem. Biophys.*, **66**, 245 (1957).

TABLE I

CHARACTERISTICS OF CROSS-LINKED TENDONS

Preparation	Cross-linking conditions; % quinone/ hr. at 25°	T_s in H_2O, °C.	T_m^1 in H_2O, °C.	$C \times 10^5$ mole cc.$^{-1}$	Equil. swelling ratio v_2^{-1} in H_2O at 80°	L_1^*/L^c
(Untreated)	0/0	61	(37)			
A	0.01/3.4	67.5	60	9	3.3	0.139
B	0.5 /0.5	65	57	8	3.3	.137
C	1.0 /1.0	72	62	11	3.2	.147
D	1.0 /20	80	64	27	2.9	.196

and amorphous phases (*cf. seq.*). After an hour at 90°, however, τ_{eq}^* diminished appreciably and the stress-strain curve was detectably lowered. These changes are plausibly attributed to hydrolytic cleavage of peptide bonds, rather than to rupture of cross linkages. Tendons cross linked with formaldehyde displayed a sustained *increase* in τ_{eq}^* at 70° in water. Change in this direction is attributable to elimination of cross linkages.

Single fibers used in the various experiments were carefully selected by visual examination for straightness and apparent uniformity of diameter.

Dynamometry.—The dynamometer was similar to that described previously by Oth and Flory.[24] In brief, the fiber was suspended vertically between clamps immersed in the aqueous medium contained in a cylindrical tube surrounded by a thermostated water-bath regulated to operate within a range of 0.1° or less. The position of the lower clamp was fixed; the upper clamp was suspended from a transducer (range of 0 to 45 g.) mounted on a rack and pinion for adjusting the length. The output of the transducer was delivered to a model G-10 Varian Graphic recording potentiometer requiring 10 mv. for full scale deflection. The length of the sample between clamps, generally 8 to 10 cm. in the native state, was measured with a cathetometer reading to ± 0.005 cm. Small temperature changes (5°) were accomplished by altering the temperature of the bath, larger changes by transferring the dynamometer assembly and sample to another thermostated water bath.

Stress–Strain Isotherms.—Stress–strain curves were established by ascertaining the length required to sustain a given tension at each of a sequence of increasing values of the tension. Approach to equilibrium was hastened, and total degradation during an experiment consequently reduced, by increasing the length sufficiently to raise the tension about 10% above the chosen value for a minute or two, then reducing the length as required to yield the desired tension. A steady value of the length was easily reached within ten minutes by this method; trial experiments demonstrated maintenance of the steady value over longer periods of time.

Stress–strain curves were determined on the same sample at a series of temperatures, usually in intervals of 5°. Repetition of the determination at one temperature after a series of measurements at other temperatures gave good agreement provided that the highest intervening temperature did not exceed 75°. When stress–strain curves were determined at higher temperatures, subsequent experiments yielded stresses which were diminished as much as 10% (90°). The isotropic lengths L_1 were not appreciably altered however.

Determination of the Shrinkage Temperature T_s.—A fiber was mounted in the dynamometer, water or aqueous KCNS solution was added to the sample container, the whole was brought to a temperature about 10° below the expected T_s, and the upper clamp was raised until the transducer registered a barely perceptible force. The length was fixed and the temperature raised at a rate of 0.1° min.$^{-1}$. The temperature at which the tension increased abruptly was recorded as T_s. It was easily fixed within one degree by this procedure.

Sample Dimensions.—The length L^c in the *crystalline* (native) state was determined by plotting tension f, against length, usually at a temperature 2 to 10° below T_s, and extrapolating to $f = 0$. Owing to the high Young's modulus (*ca.* 2×10^4 kg. cm.$^{-2}$) of the native fiber, even at temperatures approaching T_s, the maximum extension over the range of stresses applied in the phase equilibrium experiments (*cf. seq.*) did not exceed 0.5%. Hence, L^c was taken to be constant at its value for $f = 0$.

The length L_1 for the undistorted (null stress) totally *amorphous* fiber was established by plotting f/L^a against $(L^a)^{-3}$ and extrapolating to $f/L^a = 0$, L^a being the length of the amorphous sample at tension f (see Fig. 3). Upon completion of the sequence of dynamometric measurements on a single specimen, it was washed with water to remove salts if present in the solution and then cut away from the clamps precisely at the edges of the clamps. The fiber was dried in vacuum at 80° for 24 hr., then weighed on a quartz spiral balance having a sensitivity of ± 0.01 mg. The weight of the sample being about 1 mg., an accuracy of ± 1% was achieved.

The volume V^* of the anhydrous sample was calculated taking the density to be 1.35 g. cc.$^{-1}$ in both crystalline and amorphous states. (The latent volume change upon melting[5] is only about 5×10^{-3} cc. g.$^{-1}$). The mean cross sectional area for the amorphous isotropic (undistorted) state *in absence of diluent* is given by $A_1^* = V^*/L_1^*$, where L_1^* is the length in this state. L_1^* was calculated from L_1, the length in the isotropic amorphous state at swelling equilibrium with the surrounding medium, according to the relation $L_1^* = v_2^{1/3}L_1$, v_2 being the volume fraction of polymer at swelling equilibrium (*cf. seq.*).

