[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF CORNELL UNIVERSITY]

Phase Transitions in Collagen and Gelatin Systems[1]

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The transformation of native beef Achilles tendon (BAT) and rat tail tendon (RTT) collagen in admixture with ethylene glycol to the amorphous, shrunken state has been investigated over the range of volume fraction v_2 of collagen from 0 to 0.83. Precision dilatometric methods employed for $v_2 > 0.08$ reveal small but unmistakable latent volume changes (*ca.* 5×10^{-3} cc. per g. of collagen). The transformation is partially reversible, the latent volume change being slowly recovered in part upon cooling. Return to the previous liquidus occurs within $\pm 2°$ of the original transformation temperature T_m. These observations show conclusively that the transformation is properly represented as a phase transition, involving melting of crystalline regions, rather than as a rate process. Melting temperatures T_m also have been ascertained by polarizing microscopy and, at high dilution, from the decrease in viscosity. The dependence of T_m on v_2 conforms rather well with polymer melting theory, which yields 24 ± 5 cal. g.$^{-1}$ for the heat of fusion of collagen. A glass-type transition observed both in native collagen–diluent mixtures below T_m and in shrunken dry collagen at 95° probably is associated with freezing-in of side chain disorder. Melting of gelatin gels resembles the re-melting of collagen allowed to re-crystallize by cooling after the initial transformation. The identity of the processes involved is indicated by the observation that T_m for gelatin–glycol is within 2° of that for collagen–glycol of the same composition.

Introduction

The phenomenon of shrinkage of native collagen upon heating while immersed in a swelling agent such as water is well known. Diversity of opinion has persisted, however, concerning the nature of the process responsible for shrinkage. The entire shrinkage, amounting to as much as 80% of the initial length, occurs within a temperature interval of only 2 or 3° if the temperature is raised slowly. The X-ray diffraction pattern disappears simultaneously with shrinkage, but may reappear on lowering the temperature,[2] or, preferably, with drying or re-elongation.[3] Absorption of heat during shrinkage is revealed by calorimetric measurements.[4,5] A small increase in volume during shrinkage has been reported by Weir.[6,7] The shrinkage temperature has been observed to increase with the concentration of the polymer,[8,9] with the tensile force on the fiber[4,9–12] and with the degree of cross-linking introduced by tanning.[13] The last mentioned observation probably is connected with the first inasmuch as cross-linking decreases the concentration of diluent (*i.e.*, water) in the fiber in the shrunken state.

The hypothesis that thermal shrinkage of collagen represents fusion of a crystalline state of organization of oriented polypeptide chains was advanced by Wöhlisch[4] and Küntzel.[14] It was subsequently set forth in a little-known paper by Van Hook[15] in 1947. Wiederhorn and co-workers[10] employed this concept in their interpretation of the influence of stress on the transformation temperature of kangaroo tail tendon collagen. Weir,[6] on the other hand, has advanced the view that the process is a rate-controlled chemical reaction having an extraordinarily high temperature coefficient. Thies and Steinhardt[12] preferred to consider that rupture of inter-chain bonds is responsible for the shrinkage, or "denaturation," a state of equilibrium being quickly attained at each temperature.

The series of investigations reported in part in this paper was undertaken with the primary object of pursuing the hypothesis that shrinkage phenomena in fibrous proteins generally, and that displayed so strikingly by collagen in particular, are manifestations of the melting of crystalline regions, and that the process may be appropriately treated as a phase transition. It was decided to apply the experimental and theoretical methods successfuly adapted in recent years to the melting of conventional polymers.[16] Specifically, precise dilatometric measurements of volume changes with temperature in the transformation region were undertaken, first on beef Achilles tendon (BAT) and subsequently on rat tail tendon (RTT). Early in the investigation it became apparent that solvolysis of collagen in water poses a serious obstacle to experiments on aqueous mixtures. Anhydrous ethylene glycol proved to be more satisfactory on this score and hence was chosen for the principal experiments reported here.

The presence of a latent volume change associated with the transition was verified in experiments on the BAT–ethylene glycol system, and a preliminary report of some of the results of this phase of the present investigation has been pub-

(1) From a thesis submitted to the Graduate School of Cornell University, 1957, by Robert R. Garrett in partial fulfillment of the requirements for the Ph.D. degree. The paper was presented before the Division of Polymer Chemistry at the 132nd meeting of the American Chemical Society, New York, N. Y., September 10, 1957.

The work described in this paper was supported in part by the Allegany Ballistics Laboratory, Cumberland, Maryland, an establishment owned by the United States Navy and operated by the Hercules Powder Company under Contract N Ord 10431.

(2) A. L. Zaides, *Kolloid Zhur.*, **12**, 347 (1950).

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

(4) 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).

(5) A. Küntzel and K. Doehner, *Angew. Chem.*, **52**, 175 (1939).

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

(7) Owing to the occurrence of extensive hydrolysis during Weir's experiments,[6] and perhaps other chemical processes involving the functional groups, it is probable that the volume change he reported included contributions from chemical processes subsequent to shrinkage as well as to shrinkage itself.

(8) E. R. Thies, *Trans. Faraday Soc.*, **42B**, 244 (1946).

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

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

(11) N. M. Wiederhorn and G. V. Reardon, *ibid.*, **9**, 315 (1952).

(12) E. R. Thies and R. G. Steinhardt, *J. Am. Leather Chem. Assoc.*, **45**, 591 (1950).

(13) K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic Press, Inc., New York, N. Y., 1956, p. 227 ff.

(14) A. Küntzel, in "Stiasny Festschrift," Roether, Darmstadt, 1937, p. 191.

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

(16) L. Mandelkern, *Chem. Revs.*, **56**, 903 (1956).

lished.[17] The temperature of the transition was shown to vary with composition in accordance with the thermodynamic theory of polymer melting.[18] It was concluded therefore that the shrinkage of collagen is indeed a melting process not unlike that occurring in other crystalline polymers.[19] Additional results on BAT and on RTT are presented in this paper. It soon became apparent in the course of the present investigation that the abrupt changes in viscosity and optical rotation which occur in dilute solutions of native collagen upon heating to *ca.* 35° in water or to *ca.* 46° in ethylene glycol (*cf. seq.*) are related to the process of shrinkage observed on fibers at high concentrations of the polymer. These changes in properties are manifestations of the configurational transformation of the protofibrillar particles existing in dilute solutions of native collagen, this transformation corresponding in every respect to that occurring in macroscopic fibers, apart from the difference in concentration.[20] Boedtker and Doty[21] have recently adopted this point of view, according to which the dilute solution transformation is regarded as a melting process. It is in fact the low concentration limit of the shrinkage transformation, as is shown explicitly in the present paper.

