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

Helix ⇄ Coil Transitions in Dilute Aqueous Collagen Solutions[1]

BY PAUL J. FLORY[2] AND EDWIN S. WEAVER

RECEIVED NOVEMBER 12, 1959

The transformation of rat-tail tendon collagen from the native helical to the random coil form, and the process of reversion of the latter to the former, have been followed viscometrically and polarimetrically in dilute aqueous solutions, both temperature and concentration being varied. The rate of the former process increases *ca.* 130-fold from 35 to 40°. Final values of the intrinsic viscosity and specific rotation after long periods at temperatures from 35 to 38° show incomplete transformation within this range. The 3° breadth of the transformation equilibrium is attributed to minor variations among the native protofibrillar population. Reversion from coil to helix is first order in the concentration over the range $c = 0.066$ to 0.41 g./100 ml. The reversion rate displays a large *negative* temperature coefficient, the half-time *increasing ca.* 135-fold from 5 to 23°. Inasmuch as the transition temperature T_m for the reverted collagen matches that of the native material and the optical rotation of the former approaches that of the latter, their structures evidently are equivalent. The apparent unimolecularity of the reversion process can be reconciled with the generally accepted three-strand coiled-coil model for the protofibril of the native form by postulating a transitory intermediate consisting of a single chain helix, possibly of the type attributed to synthetic poly-L-proline II, the formation of this intermediate being rate determining. This hypothesis succeeds further in explaining the large negative temperature coefficient of the reversion process. Consideration of the increase in the minimum helix length required for thermodynamic stability at a given degree of supercooling $\Delta T = T_m - T$, where T_m is the equilibrium transformation temperature, leads to an expression of the form Const. $\exp(-A/kT\Delta T)$ for the specific rate where A is a constant. The observed reversion rates are compatible with this relationship.

Introduction

The remarkable thermal contraction of collagen fibers has been shown to be brought about by a transition between crystalline and amorphous phases, this transition being fully analogous to that involved in the melting of other crystalline polymers. Compelling evidence for this viewpoint has been adduced from separate studies of the influences of dilution[3,4] and of longitudinal stress[5-7] on the transition temperature. Heats of fusion independently determined by these respective methods are in satisfactory agreement.

At high dilutions the transition is manifested by marked decreases in the viscosity of the solution and in the magnitude of the optical rotation.[8-11] The native protofibrillar particles of collagen, although individually dispersed at high dilution, impart high viscosity to the solution owing to their extreme asymmetry.[10] This structure is destroyed in the course of the transformation, and the component polypeptide chains assume the form of random coils which effect the viscosity of the solution to a much smaller degree. That the dilute solution transformation and the phase transition observed at higher concentrations are one and the same is convincingly demonstrated by experiments previously reported on the system collagen–glycol.[3] In the work referred to, the melting points for mixtures covering a wide range of concentration were shown to be connected in a virtually continuous manner with the transformation temperature characteristic of extremely dilute solutions.

Reversion of transformed collagen in more concentrated mixtures with glycol was reported previously.[3] An easily discernible, although small, decrease in the specific volume was observed when the transformed mixture was maintained at a temperature of 10° or more below its melting point. The latent volume decrease corresponded to restoration of only a small fraction of the native structure. Upon re-warming, however, it was dissipated at a temperature approximating the original melting point.

Reversibility of the transformation in dilute solutions is well demonstrated by the work of Ferry and Eldridge[8] and of Robinson[9] on optical rotations of dilute aqueous solutions of gelatin and by the similar results on ichthyocol recently published by von Hippel and Harrington.[12] The specific rotation of gelatin, or transformed collagen, is about −130°. Upon cooling below the transformation range, *e.g.*, to 0°, the specific rotation increases in magnitude in the course of time, approaching −350°, the value characteristic of dispersed native collagen. Reversion to the former value occurs upon warming; the cycle may be repeated.

Doty and co-workers[10,11] have shown the elementary protofibrils of native collagen to consist of rod-like particles approximately 13.5 Å. in diameter and 3000 Å. in length and to have a molecular weight of approximately 350,000. According to prevailing views,[13] each particle is constructed from three polypeptide chains wound in a compound helix; lateral hydrogen bonds between the component chains confer stability on the particle. In the process of transformation, the compound helical structure is destroyed, and the component chains are believed to be dispersed as two or three[10] separate random-coil macromolecules.