Determination of the Equilibrium Tension for Coexistence of Crystalline and Amorphous Phases.—With the distance between clamps set about 20% less than L^c, the sample and surrounding solution were brought at once to a temperature $T > T_s$. Melting caused formation of one or more nodes of amorphous collagen transversing the fiber; the slack in the fiber was taken up, and tension developed. The temperature was promptly adjusted to the desired level ($> T_m^1$ but sometimes $< T_s$) for determination of the equilibrium stress. The length was then slightly reduced to permit further melting. The tension increased rapidly at first, then gradually approached a steady value. A sudden slight increase in length produced a temporary increase in the tension, which subsequently decreased (evidently owing to recrystallization) toward the previous value. On the other hand, a diminution in the tension consequent upon a slight decrease in length was followed by an increase in f toward its steady value f_{eq}. Attainment of the equilibrium tension was expedited by fine adjustment of the length until the recorded force became constant in time for at least 30 min.

Measurements were repeated at other temperatures on the same fiber. Owing to the imperfect reversion of collagen to the crystalline state, establishment of a new interface by reducing the total sample length for each determination proved to be necessary. The value of f_{eq} at any given temperature was found to be approximately independent of the total sample length and hence of the extent of transformation. This observation attests to the uniformity of individual fibers along their lengths.

Melting temperatures T_m^1 for the isotropic state were obtained by extrapolating T_m to zero tension.

Equilibrium Swelling.—The length and mean diameter of a sample of dried crystalline fiber, 2 to 3 mm. in length with ends cut perpendicular to the axis, were measured with a microscope using the scale of the micrometer eyepiece. The specimen was then placed in a small cell consisting of a pair of optically-flat, circular plates separated by a rubber grommet and filled with the chosen aqueous solution. The cell was placed in a pre-heated hot stage, and the relative dimensions were redetermined after shrinkage and swelling. The dimensions reached approximate constancy after about 30 min. The ratio of the volume calculated from the dry dimensions to that after melting and swelling was taken to represent the volume fraction v_2 of polymer at equilibrium.

This method was insufficiently precise for determination of relative changes in v_2 for the amorphous, isotropic fiber with temperature. The temperature coefficient of swelling was therefore computed from the temperature dependence of L_1 established by dynamometric measurements as related above. Changes in lateral dimensions with temperature were assumed to be proportional to those in L_1; *i.e.*, swelling of the amorphous state was assumed to be isotropic.

Results

Knowledge of the relationship between f, L and T for each coexisting phase is essential for interpretation of the phase equilibrium. The length L^c

(24) J. F. M. Oth and P. J. Flory, THIS JOURNAL, **80**, 1297 (1958).

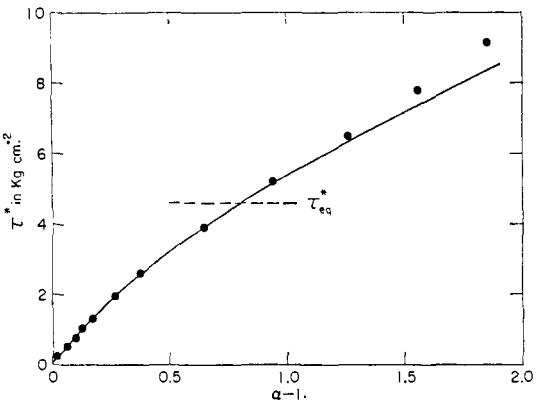

Fig. 2.—Typical stress–strain curve; preparation B in water at 70°. Curve calculated according to eq. 2′.

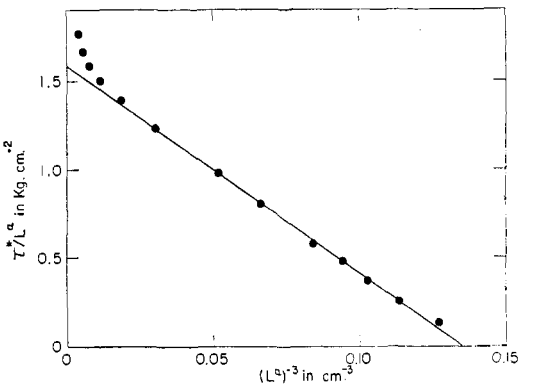

Fig. 3.—Results shown in Fig. 2 plotted as suggested by eq. 2.

of the crystalline form of the native tendon fiber is so nearly independent of f and T as to permit treatment of L^c as a constant. In the amorphous state, however, cross-linked collagen is readily deformable. Accordingly, we turn first to the "equation of state" for amorphous collagen, *i.e.*, to its stress–strain–temperature relationship while in swelling equilibrium with the surrounding aqueous solution. The equilibrium degree of swelling of amorphous collagen and its dependence on temperature will be considered also.

Relationship of Stress to Strain.—Typical stress–strain data, determined in the manner set forth in the Experimental section, are presented in Fig. 2 for an amorphous, cross-linked collagen fiber from preparation B immersed in pure water. The directly measured tensions f were converted to "stresses" $\tau^* = f/A_i^*$ referred to the unswollen cross section A_i^* of the unstretched sample calculated from its length L_i^* and "dry" weight.