In consideration of the relationship of gelatin to collagen, the former being essentially the transformation product of the latter, related experiments were conducted on gelatin gels. The setting of aqueous solutions of gelatin to elastic gels is well known.[22] The process is reversible, the rigidity of the gel vanishing upon warming to a fairly well defined temperature; this "transformation" temperature increases with the concentration of gelatin, an observation which is at least qualitatively similar to that cited for collagen itself. The transformation manifested by loss of rigidity of the gelatin gel is accompanied by small but detectable increases in volume[23] and in heat content[24,25] and by a large change in optical rotation.[22] Upon cooling the sol below the transformation temperature, the gel rigidity is not restored immediately,[22] but increases slowly with time toward its former value. Careful measurements have revealed that the volume also decreases slowly with time after cooling below the transformation temperature.[26]

Gelatin gels which have been concentrated by evaporation and allowed to cool display crystalline X-ray reflections[27] at 2.8, 7.8 and 11.5 Å. and in addition an amorphous halo at 4.4 Å. The 2.8 and 11.5 Å. reflections correspond to the crystalline spacings observed in native collagen. They disappear on heating above the transition temperature but reappear after several hours at room temperature.[23,27] At high dilutions the crystal pattern is no longer detectable, as should be expected inasmuch as the degree of crystallinity must decrease with dilution.[28]

Gels which have been oriented by stretching exhibit X-ray diffraction patterns which resemble that of collagen; the characteristic meridional reflection at 2.86 Å. is observed, and an equatorial spacing of 10 to 16 Å., depending on the degree of swelling, as well.[23,29] Concentrated gelatin–water gels which have been stretched and then cooled display a well-defined shrinkage temperature when subsequently warmed.[30] Their behavior in this respect is analogous to the thermal shrinkage of collagen.

Boedtker and Doty[31] concluded from light scattering studies on gelatin sols and gels that aggregation of molecular chains with formation of crystalline bundles is responsible for the development of a gel structure on cooling. They thus reach the conclusion that the gel–sol transformation in gelatin is essentially a melting process.

Long ago (1911) Dhére[32] made the interesting observation that a fibrous precipitate may be caused to form from dilute water–gelatin gels. We have confirmed this finding. Dialyzed aqueous gels ($w_2 = 0.01$) exhibit a gradual increase in turbidity with time at temperatures of 0 to 5°. If the gel is subjected repeatedly to warming to 20° followed by cooling to near 0°, a fibrous, birefringent (when dry) precipitate gradually forms at the expense of the gel phase. As the concentration of residual gelatin is further reduced by precipitation, the latter ultimately disappears in favor of a viscous solution reminiscent of protofibrillar solutions of collagen.

The foregoing observations clearly indicate a close correlation between the crystallinity responsible for the gel structure of gelatin and the crystalline state in native collagen, or in recrystallized collagen (*vide infra*). The experiments reported below provide quantitative comparisons with the melting and recrystallization behavior of collagen.

Experimental

Bovine Tendo Achillis.—Tendons were obtained from mature animals directly after slaughter. A 3-inch length of the thickest part of the tendon was chosen, situated directly between the point of division of the tendon (above the hoof) and the muscle insertion. The elastic sheaths were removed with a knife under running tap water. The bared tendons were agitated in 1 *M* aqueous NaBr, the solution being replaced by successive fresh portions until the tendons were free of blood. A minimum of three such one-hour extractions was employed for removal of globular proteins. The tendons were then soaked in successive portions of cold distilled water until bromide ion disappeared from the rinse. The washed lengths were air dried, and fats were removed by extraction with diethyl ether. The moisture content was thereupon adjusted to a weight fraction of water w_1 of about 0.2 to 0.3. Within this swelling range

(17) R. R. Garrett and P. J. Flory, *Nature*, **177**, 176 (1956).
(18) P. J. Flory, *J. Cell. and Comp. Physiol.*, **49**, 175 (1957).
(19) P. J. Flory, *Science*, **124**, 53 (1956).
(20) J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Serie Chem.*, **29**, 223 (1955).
(21) H. Boedtker and P. M. Doty, THIS JOURNAL, **78**, 4267 (1956); **77**, 248 (1955).
(22) J. D. Ferry, in "Advances in Protein Chemistry," Vol. VI, Academic Press, Inc., New York, N. Y., 1948, p. 1.
(23) J. C. Derksen, Thesis, Amsterdam, 1935.
(24) J. R. Katz, *Rec. trav. chim. Pays-Bas*, **51**, 835 (1932).
(25) W. B. Pleass, *Proc. Roy. Soc. (London)*, **A126**, 406 (1930).
(26) R. E. Neiman, *Colloid J. (USSR)* **14**, 170 (1952); **16**, 280 (1954).
(27) J. R. Katz, J. C. Derksen and W. F. Bon, *Rec. trav. chim. Pays-Bas*, **50**, 725 (1931).
(28) O. Gerngross, K. Hermann and R. Lindemann, *Kolloid Z.*, **60**, 276 (1932).
(29) K. Hermann, G. Gerngross and W. Z. Abitz, *Z. physik. Chem.*, **B10**, 371 (1930).
(30) N. Hirai, *J. Chem. Soc. Japan*, **74**, 347, 441, 443, 539, 593, 810 (1953).
(31) H. Boedtker and P. M. Doty, *J. Phys. Chem.*, **58**, 968 (1954).
(32) C. Dhére, *J. Physiol. Path. Gen.*, **13**, 157, 167 (1911).

BAT is somewhat flexible and can be cut fairly easily. Samples of BAT for dilatometric experiments were cut into transverse sections 0.015 to 0.018 inch thick (*e.g.*, samples for which data appear in Figs. 2 and 3) with a slicing machine.

A crude extract of testicular hyaluronidase was obtained from 5-fold diluted whole frozen bull semen by addition of 8% by volume of toluene. The extract was aged at room temperature for 3–4 hours, stirred, centrifuged and decanted.

Tendon sections prepared as described above were placed in approximately ten times their volume of the crude hyaluronidase solution and allowed to digest for 12 hours at 30° in order to remove such of the mucopolysaccharide from the BAT as is solubilized by this enzyme. They were subsequently washed repeatedly in distilled water, and portions of the sample were tested for the disappearance of metachromasia with toluidine blue.

The purified BAT sections were air dried and sorted by hand, damaged sections being discarded. The sections were then dried to constant weight *in vacuo* at temperatures increasing from 30 to 60° as drying progressed. If the hyaluronidase treatment is omitted, cementation of the sections prevents separation of the sections prior to drying and causes further difficulties in the swelling step.

A weighed portion of the dried sample, supported on a fine screen, was placed in a vacuum oven over an evaporating dish containing ethylene glycol (Matheson, Coleman and Bell, b.p. 195–197°) which had been shown by Karl Fischer titration to contain less than 1% of water. The temperature in most instances was maintained initially at 55° and was lowered to 45° as the desired weight fraction of collagen w_2 was approached.

Rat Tail Tendon.—The tendons were excised from the tails, immersed in 0.5 *M* NaH_2PO_4 overnight, rinsed alternately with distilled water and methanol and dried *in vacuo* at 40–45° in accordance with the procedures, described in detail by Dumitru and Garrett,[33] for solubilizing RTT. Samples for which the weight fraction w_1 of diluent exceeded 0.5 were prepared by soaking portions of the dried collagen in glycol until the desired degrees of swelling were indicated by the fiber diameters.[34] The samples were subsequently enclosed in desiccators for three weeks for the purpose of allowing the diluent to become more evenly distributed. The sample composition, and the uniformity of the glycol distribution as well, were established by evaporating small weighed portions in a vacuum oven at 100°, the residue being weighed thereafter.

The dilute solution used for the viscometric observations was prepared by agitating a weighed sample with the diluent for several hours at room temperature, after which the resulting viscous solution was filtered through a coarse glass frit. The concentration was determined by evaporation as above.