(1) Abstracted in part from the dissertation presented by Edwin S. Weaver to the Graduate School of Cornell University in partial fulfillment of the requirements for the Ph.D. degree, 1959.

(2) Mellon Institute, Pittsburgh 13, Pa.

(3) P. J. Flory and R. R. Garrett, THIS JOURNAL, **80**, 4836 (1958).

(4) R. R. Garrett and P. J. Flory, *Nature*, **177**, 176 (1956).

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

(6) O. K. Spurr, Ph.D. dissertation, Cornell University, 1958.

(7) P. J. Flory, THIS JOURNAL, **78**, 5222 (1956).

(8) J. D. Ferry and J. E. Eldridge, *J. Phys. Chem.*, **53**, 184 (1949).

(9) C. Robinson, in "The Nature and Structure of Collagen," Ed. by J. T. Randall, Academic Press, Inc., New York, N. Y., 1953, p. 96.

(10) H. Boedtker and P. Doty, THIS JOURNAL, **77**, 248 (1955); **78**, 4267 (1956).

(11) 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.

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

(13) (a) G. N. Ramachandran and G. Kartha, *Nature*, **174**, 269 (1954); *ibid.*, **176**, 593 (1955); G. N. Ramachandran, *ibid.*, **177**, 710 (1956). (b) A. Rich and F. H. C. Crick, *ibid.*, **176**, 915 (1955); see also ref. 11, p. 20. (c) P. M. Cowan, S. McGavin and A. C. T. North, *Nature*, **176**, 1062 (1955).

In view of the extraordinary features of the structure postulated for native collagen, the processes of transformation and reversion manifested by this protein seemed especially worthy of investigation. The work reported here has been concerned primarily with rates of the transformation and the reversion processes in dilute aqueous solutions of collagen and with the dependences of these rates on concentration and temperature.

Experimental

Tendons from the tails of freshly killed rats provided the source of collagen. Solutions in distilled water were prepared according to the method of Dumitru and Garrett.[14] Their procedure offers the dual advantages of virtual complete dissolution of the tendon collagen and an extraordinarily mild treatment consisting of immersion in 0.5 *M* NaH_2PO_4 at 5° for 12 hr. followed by washing and dissolving in distilled water at the same temperature. After removal of extraneous material by filtration through a coarse sintered Pyrex frit, the highly viscous solution was stored at 5°. No evidence of microbiological contamination or of other changes was apparent for periods up to one month. Longer periods of storage were avoided.

Concentrations of the solutions, determined by evaporating aliquots to constant weight in vacuum at 50°, were usually in the range 0.2 to 0.4 g./100 ml. Solutions of lower concentrations were obtained by dilution. Samples employed for viscosity and optical rotation measurements were filtered immediately prior to use. Except as noted otherwise, the solutions were unbuffered; the *p*H was *ca.* 5.

Viscosities were measured with Ubbelohde viscometers placed in thermostat baths regulated within ±0.01°. Flow times in all cases were of the order of 100 sec. or greater. Kinetic energy corrections were applied where low flow times rendered them significant.

Optical rotations were determined with a Schmidt and Haensch polarimeter, a sodium vapor lamp being used for the source of illumination. The polarimeter tube was 20 cm. in length. Its temperature was controlled within ±0.02° by circulating water from a thermostat bath through the jacket surrounding the tube. The instrument permitted an accuracy of ±0.02° in the measured rotation angle. Turbidity of the *native* collagen solutions prevented realization of this precision. The *transformed* collagen solutions used in experiments on the reversion process were satisfactorily transparent, however.