The curve shown in Fig. 2 was calculated from the elastic equation of state for rubber elasticity of Gaussian networks[21,25]

$$\tau^* = CRT[(L^a/L_i^*) - (L^a/L_i^*)^{-2}v_2^{-1}] \quad (2)$$

$$= CRT\, v_2^{-1/3}(\alpha - \alpha^{-2}) \quad (2')$$

where R is the gas constant, $\alpha = L^a/L_i = L^a v_2^{1/3}/L_i^*$ is the extension ratio, and C, a function of the structure of the network and of the temperature, is defined by

$$C = (\nu/V^*)\langle r^2\rangle_i^*/\langle r^2\rangle_0 \quad (3)$$

ν is the number of moles of chains in the network, $\langle r^2\rangle_i^*$ is the mean-square end-to-end distance for the chains of the network in the undistorted state of volume V^* and $\langle r^2\rangle_0$ is the mean-square end-to-end distance for the free chains (unconstrained by cross linkages or otherwise) at temperature T. In general, $\langle r^2\rangle_0$ will depend on the temperature.

The same data are plotted in Fig. 3 in such a way as to linearize the relationship according to eq. 2. The curve in Fig. 2 corresponds to the straight line in Fig. 3. Deviations at higher elongations are evident in each figure. The fact that they occur at elongations above that at which $\tau^* = \tau_{eq}^*$, the stress required to maintain a state of equilibrium between amorphous and crystalline zones along the fiber (*cf. seq.*), suggests crystallization induced by stretching as the cause of deviation. In any event, the form of the stress–strain curve agrees satisfactorily at moderate elongations with the theory of elasticity for Gaussian networks.[26] A similar conclusion was drawn by Wiederhorn and Reardon[27] from the stress–strain curves for kangaroo tail tendons at much lower levels of cross linking.

The results shown in Figs. 2 and 3 are typical of stress–strain curves observed for the various samples listed in Table I in pure water. Similar results were obtained also for samples of preparation A in 1 M and in 3 M KCNS.

Values of C for the various quinone cross-linked networks are given in Table I. Also included are the approximate swelling ratios, equal to $1/v_2$, as estimated from measurements of linear dimensions using the microscope. A further indication of the degree of cross linking is afforded by the magnitude of shrinkage in length upon melting. The ratio of the amorphous length L_i in absence of tension to the length L^c in the native state should, according to theory,[21] increase with incorporation of cross linkages in the highly oriented, native state. Inasmuch as L_i is affected by the degree of swelling, a better index is afforded by L_i^*/L^c. These ratios are given in the last column of Table I. They are qualitatively in accord with the degrees of cross linking as represented by the values of C. The results are inadequate however for significant test of the theoretical prediction of proportionality between L_i^*/L^c and the square-root of the degree of cross linking.[21]

Dependence of the Isotropic Length and the Stress on Temperature.—Values of L_i for a series of temperatures were determined by extrapolation of τ^*/L^a to zero as in Fig. 3. Measurements within the range of small stresses sufficed for this purpose. Relative values of L_i so determined for samples of preparation A in water, in 1 M KCNS and in 3 M KCNS are presented in Fig. 4. A considerable spontaneous extension with reduction in tempera-

(25) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, New York, 1953.

(26) Inasmuch as elongation is accompanied by dilation and L_i is defined as the isotropic length at the prevailing volume V, allowance should be made for the consequent increase in L_i with stretching. The effect is not large and for the purposes of the present study may be neglected.

(27) N. M. Wiederhorn and G. V. Reardon, *J. Polymer Sci.*, **9**, 315 (1952). N. M. Wiederhorn, G. V. Reardon and A. R. Browne, *J. Am. Leather Chemists' Assoc.*, **48**, 7 (1953).

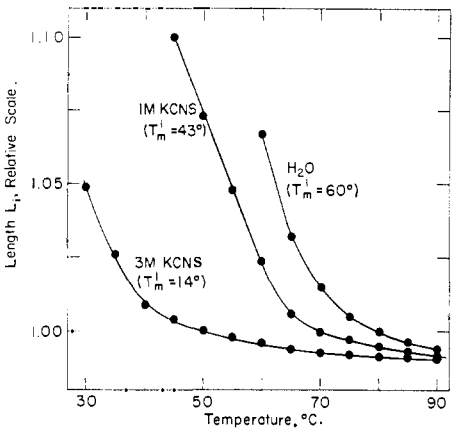

Fig. 4.—Isotropic lengths (undistorted state) for amorphous, cross-linked RTT (preparation A) in the media indicated.

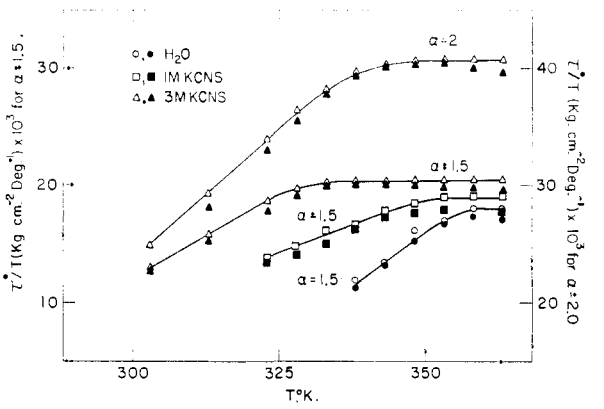

Fig. 5.—Stress-temperature ratios for amorphous, cross-linked RTT (preparation A) at fixed extension ratio α as functions of the temperature under the conditions indicated. Open points represent initial measurements taken in order of decreasing temperature; filled points represent subsequent determinations with rising temperatures.

ture is evident in each case. Presence of KCNS reduces the temperature coefficient at elevated temperatures and shifts the location of the steep rise in L_i toward lower temperatures.