Samples for which the weight fraction of diluent was less than 0.5 were prepared by swelling dried tendons in glycol vapor at 45° in the manner described for BAT. Formaldehyde tanning of one sample was accomplished by placing the fresh tendons in 10% aqueous formaldehyde at *p*H 6.5 for 24 hr. at room temperature.

Gelatin.—Knox Special Gelatin Type B (Lot B-136-1) was used. It has been characterized by Gouinlock.[35] The water content was shown by Karl Fischer titration to be reduced to less than one weight per cent. by drying overnight in a vacuum oven at 45°. Samples so dried were swollen in measured quantities of diluent (either freshly distilled water or ethylene glycol) and subsequently heated mildly until dissolved. After careful mixing the samples were ready, without further preparation, for introduction into the dilatometers.

Dilatometry.—The volume changes of interest in this work were so small as to require dilatometers having sensitivities at least an order of magnitude greater than those used in previous studies.[36] In addition, the temperature range investigated is rather broad. Dilatometers of the types illustrated in Fig. 1 were designed to meet these requirements. They were made of Pyrex glass. Sample chambers of dilatometers used for collagen–diluent mixtures had volumes of about 75 cc. Those used for the more dilute gelatin mixtures had capacities of about 200 cc. and were pear-shaped, with pointed end upwards. An additional stopcock of large bore (3 mm.) was attached immediately above the pear-shaped bulb in this modification of the dilatometer for the purpose of facilitating introduction and removal of the more fluid gelatin solutions for which it was designed. All stopcocks were ground by hand using No. 600 carborundum in light oil. They were lubricated with "Apiezon-M" stopcock lubricant. "Trubore" capillary tubing of either 1 mm. or 0.5 mm. i.d. was used, the capillary being graduated in millimeters for a length of about 25 cm.

In most of the experiments reported here, a "dummy" dilatometer was used in conjunction with the dilatometer containing the sample. The former was similar in design to the latter and contained very nearly the same amount of mercury. The difference between the changes in the capillary heights represented (approximately) the volume expansion of the sample.

Dilute gelatin sols were poured into the dilatometer through a funnel having a sufficiently narrow stem to pass directly into the chamber. In the case of more concentrated sols, a large hypodermic syringe, warmed before use, was employed for this purpose. In each case the sol was prevented from entering the capillary tubing by a little mercury previously poured into the sample chamber. After the chamber had been filled with sol, and the stopcock above the chamber closed, the mercury was allowed to enter the dilatometer from the mercury reservoir as far as the tops of both capillaries, and the reservoir stopcock was closed.

The foregoing procedure was not practical in the case of collagen samples. These were introduced directly, under dry nitrogen, into the open end of the sample chamber of the dilatometer (Fig. 1). A spacer bulb formed from a three-inch section of Pyrex tubing, closed at both ends, and of an outside diameter about a millimeter less than the inner dimension of the sample chamber tube, was inserted above the sample. This prevented the sample from being overheated when the dilatometer was sealed. After sealing, the graduated capillary was capped, and the dilatometer was evacuated through the ground glass joint. Only negligible quantities of ethylene glycol were lost upon evacuation for 4 hr. at room temperature. Redistilled mercury was then poured into the dilatometer, and the latter was transferred to a thermostat.

Liquid (water or paraffin oil) thermostat baths were used. Temperature control was provided by thyratron relays[36] used in conjunction with mercury-in-glass regulators of a size affording temperature regulation within ±0.01° or better. Temperatures were measured either directly with a suitable thermometer or, in some cases, more conveniently by observing the mercury column of the "dummy" dilatometer.

After placing the dilatometer in the thermostat at the desired initial temperature, the mercury level was lowered to the bottom of the capillary by applying suction to the mercury reservoir (Fig. 1). The position of the mercury level was recorded and the temperature was altered by an increment of two or three degrees. It was necessary at each stage to withdraw mercury in order to maintain the mercury level within the capillary section of the graduated tube. The quantity removed was of course evident from the change in height brought about by readjustment in the aforementioned manner under constant conditions.[37]

Heating Schedules.—The establishment of equilibrium along the solidus for collagen–diluent mixtures required the presence of melted sections of the fibers in coexistence with

(33) E. T. Dumitru and R. R. Garrett, *Arch. Biochem. Biophys* **66**, 245 (1917).

(34) The length does not change greatly on swelling, hence the swelling ratio $1/v_2$, where v_2 is the volume fraction of collagen, is indicated by the square of the ratio of the swollen to the unswollen fiber diameters.

(35) E. V. Gouinlock, P. J. Flory and H. A. Scheraga, *J. Poly. Sci.*, **16**, 383 (1955).

(36) L. Mandelkern, R. R. Garrett and P. J. Flory, THIS JOURNAL **74**, 3949 (1952); P. J. Flory, R. R. Garrett, S. Newman and L. Mandelkern, *J. Poly. Sci.*, **12**, 97 (1954).

(37) A later modification of the dilatometer substitutes weighing of mercury issuing, or entering, through a capillary tip for reading of the column height. The weighing dilatometer offers the advantage of greater convenience through elimination of the necessity for readjustment every few degrees.

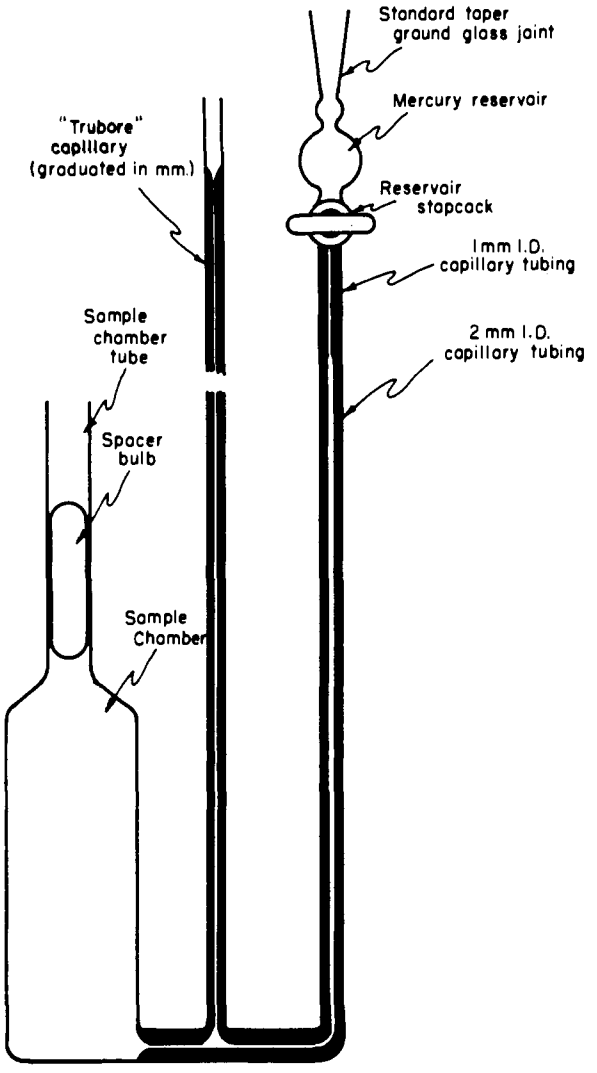

Fig. 1.—Dilatometer design. Dimensions are approximately to scale except over-all length, which was 25 to 30 cm.

the unmelted material. As previously reported,[38] initiation of melting requires superheating above the equilibrium melting temperature T_m. Dilatometers containing collagen were therefore heated several degrees above T_m until partial melting occurred, as indicated by wetting of the dilatometer wall by the sample. The dilatometer was cooled immediately by removing it from the thermostat, whereupon the series of observations designed to establish T_m was commenced.

The susceptibility of collagen to decomposition at elevated temperatures necessitated minimization of the time a given sample was held at each of the higher temperatures. On the other hand, reasonable approach to equilibrium required that the sample be maintained for long periods at each temperature more than 3° below T_m. At all higher temperatures equilibrium was promptly established. In general, the sample was brought rapidly up to a temperature within about 10° of T_m. Volumes were then determined at successively increasing temperatures separated by intervals of one to three degrees. Observations on cooling were conducted as described later.

Gelatin–diluent gels were similarly observed at successive temperature intervals. They were permitted to remain at each temperature for approximately 0.5 hr. over the entire temperature range except for a region covering about 10° below the melting temperature. In the latter region a period of several hours was allowed at each temperature to permit volume equilibration.

Optical Determination of Melting Temperatures.—A polarizing microscope (Leitz, Model CM) equipped with its auxiliary electrically heated hot stage was used. The temperature was controlled to within ±0.5°, and the magnification was 20×. The sample was contained in an air-tight cell having a disk-shaped cavity approximately 2 mm. in thickness and 5 mm. in diameter. Shrinkage of the collagen fibers could be observed directly. Crystallinity was evidenced by depolarization and melting by its disappearance.

In the case of collagen fibers it was necessary, as in the dilatometric experiments, to initiate melting by pre-heating above the shrinkage temperature,[38] complete melting being avoided, however. The desired co-existence of crystalline and amorphous regions in samples of RTT was conveniently achieved by heating a part of the length of the specimen on a spatula.

Viscometry.—Ubbelohde viscometers were used, the temperature being controlled to ±0.003°.

Results

Melting of Collagen–Glycol Mixtures.—The relative volume–temperature relationship depicted in Fig. 2 is typical of native collagen–glycol mixtures in the higher concentration range. A "dummy" dilatometer was used in conjunction with the dilatometer containing the sample, the quantities of mercury in each being very nearly equal. The difference between the two dilatometer readings plotted along the ordinate axis in Fig. 2 represents a relative measure of the sample volume. The various points were taken in the order indicated.

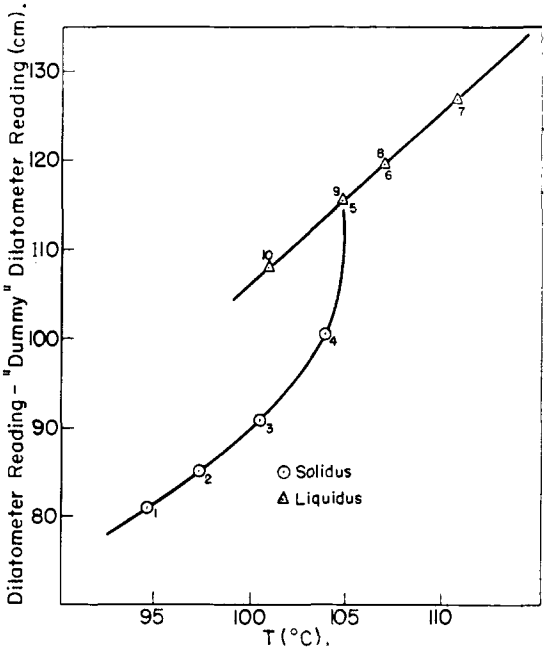

Fig. 2.—Dilatometric results for BAT collagen–ethylene glycol mixture at weight fraction w_2 = 0.775. Sample weight, 8.6 g.; capillary diameter 0.50 mm. Points taken in the sequence indicated by numbers.

Observed volumes along the initial solidus (points 1 to 4 in Fig. 2, for example) invariably diminished gradually with time. Although the drift was reduced by raising the temperature about 5 to 10° above the temperature T of observation for 5 to 10 min. before placing the dilatometer in the bath regulated at T, a very small decrease in vol-

(38) J. F. M. Oth, E. T. Dumitrn, O. K. Spurr, Jr., and P. J. Flory, THIS JOURNAL, **79**, 3288 (1957).

ume persisted even after two days. Within 3° of the melting point T_m and along the liquidus the volume came to equilibrium promptly within 30 min. Although the liquidus could therefore be traversed rapidly, the prolonged exposure of the sample to the fairly high temperatures involved along the solidus in Fig. 2 brought on the commencement of decomposition, with attendant generation of gas bubbles, after point 10; the experiment was therefore discontinued.

The gradual diminution of volume along the initially observed solidus appears to be due to the presence in the native collagen of voids too small to be occupied by mercury. The presence of voids has been demonstrated recently by Stromberg[39] who observed changes in volume when the system comprising mammalian tendon collagen immersed in mercury was subjected to pressures up to 10,000 atmospheres. As a consequence of the pore structure, the volumes observed along the initial solidus in our experiments are fictitiously large; the extrapolation of the liquidus to lower temperatures may even intersect the observed solidus on this account. The slow penetration of mercury into these voids as the temperature is raised, and/or their gradual collapse, is believed to account for the observed decreases in volume with time.

In spite of these difficulties, a small *positive* latent volume change is readily apparent in Fig. 2 at the melting temperature T_m = 104–105°. It amounts to about 18 cm. of column height; this may be taken as a minimum value in consideration of the aforementioned source of error affecting points on the solidus. According to the capillary cross-section and sample weight given in the legend to Fig. 2, the dilatometer displacement corresponds to a latent volume change of about 5×10^{-3} cc. per g. of collagen. Precision dilatometry, involving large samples, small capillaries and good temperature control was required because of this unusually small latent volume change. Of primary significance is the successful demonstration of an unmistakable latent volume change, and its occurrence identifies the location of the melting temperature

A method for plotting the dilatometric results to better advantage is shown for another experiment in Fig. 3. Here a linear volume–temperature relationship was first chosen to represent the liquidus, and the difference between the value of the volume given by this relationship at the temperature T and the observed volume is plotted as "Δy," expressed along the ordinate as cm. of column height. The point at which Δy becomes zero is the melting point; positive values at lower temperatures indicate diminution of the volume owing to the presence of a crystalline phase.

Each of the points 1 to 6 inclusive was obtained by heating to $T + 5°$ for *ca.* 5 min., then holding at temperature T for about 24 hr. The increase in Δy (decrease in sample volume relative to the extrapolated liquid) up to point 6 is attributed to collapse of voids. The volume became constant within 2 hr. at points 7 to 9, hence further exposure to these temperatures was unnecessary. The sample was cooled quickly to point 10, and the reading was recorded immediately upon temperature equilibration. During 63 hr. at this temperature the volume decreased (*i.e.*, Δy increased) to point 11. Recrystallization is thus in evidence. The temperature was then raised in increments of about one degree to point 27, the sample being held at each temperature for 0.5 hr. (Some of the points are omitted for clarity.) Remelting occurred at approximately the same temperature as that initially observed.

Similar evidence for recrystallization was observed for BAT–glycol mixtures having w_2 = 0.56, 0.72 and 0.86, and in the latter two instances remelting with return to the previous liquidus was observed as well. In every instance the melting point obtained on remelting was within ±2° of that found initially.