The stress–strain curves determined at various temperatures provided, by interpolation, values for the stress at fixed extension ratio α as a function of temperature. Improved results were secured, however, through performance of separate experiments in which the tension was measured as the temperature was varied at fixed $\alpha = L^a/L_i$. Fulfillment of this condition required alteration of L^a in proportion to the change in L_i with temperature, as prescribed by results such as those shown in Fig. 4. Stresses thus determined at fixed extension ratios are presented in Fig. 5, where τ^*/T is plotted against temperature.

The marked increases in length (Fig. 4) and decreases in τ^*/T (Fig. 5) with reduction of temperature in the lower temperature ranges suggest onset of crystallization. That crystallization should occur at temperatures well above the equilibrium melting points T_m^i established for the respective media as noted in parentheses on Fig. 4 is surprising. The degree of crystallinity required to account for the changes observed is small, however, and may be restricted to selected portions of the polymer chains which, by virtue of the amino acid residue sequence or of the placement of cross linkages, are especially inclined to adopt the crystalline arrangement. (If the structure were uniform throughout, crystallinity should not occur in detectable amounts at temperatures appreciably above T_m.) The fact that the anomaly extends up to a higher temperature for $\alpha = 2.0$ than for $\alpha = 1.5$ (Fig. 5) is further evidence pointing to crystallization as the cause.

More significant is the fact that at sufficiently high temperatures τ^*/T reaches constancy with temperature, within experimental error, in each of the three media. Simultaneously, $d L_i/dT$ becomes very small. Thus, it follows from eq. 2′ that C is in each case sensibly constant. Moreover, the limiting values of τ^*/T for the three media are similar; the agreement is improved if account is taken of the factor $v_2^{-1/3}$ in eq. 2′ using the data of Table II. Thus, C is independent also of the medium, in compliance with theory. This constancy of C implies insensitivity of $\langle r^2\rangle_0$ to temperature and to presence of thiocyanate. This inference finds support in the observation that the intrinsic viscosity of soluble transformed collagen is independent of temperature between 40 and 70° in each of the three media. The results given in Fig. 5 are too crude, however, in comparison with others on systems more amenable to exact measurement, to warrant the conclusion that amorphous collagen fulfills precisely the condition $[\partial(\tau^*/T)/\partial T]_{V,\alpha} = 0$ for ideal rubber elasticity.

TABLE II

SUMMARY OF SWELLING RESULTS

Preparation A

Solvent medium	v_2 at swelling equil. at 80°	χ_1	$(d\, v_2/dT)^a \times 10^4$	$-\chi_1 = T(d\chi_1/dT)$
H_2O	0.30	0.62	4	0.08
1 *M* KCNS	.254	.59	3	.05
3 *M* KCNS	.246	.59	0.8	.01$_5$

[a] Calculated from limiting slopes of Fig. 4 at highest temperatures of measurement.

Swelling Equilibrium and the Enthalpy of Dilution.—The theoretical equation for swelling equilibrium in a tetrafunctionally cross-linked network may be written[21,25]

$$C\nu_1[v_2^{1/3} - (v_2/2)(\langle r^2\rangle_i^*/\langle r^2\rangle_0)^{-1}] = -[\ln(1 - v_2) + v_2 + \chi_1 v_2^2] \quad (4)$$

where ν_1 is the molar volume of the diluent and χ_1 is the parameter expressing the ratio of the polymer-solvent interaction free energy to RT. The value of C, furnished by elasticity measurements, is given in Table I. Values of v_2 are presented in Table II. Since the term in v_2 occurring in the left hand member of eq. 4 is comparatively small, $\langle r^2\rangle_i^*/\langle r^2\rangle_0$ therein may be replaced by unity without appreciable error. The values for χ_1 recorded in the third column of Table II have been thus computed using eq. 4 taking $\nu_1 = 18$ cc. in each instance.

Equation 4 has been derived for binary systems consisting of a polymer network and a single diluent. In applying it to the calculation of χ_1 for the thiocyanate solutions we disregard selective binding of

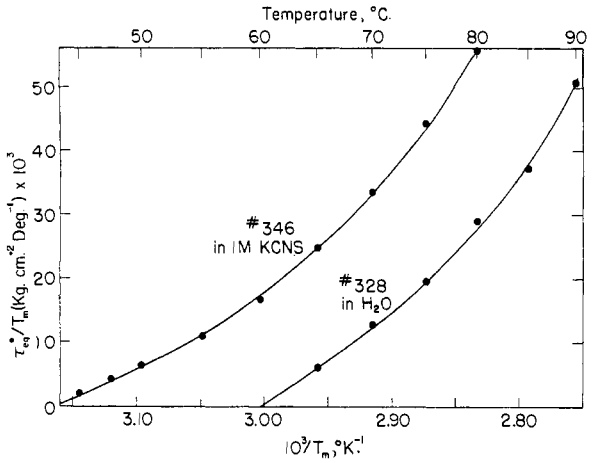

Fig. 6.—Equilibrium stress τ_{eq}^* divided by T_m plotted against $1/T_m$ for two samples, preparation A.