It is to be noted that the liquidus is not well defined by our measurements; minimization of exposure to elevated temperatures seemed more important than securing the necessary points above the melting temperature. Hence, the linear relationships assumed to represent the liquidus for the purpose of calculating Δy may be in error. This uncertainty does not affect the location of T_m, nor does it invalidate the observation of gradual diminution of the volume after cooling the melt to a temperature some 10 to 15° below T_m. It would be unwarranted however to assert, as Fig. 3 suggests, that cooling from point 9 to 10 occurred without diminution of volume below the liquidus, *i.e.*, that recrystallization did not occur in this interval. A different choice of volume–temperature relationship for the liquid, and its use in computing Δy, would raise point 10, for example, above the abscissa. Whether or not melted collagen can be supercooled without incidence of any recrystallization whatever remains unanswered by our experiments.

Dilatometric experiments on BAT with glycol at concentrations of collagen less than w_2 = 0.5 to 0.6 were not altogether successful owing to failure of the glycol to disperse throughout the sample, with the result that a portion of the diluent was observed to rise to the top of the dilatometer on melting. Evidently, the interfibrillar interstices can accommodate a weight fraction of only about 0.4 of glycol in the native state. The glycol in excess of this amount may not be completely available to the amorphous polymer and therefore may be partially ineffective in lowering T_m. Melting points in the aforementioned range are believed to be anomalously high on this account. Rat tail tendon, which can be dispersed uniformly at all dilutions, was therefore used for determinations at lower concentrations.

In order to establish a comparison between melting points of RTT and of BAT, a dilatometric experiment on the former was performed within the higher concentration range of the experiments on the latter. The experiment represented in Fig. 4 at w_2 = 0.64 was carried out for this purpose. The RTT was first tanned in aqueous formaldehyde as described in the experimental section. The total elapsed time during cooling of the melted sample from points 13 to 18 was *ca.* 2.5 hr. Re-

(39) R. R. Stromberg, *J. Research Natl. Bur. Standards*, **54**, 73 (1955).

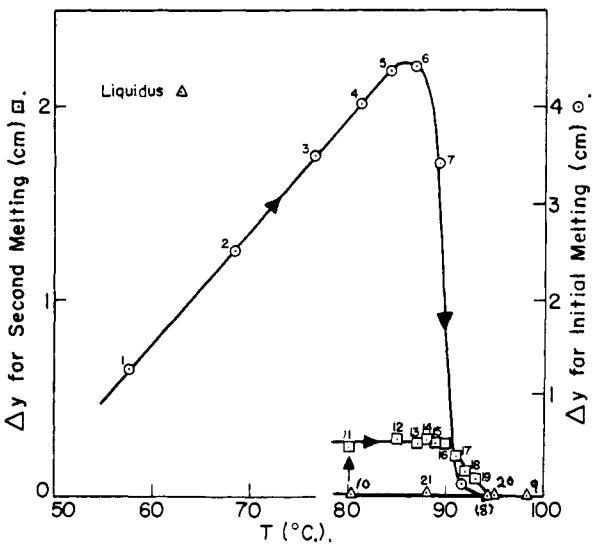

Fig. 3.—BAT collagen–ethylene glycol, w_2 = 0.70. Sample weight 7.5 g.; capillary diameter 1.00 mm. Points taken in the order indicated.

crystallization occurred during 45 hr. between points 18 and 21. Remelting is observed to have been completed at the original melting point. The melting point, 89°, agrees fairly well with that interpolated from the results for BAT (see Table I and Fig. 7).

The experiment shown in Fig. 5 was conducted on a large sample (*ca.* 100 g.) at a weight fraction of RTT of 0.10. The sample was first heated at 55° for a few minutes to initiate melting, after which it

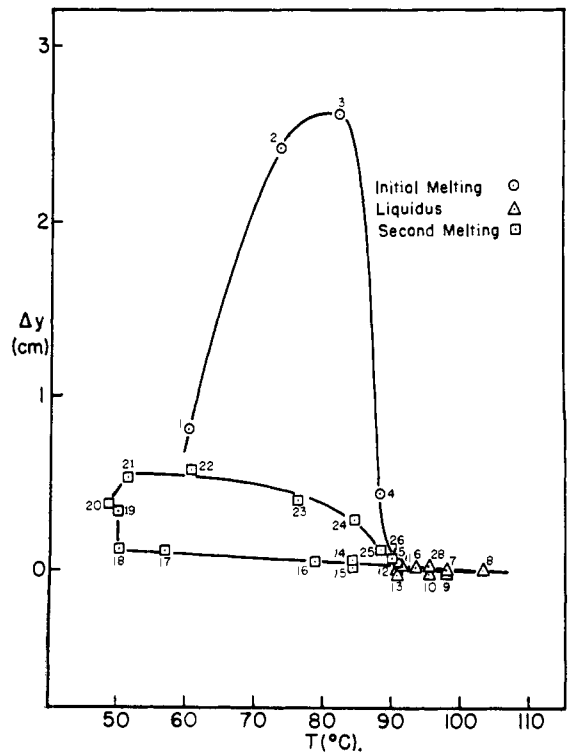

Fig. 4.—Tanned RTT collagen–ethylene glycol, w_2 = 0.64. Sample weight, 7.8 g.; capillary diameter 1.00 mm. Points taken in the order indicated.

was maintained for about 2 hr. at each temperature before recording the dilatometer level. Decreases in volume with time were observed along the solidus. The melting point is well defined.

Melting points for two RTT–glycol mixtures, w_2 = 0.72 and 0.44, respectively, were determined by observing the disappearance of birefringence. Short pieces of tendon were used in each case, shrinkage being initiated over part of the length prior to insertion of the swollen sample in the cell previously described. Preliminary observations indicated the approximate melting point. Thereafter fresh samples were brought immediately to temperatures within one or two degrees of the anticipated melting point, and the temperature at which the birefringence disappeared was determined. Thus, for example, birefringence remained unchanged for 3 hr. at 66° at the higher concentration but disappeared within 1 hr. at 67.5 to 68°. At the lower concentration birefringence persisted at 47° but vanished at 48°.

The relative viscosity of a dilute ($w_2 \cong 5 \times 10^{-4}$) solution of RTT in glycol was measured at a series of temperatures in order to ascertain the transformation temperature at the infinite dilution limit and to observe its relation to the melting

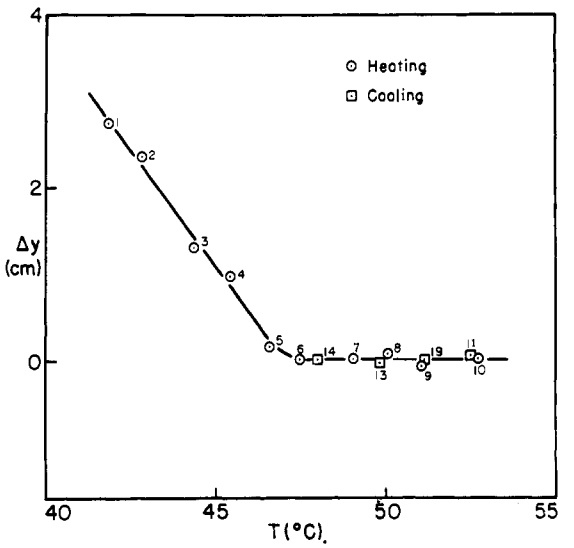

Fig. 5.—RTT collagen–ethylene glycol, w_2 = 0.10. Sample weight, *ca.* 100 g.; capillary diameter 0.50 mm.

temperatures determined at higher concentrations. To this end, RTT washed with monosodium phosphate as previously described, was dispersed in ethylene glycol and the viscosity of the solution was measured after 1 hr. at each temperature. The viscosity of pure glycol also was determined at each temperature. In Fig. 6 the relative viscosity is plotted against the temperature. The decrease in the relative viscosity in the melting range is striking; the similar viscosity decrease for aqueous solutions of native collagen is well known and is attributed to transformation from protofibrillar to random coil form.[21] This process should be fully equivalent to melting at higher concentrations, except for the possible effect of the proximity of protofibrillar elements in the latter case.