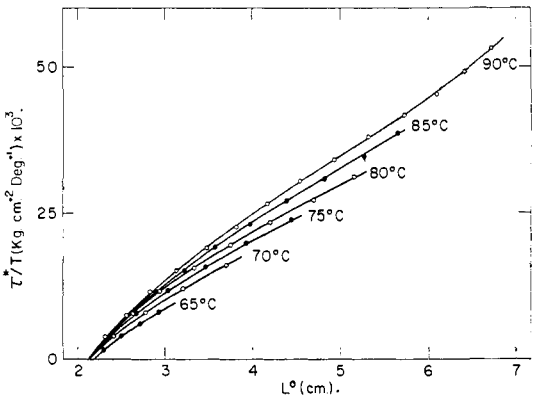

Fig. 7.—Typical set of stress–strain curves for the same sample (#328, preparation A) in water at the temperatures indicated.

the thiocyanate by collagen. As we are primarily concerned with the temperature coefficient and profound changes in binding with temperature are improbable, the error in the absolute values of χ_1 for the thiocyanate solutions arising from the single liquid approximation implicit in use of eq. 4 is of small consequence. Likewise, the arbitrary retention of $v_1 = 18$ cc. for these solutions is a legitimate expedient.

Assuming the length changes occurring in the higher temperature ranges of Fig. 4 to represent isotropic change in the degree of swelling, we calculate from them the changes dv_2/dT in concentration at swelling equilibrium given in the fourth column of Table II.

The enthalpy parameters[25] $\kappa_1 = -T\, d\chi_1/dT$ in the last column of Table II for the various media have been calculated according to eq. 4 from these values of dv_2/dT. While the change of L_i with T from which dv_2/dT is calculated reaches constancy for 3 M KCNS within the range of experiment, the experiments fail to show decisively that the limiting slope has been reached in 1 M KCNS and in pure water. Hence, the values given for dv_2/dT, and likewise for $-\kappa_1$, are to be regarded as upper limits.

The Stress τ_{eq}* for Phase Equilibrium.—Tensions f_{eq} required to maintain equilibrium between crystalline and amorphous phases, visible to the eye as distinct zones transversing the fiber, were determined as described in the Experimental section over the widest feasible temperature ranges. Determinations were carried out in pure water on the various preparations characterized in Table I; preparation A was investigated also in 1 M KCNS and in 3 M KCNS. Typical results are presented in Table III; the data for two of these experiments are also shown in Fig. 6, where the ratio τ_{eq}^*/T_m of the equilibrium stress to the absolute temperature is plotted against the reciprocal of the temperature.

The series of determinations on a single specimen was carried out in order of decreasing temperatures, a fresh interface being established by diminishing the total length upon proceeding to the next lower temperature. In several of the experiments, including the last one presented in Table III, a second set of determinations was carried out after completion of one sequence. The temperature was raised to its previous highest value and the sequence repeated. Comparison with the first set demonstrated the reproducibility of the results and the approximate uniformity of the collagen fiber throughout its length. Appreciable differences in thermodynamic stability of the native form (or in cross-section) along the fiber would have resulted in preferential melting of the less stable zone(s) during the first series. Greater stability of those regions undergoing melting in the second series would have been manifested in lower τ_{eq}^* values, contrary to observation.

Immediately following completion of the phase equilibrium stress determinations, residual crystalline regions were melted and stress–strain curves were determined for the amorphous state in the same medium and at selected temperatures over the range covered in the phase equilibrium experiments. A typical set of stress–strain curves, determined in the order of decreasing temperatures at 5° intervals using the same specimen, is shown in Fig. 7. The lengths for the totally amorphous samples under conditions (T, solvent medium and $\tau^* = \tau_{eq}^*$) equivalent to those prevailing at each point in the phase equilibrium determinations were found by interpolation. They are given in the third column of Table III.

By adoption of this procedure, the length L^a for evaluating $\Delta\bar{L} = -(L^c - L^a)$ was secured from measurements on the same specimen used to establish the relationship of τ_{eq}^* to T_m. Errors arising from differences between fibers from the same preparation, which otherwise would have affected the values for the enthalpy of transformation deduced (see below) according to eq. 1, were thereby avoided.

The enthalpy changes $\Delta\bar{H}$ were calculated according to eq. 1 from the slopes of smooth curves drawn through the points in plots such as those shown in Fig. 6 and from $\Delta\bar{L} = L^a - L^c$, L^c being treated as constant with temperature and stress. Values determined in this manner at the location of each measured equilibrium stress are tabulated in the last column of Table III, the results being expressed in cal. per cc. of dry collagen. Averages over the range of measured stresses for each experi-

TABLE III

CRYSTALLINE-AMORPHOUS EQUILIBRIA—TYPICAL RESULTS

T_m, °C.	τ_{eq}^*, kg. cm.$^{-2}$	L^e, cm.	$\Delta\bar{H}/V^*$, cal. cc.$^{-1}$
Fiber 328, preparation A in H_2O			
$V^* = 1.02 \times 10^{-3}$ cc.; $L_i^* = 1.40$ cm.; $L^o = 9.93$ cm.			
90.0	18.3	6.51	17.9
85.0	13.3	5.55	18.2
80.0	10.3	4.90	19.1
75.0	6.9	3.92	19.2
70.0	4.4	3.28	17.6
65.0	2.0_5	2.70	16.9
Fiber 346, preparation A in 1 M KCNS			
$V^* = 0.94 \times 10^{-3}$ cc.; $L_i^* = 1.32$ cm.; $L^o = 9.59$ cm.			
80.0	19.7	8.00	11.6
75.0	15.4	6.90	13.8
70.0	11.5	5.80	13.1
65.0	8.4	5.00	12.2
60.0	5.6	4.13	12.8
55.0	3.6_5	3.55	12.5
50.0	2.1	3.15	12.0
47.5	1.4	2.95	12.0
45.0	0.7	2.78	11.8
Fiber 116, preparation D in H_2O			
$V^* = 1.32 \times 10^{-3}$ cc.; $L_i^* = 1.81$ cm.; $L^o = 9.07$ cm.			
80.1	6.4_5	3.22	12.6
75.2	4.2_5	3.05	11.8
71.0	2.3_5	2.89	10.9
67.5	0.9_5	2.78	9.0
80.0	6.3	3.21	12.6[a]
74.6	3.7	3.00	11.9[a]
70.9	2.3	2.88	10.9[a]
67.0	0.8	2.77	9.0[a]