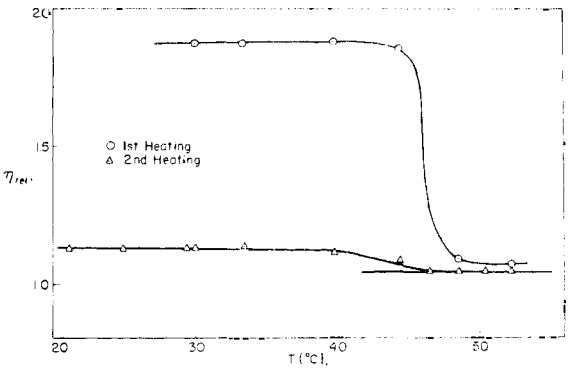

Fig. 6.—Relative viscosity of collagen-glycol solution, $w_2 \cong 5 \times 10^{-4}$ as a function of temperature.

Upon cooling the transformed solution to 21° and holding overnight, a small but definite increase in viscosity was noted. In analogy with the dilatometric experiments, reversion to the protofibrillar (crystalline) form is suggested. Upon warming, the sample was observed to re-melt at a temperature within ±2° of the original T_m. The observation of slightly lower viscosities above T_m after the second melting may be due to solvolytic degradation.

Table I presents a summary of the melting points for various collagen–glycol mixtures. Weight fractions have been converted to volume fractions calculated for the amorphous mixture at 25° using for the densities of glycol and collagen (amorphous) the respective values 1.11 and 1.35. Null volume changes on mixing are implicitly assumed. Also, possible consequences of changes in the volume fraction with temperature are disregarded. Errors of interpretation arising from these sources are trivial.

The melting temperatures are plotted against the volume fraction in Fig. 7. Numbers identify the points with entries in Table I. The points fall smoothly on the curve, (calculated, *cf. seq.*), with the exception of points 5, 6 and 10. Point 6 is believed to be high on account of non-uniform dispersion of glycol through the BAT sample (see above). Point 5 may be in error for a similar reason, or on account of the specific effect of the cross linkages introduced with formaldehyde. The basis for the deviation of point 10 will be postponed for later consideration.

Melting of Gelatin Gels.—The melting of a gelatin–ethylene glycol mixture at $w_2 = 0.10$ is represented in Fig. 8. Several hours was allowed for equilibration at each temperature, except at temperatures above the melting point where readings were taken immediately following thermal equilibration. The latent volume change, though small, is unmistakable. The melting point is only a degree below that found for the RTT collagen-glycol mixture of the same concentration. Thus, while the degree of crystallinity is less for the gelatin, the melting point appears to be nearly the same as for collagen at the same concentration.

TABLE I

MELTING TEMPERATURES FOR COLLAGEN-GLYCOL MIXTURES

	Sample	Method	w_2	v_2	T_m, °C.
1	BAT	Dilatometry	0.86	0.835	118
2	BAT	Dilatometry	.775	.74	104
3	RTT	Polarizing micr.	.72	.68	97
4	BAT	Dilatometry	.70	.66	93
5	RTT-CH_2O tanned	Dilatometry	.64	.595	89
6	BAT	Dilatometry	.56	.51	83
7	RTT	Polarizing micr.	.44	.39	68
8	RTT	Polarizing micr.	.12	.10	48
9	RTT	Dilatometry	.10	.084	47
10	RTT	Viscometry	.0005	.0004	45–48

Melting temperatures similarly determined for gelatin–water mixtures are given in Table II. The determination at the highest temperature is somewhat in doubt owing to evidence of decomposition. Latent volume changes were small, and the forms of the volume–temperature curves were suggestive of those obtained on second melting of the collagen–glycol gels. These observations accord with the presumed low crystallinity in gelatin. The latent volume change is easily observable, however, by present methods.

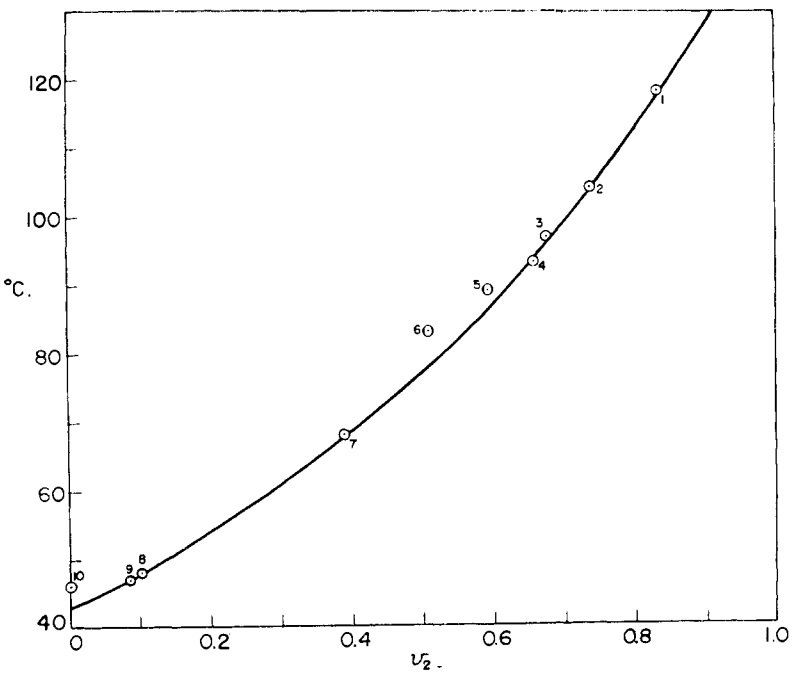

Fig. 7.—Melting temperatures plotted against composition for collagen-glycol mixtures. Numbers identify points with entries in Table I. The curve has been calculated from eq. 3; *cf. seq.*

TABLE II

MELTING TEMPERATURES FOR GELATIN-WATER GELS

w_2	0.040	0.110	0.27	0.45
T_m, °C.	26	31	37	45

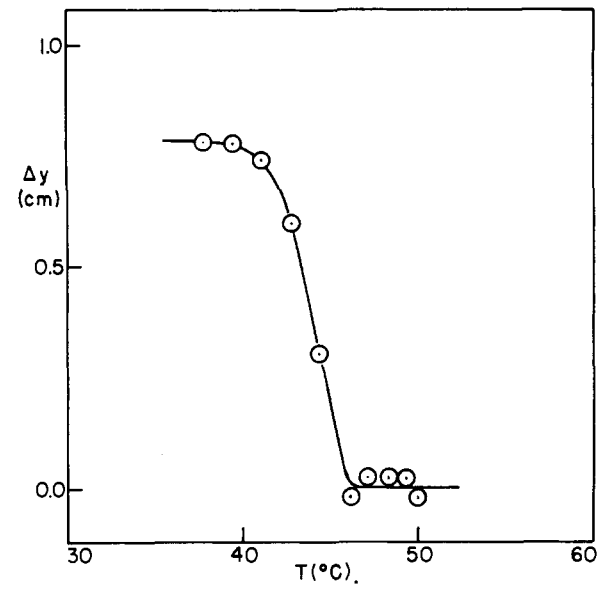

Fig. 8.—Gelatin–ethylene glycol, $w_2 = 0.10$. Sample weight *ca.* 190 g.; capillary diameter 1.00 mm.