[a] This set of values was taken from the curve drawn through both sets of points; hence the two sets of enthalpies are not independent.

ment are given in the second column of Table IV. Averages of these results are given in the third column, and heats of transformation $\Delta\bar{H}_u$ per peptide unit (average molecular weight 93) calculated from them are recorded in the last column of this table.

TABLE IV

ENTHALPIES OF MELTING

Preparation and diluent	$\Delta\bar{H}/V^*$ in cal. cc.$^{-1}$: Separate expts.	Average	$\Delta\bar{H}_u$, kcal. mole^{-1}
A, Water	18.2, 17.6, 16.7	17.5	1.21
B, Water	16.5, 16.7, 19.5	17.5	1.21
C, Water	16.1, 18.7	17.4	1.20
D, Water	11.1, 12.8, 12.4	12.1	0.83
A, 1 M KCNS	12.4, 11.8, 13.6	12.6	.87
A, 3 M KCNS	7.5, 5.1, 6.0	6.2	.43

An alternative method for deducing $\Delta\bar{H}$ involves use of the expression obtained by eliminating τ_{eq}^* through substitution of eq. 2 (for the elastic equation of state of the amorphous polymer) in eq. 1 and integrating the resulting differential expression.[21] Results deduced in this manner did not differ significantly from those of Table IV obtained by the slope method set forth above. The integral method is open to objection on the grounds that it presumes adherence to the equation of state 2, C and L_i^* being constant with both temperature and strain. That cross-linked amorphous collagen deviates from this relationship at lower temperatures is apparent from the results shown in Figs. 4 and 5. It is possible to show, however, that these deviations in L_i and τ^*/T_m, respectively, affect the value of $\Delta\bar{H}_u$ oppositely. Their mutual compensation may explain the otherwise fortuitous agreement between the two methods for determining the enthalpy change. The slope method has been preferred inasmuch as it involves directly measured quantities only.

The results on samples of preparation A are of greater accuracy than those reported in Table IV for the other preparations. This is in part due to the greater stress ranges covered—up to 10 to 20 kg. cm.$^{-2}$ for A, compared with 5 to 8 kg. cm.$^{-2}$ for B, C and D. The low value of $\Delta\bar{H}_u$ found for preparation D is considered spurious rather than indicative of a genuine reduction in the enthalpy change owing to the higher degree of cross-linking of this preparation (see Table I). It has therefore been ignored in summarizing the results in Table V. The mean values given for $\Delta\bar{H}_u$ are believed to be correct to ± 15%.

TABLE V

SUMMARY OF RESULTS

Diluent	$\Delta\bar{H}_u$, kcal. mole^{-1}	$\Delta H_{sol.}$, kcal. mole^{-1}	ΔH_u, kcal. mole^{-1}	T_m^i, °C.	ΔS_u, cal. deg.$^{-1}$ mole^{-1}
Water	1.2	−0.15	1.3_5	60	4.1
1 M KCNS	0.8_7	− .10	0.9_7	43	3.1
3 M KCNS	0.4_2	− .03	0.4_6	14	1.6

Correction for Heat of Dilution.—Under the conditions of determination of the stress for phase equilibrium the sample functions as an open system in equilibrium with surrounding diluent. The enthalpy change $\Delta\bar{H}_u$ therefore includes a contribution from the integral heat for the dilution accompanying transformation. Before proceeding with the estimation of this contribution from the enthalpy of dilution parameter κ_1 given in Table II, the role of specific interactions between functional groups of the protein and the components of the diluent requires examination.

The collagen fibers in their native state contain appreciable quantities of liquid; in fact, the measured volume fraction of water may approach that for swelling equilibrium in the amorphous state. Most of the water present in native collagen is loosely held between the collagen helices (protofibrils) and perhaps also in structural voids. Only about 10% is tenaciously combined,[28] presumably through hydrogen bonds with polar groups, and consequently more difficult to remove.

Native collagen immersed in water may therefore be regarded as a crystalline protein solvated with a small proportion of firmly bound water within the crystal lattice. The status of additional water retained by the structure differs little from that of the surrounding solvent, hence it need not be distinguished from the latter. A similar distinction between bound solvent species and those loosely retained by the structure may be presumed to apply to native collagen immersed in aqueous thiocyanate

(28) H. B. Bull, THIS JOURNAL, 66, 1499 (1944).

solutions, except that the solvate then may include ionic complexes.

In the amorphous state the polypeptide chains enjoy greater freedom to interact with components of the solvent. Hence, solvation should occur to a greater degree than in the crystalline state. The measured heat of transformation $\Delta\bar{\bar{H}}$ may be considered to consist of a latent heat ΔH for conversion of the crystalline solvate to the amorphous solvate of the same composition and the enthalpy change ΔH_{sol} accompanying dilution of this solvate with additional solvent. Thus

$$\Delta\bar{\bar{H}} = \Delta H + \Delta H_{sol} \qquad (5)$$

Owing to solvation in both crystalline and amorphous states, ΔH may be expected to depend on the solvating medium.