The results shown in Table II are in substantial agreement with those of Neiman[26] covering approximately the same composition range. They accord also with melting temperatures obtained by Hirai,[30] who applied a variety of methods at compositions ranging up to $w_2 = 0.75$.

A Glass-like Transition in Collagen.—Preliminary dilatometric studies on native BAT containing various quantities of water revealed discontinuities in the slope of the dilatometer reading plotted against the temperature. An example of such an experiment is presented in Fig. 9. A two-inch length of tendon was placed in a dilatometer after washing the sample with distilled water. Observed volumes were constant with time over the range investigated, and, as indicated in Fig. 9, the volume–temperature relationship was accurately reproducible in succeeding cycles.

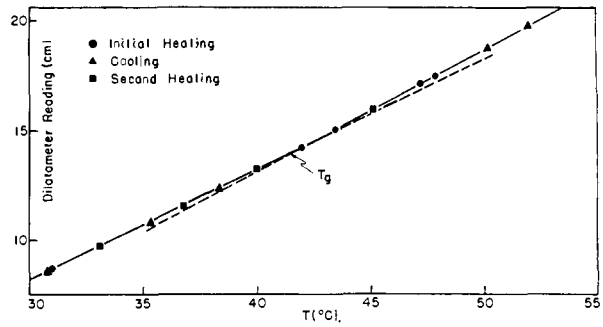

Fig. 9.—Volume–temperature experiment indicating a glass-like transition in native BAT–water mixture; $w_2 \cong 0.4$.

Inasmuch as the melting temperature lies above 60°, the observations relate to the tendon in its native, largely crystalline condition. While the change of slope is small at the point indicated by T_g, a significant change evidently occurs in the neighborhood of 40 to 45°. (The error is much less than the size of one of the points.)

The observation of a glass type transition in native collagen was unexpected in view of the high degree of crystallinity in this form. While it is possible that the transition is confined to the very small fraction of the material which may be in the amorphous condition, it appears more likely to us that the side chains of the collagen molecules are responsible for the transition. According to this interpretation, the side chains lack sufficient mobility below T_g to achieve a state of equilibrium.

The results of an experiment conducted on dried, shrunken BAT collagen are shown in Fig. 10. A sample of tendon similar to that used in the experiment previously tried was dried by heating at 175° for several minutes. Shrinkage was observed to occur during drying. The data were taken rapidly in order to complete the experiment before decomposition became excessive.

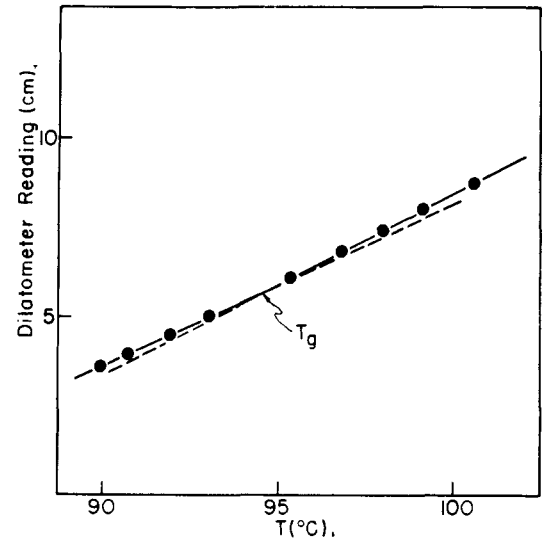

Fig. 10.—Evidence for a glass-like transition in air dried, shrunken BAT in absence of diluent.

An attempt to conduct a similar experiment on the tendon prior to shrinkage failed owing to instability of the volume at each temperature. The slow disappearance of voids is presumed to be responsible for this difficulty.

The observed glass transition temperature in the neighborhood of 95° for dry, shrunken collagen agrees within experimental error with that reported recently by Hirai[30] for dry gelatin.

Discussion

The process of melting of collagen–diluent mixtures presents a complicating feature not ordinarily encountered in the melting of other polymers. This complication arises from the fact that the crystalline phase contains diluent. We do not refer here to diluent present in the interfibrillar cavities, which may indeed serve as repositories of diluent (reluctantly, perhaps, as previously noted), but to the interprotofibrillar spaces within the crystalline phase itself. Absorption of solvent by the ordered phase in this manner is evidenced by the increase in equatorial X-ray spacings with solvation (in water).[3,40] In prescribing the thermodynamic con-

(40) A. L. Zaides, *Kolloid Zhur.*, **16**, 265 (1954).

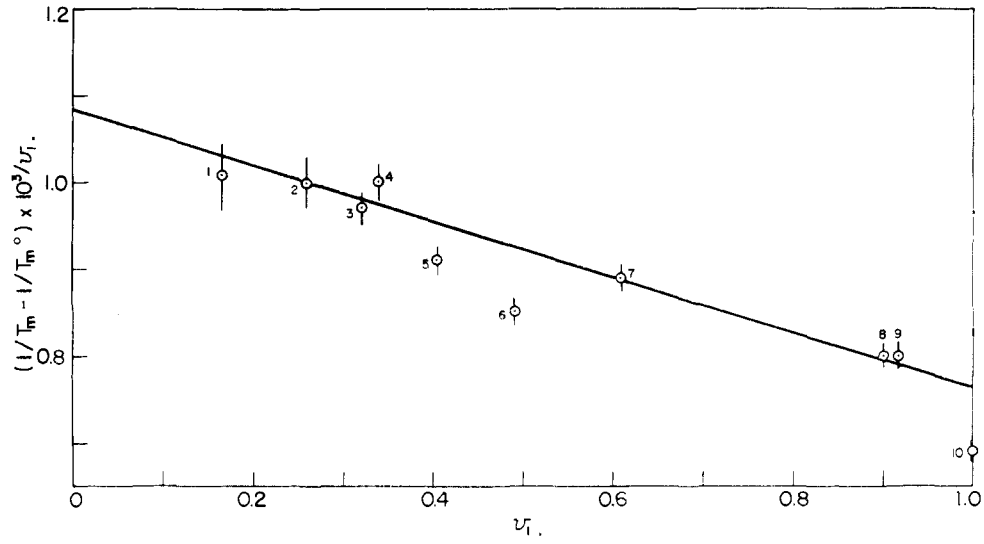

Fig. 11.—Plot of melting temperatures for the collagen-glycol system according to eq. 3. Numbers identify points with entries in Table I and with their representation in Fig. 7.

ditions for phase equilibrium, it does not suffice, as in other cases, merely to stipulate

$$\mu_2 = \mu_2' \qquad (1)$$

where μ_2 and μ_2' are chemical potentials of the solute in the amorphous and crystalline phases, respectively; it is required also that

$$\mu_1 = \mu_1' \qquad (2)$$

where the subscript 1 refers to solvent, which occurs in both phases.