So sharp a distinction between solvent components bound firmly to the protein and those present in the category of "excess" solvent is of course artificial. The fact that measurements on collagen immersed in excess diluent are necessarily confined to composition ranges in which the complexing of polar groups attains saturation justifies its adoption nevertheless. Thermodynamic measurements, including determinations of equilibrium swelling, carried out in the presence of large proportions of diluent necessarily reflect only interactions of the solvated species with solvent. The viewpoint set forth above is operationally valid and free of significant error for quantitative interpretation of present results.

Integration of the familiar van Laar quadratic expression for the partial molar heat of dilution[25]

$$\Delta\bar{H}_1 = RT\kappa_1 v_2^2$$

yields for the integral heat of solution

$$\Delta H_{sol}/V^* = RT(\kappa_1/v_1)(1 - v_2) \qquad (6)$$

The values of ΔH_{sol} given in the third column of Table V have been calculated from this equation using the κ_1 and v_2 values of Table II. Heats of fusion given in the fourth column are derived from data given in preceding columns using eq. 5.

Strict adherence to the concept of a solvate would require v_2 of eq. 6 to refer to the volume fraction of the solvate, rather than of anhydrous protein. Presuming the solvate to consist of a preponderance of protein, the correction involved would not be significant; moreover, the manner of calculation of ΔH_{sol} from v_2, with neglect of the unknown correction, is consistent with the procedure used to calculate κ_1, where the uncorrected v_2 was likewise used.

Errors arising from the difficulties attending determinations of the swelling-temperature coefficient (Table II) contribute a greater uncertainty than that accruing from the issue raised in the preceding paragraph. They are in the direction to render $-\Delta H_{sol}$ too large, the uncertainty being greatest for water for which $-\Delta H_{sol}$ has the largest value. Even in this instance the correction does not exceed the experimental error in $\Delta\bar{\bar{H}}_u$.

Remelting of Recrystallized Collagen.—When appropriately cross-linked collagen fibers are shrunken by heating in a liquid such as water and then cooled, spontaneous re-elongation may be observed.[29] The recrystallization responsible for this partial reversal of the initial transformation may be augmented by application of a very small tension.

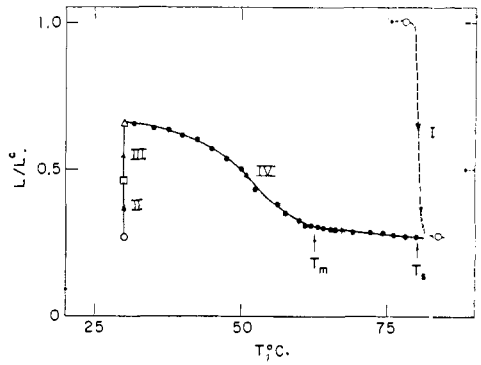

Fig. 8.—Relative length changes in water during initial shrinkage (I), spontaneous recrystallization at 30° (II), strain induced recrystallization at 30° (III) and re-warming (IV). Steps I and IV were conducted under a small tension of 0.2 g. (Sample from preparation D.)

An experiment conducted in pure water to determine the fate of the regenerated crystallinity upon re-warming the sample is set forth in Fig. 8. In contrast to initial melting, the length diminishes gradually over a range of temperature. However, completion of melting, as indicated by convergence of the length to that for the "liquidous," is reached at a temperature within 2° of the initial equilibrium T_m, the location of which is indicated on Fig. 8.

These results parallel those of Garrett[5] and of Weaver.[17] The former investigator observed melting of collagen–ethylene glycol mixtures of intermediate composition by means of dilatometry; the latter studied the transformation in dilute aqueous solutions. In both cases, re-melting was observed to take place over a range of temperature, but completion of the process coincided with the initial melting temperature.

Concluding Remarks.—Wöhlisch and de Rochemont[7] observed increases in the *shrinkage* temperature of bovine tendon in pure water, which amounted to about 20° for a stress of 10 kg. cm.$^{-2}$. In evident agreement with this result, Tobolsky, Haselkorn and Catsiff[13] reported values of 0.63 and 0.33 kg. cm.$^{-2}$ for "df/dT_s," depending on the method of determination. The stresses in both instances were referred to the cross section of the native tendon, whereas we have chosen the cross section for the undistorted, unswollen, amorphous state for expression of our results. The basis of computation of the results quoted is not presented in detail in either instance. The stress-temperature coefficients appear, however, to be nearly an order of magnitude smaller than our values of $d\tau_{eq}/dT_m$ for phase *equilibrium*. Tobolsky and co-workers calculated the entropy of fusion to be 0.005 cal. deg.$^{-1}$ g., which corresponds to $\Delta\bar{\bar{H}}_u = 0.18$ kcal. mole^{-1}. This value is about one-sixth of our result, as recorded in Table V, for collagen in water. The latter is supported by direct calorimetric determinations of the heats of transformation of collagen. Thus, Wöhlisch[7] found $\Delta\bar{\bar{H}}_u = 1.6$ kcal. mole^{-1} for

(29) A. Ewald, *Z. physiol. Chem.*, Hoppe-Seyler's, **105**, 135 (1919).

beef tendon in water, and Küntzel and Doehner[8] found 1.18 kcal. mole^{-1} for hide powder in water.