If, as seems likely,[40] a part of the diluent is firmly solvated to certain polar groups and *the remainder is loosely held*, then we may approximate the actual situation prevailing at completion of melting by postulating removal of the latter into the solution (*i.e.*, amorphous phase) surrounding the last remaining crystalline regions, and retention by these regions of their complement of firmly held solvent. Assuming this to be the case throughout the composition range investigated, we may rely on condition 1 only and take the chemical potential μ_2' of the solute in the crystalline phase to be constant. With these simplifications, we obtain the usual polymer melting point relation[16,41]

$$1/T_m - 1/T_m^\circ = (R/\rho_2 v_1 \Delta h)(v_1 - \chi_1 v_1^2) \qquad (3)$$

where ρ_2 is the density of the amorphous polymer, Δh its heat of fusion per gram of crystalline polymer, v_1 the molar volume of the solvent and χ_1 the parameter characterizing the interaction between solvent and (amorphous) polymer. Ordinarily, T_m° represents the melting point of the pure polymer, but in view of our recognition of the role of solvation it must refer, somewhat ambiguously perhaps, to "pure" polymer in a condition equivalent to its solvated state. At any rate, according to our postulate, the value indicated for T_m° by extrapolating the data in Fig. 7, namely, 145°, is to be regarded as dependent on the diluent which provides the solvation in the experiments yielding the extrapolated result.

(41) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 568 ff.

In Fig. 11, the data of Fig. 7 are plotted according to eq. 3, with T_m° being assigned the value 418°K. The data are reasonably well represented by the straight line yielding $R/\rho_2 v_1 \Delta h = 1.08 \times 10^{-3}$ in °K.$^{-1}$, and $\chi_1 = 0.30$. Taking $\rho_2 = 1.35$ g. cc.$^{-1}$ and $v_1 = 56$ cc., we obtain $\Delta h = 24$ cal. g.$^{-1}$, or 2250 cal. per peptide unit.[17] The curve shown in Fig. 7 has been calculated from eq. 3 using the values of the parameters given above.

The validity of the heat of fusion thus obtained is of course contingent on the manner in which we have dealt with conditions 1 and 2. A more reliable procedure would require experimental determination of the activity of the diluent in the native collagen phase as a function of composition, and hence the determination of the dependences of the chemical potentials μ_1' and μ_2' on composition. Even with such refinement, there would remain the more fundamental difficulty of establishing T_m°. Alteration of the value here assumed by ±5° would change Δh by *ca.* ± 20%.

The deviation of points 5 and 6 from the line drawn in Fig. 10 has been ascribed to experimental causes (see above). Deviation of point 10, representing the transformation in dilute solution, appears to be explicable on theoretical grounds, which will be indicated here in outline only.

The geometrical form of the protofibrillar collagen "molecule"[21] undoubtedly is of primary importance with reference to the reversible formation of stable aggregates, or fibrils, from dilute collagen dispersions. Space requirements of the highly asymmetric particles virtually dictate parallelization of them, except at high dilution, with the formation of an anisotropic phase.[42] This phase is readily reduced to a well-ordered fibrillar array by the agency of intermolecular forces. These forces need not be large,[42] and therefore may contribute relatively little to the thermodynamic stability of the aggregate (*i.e.*, fibril). The ease with which fibrillar collagen (*e.g.*, native RTT or reconstituted

(42) P. J. Flory, *Proc. Roy. Soc. (London)*, **A234**, 73 (1956).

fibrils) may be dispersed in dilute solution, and subsequently re-aggregated to fibrils, would be difficult to reconcile with the formation of strong inter-protofibrillar bonds. A few such bonds may, of course, be present in the less soluble collagens,[13] *e.g.*, in BAT.

Theoretical considerations of the thermodynamics of mixtures of rod-like and random coil particles, these being interconvertible, show that the transformation of one form to the other should be independent of the total concentration, provided that the rod-like species are randomly dispersed both in space and in orientation. It follows therefore that when the concentration of the collagen–glycol system reaches a dilution such that the fibrillar organization disintegrates and the protofibrillar particles disperse, then the transformation temperature for conversion from protofibrillar (rod-like) form to random coils should remain substantially unchanged with further dilution. Accordingly, the temperature for the transformation at high dilution (determined viscometrically) is above the curve drawn through the remainder of the points in Fig. 7, and therefore below the line of Fig. 11.

The fact that the melting point for gelatin–glycol agrees almost exactly with that for collagen–glycol at the same composition affords convincing evidence that the aggregation process responsible for gelation in gelatin is none other than a reversion to the native (collagen) crystalline form. This point of view is supported by the correspondences in the X-ray crystal reflection for gelatin and collagen.[27,29] To be sure, the degree of crystallinity in gelatin gels never approaches that of native collagen. In this respect, however, gelatin appears to resemble collagen–glycol samples which have been allowed to recrystallize following initial melting. Notwithstanding the low degree of crystallinity in gelatin, and the often cited chemical changes believed to accompany conversion of collagen to gelatin, the thermodynamic stability of the crystalline aggregates, indicated by the melting temperature, is quantitatively almost identical with that in native collagen according to our results.

Witnauer and Fee[43] recently reported shrinkage temperatures for samples of cowhide swollen by various diluents: glycol, formamide, phenol and water at various concentrations. For reasons presented earlier and on the basis of our observations on tendon collagen, the temperatures T_s they have observed for spontaneous shrinkage should not be identified with the equilibrium temperature T_m; the disparity between T_s and T_m may, of course, be insensitive to composition. It is perhaps somewhat fortuitous that Witnauer and Fee find shrinkage temperatures for cowhide collagen–glycol mixtures very close to our T_m's for tendon collagen–glycol mixtures of the same composition. They obtain heats of fusion in the range 1600 to 2200 cal. per mole of peptide units through application of eq. 3 to their results on mixtures containing the first three of the diluents in the order stated above. The results for compositions with water yield the much higher value of 7200 cal. per mole, *the same value of* $T_m°$, namely, 145°, being used as for the other diluents.

The disparity between the heats of fusion thus calculated from the melting points for the collagen–water and for the collagen–glycol systems may be traced to the similarity in melting points for compositions at the same weight concentration in the respective diluents. If the same value of $T_m°$ is appropriate, then in view of the much smaller molar volume of water it follows from eq. 3 that the heat of fusion calculated from results for this diluent must be correspondingly greater. The results of Hirai[30] on gelatin–water systems appear to corroborate those of Witnauer and Fee[43] in yielding melting points only some 15 to 20 degrees lower than those for collagen–glycol mixtures of corresponding weight fractions. It is to be noted, however, that solvolytic degradation becomes severe in water at elevated temperatures. Melting points observed in water at higher concentrations are therefore open to serious question, and in particular the extrapolation to $T_m°$ cannot be regarded as trustworthy. It is at least plausible that a much higher melting point ($T_m°$) should be ascribed to the crystalline collagen "hydrate." The apparent anomaly arising from the fact that water yields approximately the same depression as glycol at the same weight concentration over the experimentally feasible range would thus be resolved.

The foregoing explanation is admittedly speculative. More important, we feel, is the realization of the experimental difficulties attending measurements in the collagen–water system at elevated temperatures and the limitations thus imposed on interpretation of results for this system.

(43) L. P. Witnauer and J. G. Fee, *J. Polymer Sci.*, **26**, 141 (1957).

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