The tendons used by Wöhlisch and de Rochemont, and those used by Tobolsky and co-workers as well, were not cross linked, apart from cross linkages present in the native tendon or formed adventitiously in the course of the procedure. As noted above, they determined T_s rather than T_m. For these reasons we consider their stress-temperature coefficients to be unsuitable for the application of the thermodynamic equation 1 and the low value of ΔS reported by Tobolsky and co-workers consequently to be in error. The entropies of fusion recorded in the last column of Table V compare favorably with those for other polymers. They are not abnormally low, as claimed by Tobolsky and co-workers.[13]

All of the results cited, in common with those of the present paper, represent enthalpy changes per mole of peptide units present in native collagen rather than per mole of those units which are *crystalline*. To the extent that the native collagen may contain non-crystalline regions, correction is required to obtain the heat of fusion per mole of units which undergo melting. The percentage of crystallinity in collagen is unknown. The physical properties (*e.g.*, elastic modulus) of the native fiber suggest that native collagen is predominantly crystalline, and hence that the mentioned correction is small, if not negligible.

The diluent melting method applied by Garrett[5] to the ethylene glycol–collagen system gives directly the heat of fusion per mole of crystalline polymer. Unfortunately, application of this method to the collagen–water system is fraught with difficulties posed by extensive solvolysis during the long periods of time during which the sample must be subjected to elevated temperatures.[30] For collagen–ethylene glycol, Garrett obtained ΔH_u = 2.25 kcal. mole^{-1}. The smaller value for ΔH_u found by us could conceivably be due to the presence of an appreciable proportion of amorphous material in native collagen. We are inclined to reject this explanation in consideration of the evident high degree of crystallinity of native collagen. We suggest instead that the smaller value of ΔH_u for collagen in water is attributable to an intrinsic difference in solvating effects of the two solvents.

The reduction in the enthalpy of fusion by KCNS is striking. Complexing of collagen with thiocyanate evidently reduces the enthalpy in the amorphous state considerably more than in the native (crystalline) state. This is not surprising in view of the greater accessibility of functional groups in the amorphous (dissolved) state.

It will be observed that whereas 3 M KCNS reduces ΔH_u by a factor of nearly three, it lowers T_m^i only from 333 °K. (in water) to 287 °K. The entropy change ΔS_u accompanying melting, given in the last column of Table V, is reduced proportionately almost as much as ΔH_u. This result is consistent with formation of ionic complexes in the amorphous state, with resultant decrease in entropy.

Finally, we note the somewhat surprising observation of a *decrease* in exothermicity of dilution with addition of KCNS to the medium (see values of κ_1 in Table II). It is as if the protein complex with thiocyanate is *less* hydrophyllic than the hydrate formed with water alone. More effective saturation of polar functional groups by KCNS could conceivably account for this observation.

(30) L. P. Witnauer and J. G. Fee, *J. Polymer Sci.*, **26**, 141 (1957), reported ΔH_u = 7.2 – 8.0 kcal./mole based on the dependence of T_s^i under zero stress on the concentration of water in cross-linked cowhide collagen. Again, the use of T_s^i rather than T_m^i casts doubt on the results.

[CONTRIBUTION FROM THE RESEARCH AND DEVELOPMENT DIVISION, AMERICAN VISCOSE CORPORATION, MARCUS HOOK, PENNSYLVANIA]

Non-Newtonian Viscosity and Flow Birefringence of Rigid Particles: Tobacco Mosaic Virus

BY JEN TSI YANG[1]

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The non-Newtonian viscosity and flow birefringence of tobacco mosaic virus (TMV) were measured over a three-decade range of shearing stress, τ. The hydrodynamic lengths, L, as determined from the two methods were comparable at high τ (after due consideration of the different averages involved). A striking difference in L, however, was observed as τ approached zero, where the viscosity curve went through a shallow maximum as contrasted with the sharp upward curvature for flow birefringence. The concept of equivalent ellipsoid was found applicable even for "fat" rods like TMV, although its equivalent length could differ by about 15% from the true molecular dimension.

Introduction

The theories of non-Newtonian viscosities of rigid particles developed by Saito[2,3] and by Kirkwood and his co-workers[4] afford a new method for the determination of the rotary diffusion coefficient, θ. The validity of the theories were confirmed experimentally in previous publications.[5] These earlier studies, however, were designed to cover as wide a range of shearing stresses as possible (up to 10^5 dynes cm.$^{-2}$) at the expense of precision. For very elongated or flattened particles this is neither necessary nor practical, and it seems desirable, therefore, to further explore this subject by using

(1) Cardiovascular Research Institute, University of California Medical Center, San Francisco 22, California.

(2) N. Saito, *J. Phys. Soc. Japan*, **6**, 297 (1951).

(3) H. A. Scheraga, *J. Chem. Phys.*, **23**, 1526 (1956).

(4) J. G. Kirkwood, *Rec. trav. chim.*, **68**, 649 (1949); J. G. Kirkwood and P. L. Auer, *J. Chem. Phys.*, **19**, 281 (1951); J. G. Kirkwood and R. J. Plock, *ibid.*, **24**, 665 (1956).

(5) J. T. Yang, *J. Am. Chem. Soc.*, **80**, 1783 (1958); *ibid.*, **81**, 3902 (1959).