Characterization of Materials. Native Collagen.—Viscosity measurements on dilute solutions of native collagen in *pure water* yielded erratic results. Reduced viscosities η_{sp}/c were determined over the range c = 0.005 to 0.020 g./100 ml. Extrapolation to infinite dilution indicated a value of approximately 90 dl./g. for the intrinsic viscosity $[\eta]$ at 20°. Through the use of 0.4% (*v/v*) aqueous acetic acid or of 0.15 *M* citrate buffer (0.10 *M* citric acid, 0.05 *M* sodium citrate) as the solvent medium, more concordant results, and lower reduced viscosities, were obtained. The intrinsic viscosities at 20° found by extrapolation were 15.8 and 14.5 dl./g. for the two solvent media, respectively. These values agree well with those reported by M'Ewen and Pratt[15] for tendon collagen, by Doty and Nishihara[11] for soluble calf skin collagen and by Burge and Hynes[16] for rat skin collagen and various fish swim bladder collagens. We attribute the much larger viscosities observed in pure water to association, which is suppressed by the electrolytes present in the acetic acid and in the citrate buffer solutions.

The specific rotation $[\alpha]_D$ found for native collagen was approximately −340 ±30°, the value being independent of temperature from 5 to 26°. This result is in good agreement with the value of −350° reported by Cohen,[17] but smaller than Doty and Nishihara's[11] −415° and Burge and Hynes[16] −370 to −420°. Turbidity of the solutions limited the accuracy of polarimetric measurements to about ± 10%.

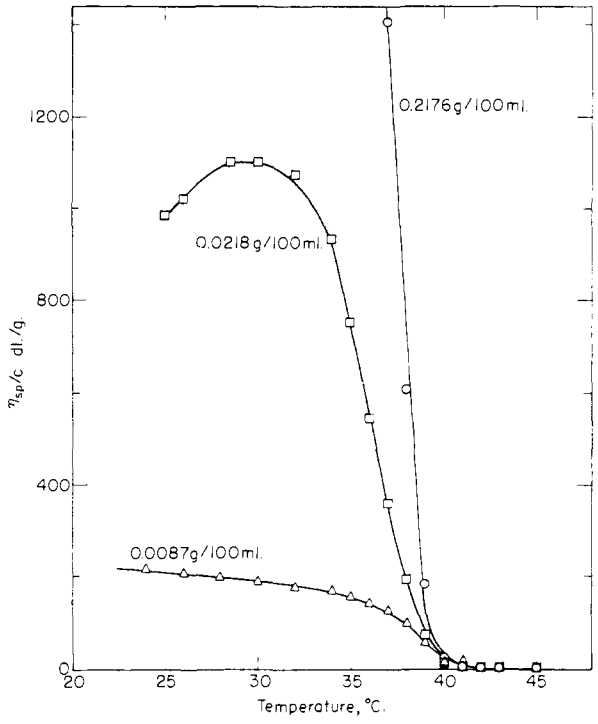

Fig. 1.—η_{sp}/c *versus* temperature for native collagen in water. Temperature raised at the rate of 1°/8 min.

Transformed Collagen.—Native collagen in dilute aqueous solutions is readily transformed ("denatured") to the random coil form ("parent gelatin") by temperatures above 40°. Solutions suitable for the characterization of transformed collagen were obtained by subjecting native collagen solutions to 80° for 2 hr. Viscosity and optical rotation measurements for characterization of this material were performed at 40°, which is well above the temperature at which reversion sets in (*cf. seq.*).

Viscosity measurements on solutions of transformed collagen in pure water (40°) led to spurious results. In general an ill-defined maximum in the reduced viscosity was indicated to occur in the vicinity of 0.1 g./dl., with $(\eta_{sp}/c)_{max}$ = 1.5 to 3.0 dl./g. Extrapolation to infinite dilution indicated $[\eta] \cong$ 1.0 to 1.5 dl./g. A substantial decrease in the reduced viscosity with increase in concentration beyond the location of the maximum was clearly evident. This latter behavior is suggestive of the well-known influence of charges on a polyelectrolyte chain in the absence of added electrolytes. In confirmation of this surmise, addition of sodium chloride to a concentration of 0.1 *M* lowered the reduced viscosity and eliminated all vestiges of the maximum in η_{sp}/c. The intrinsic viscosity $[\eta]$ obtained by extrapolation was 0.39 dl./g. at 40°. In 2 *M* KCNS, in which transformation occurs without heating, $[\eta]$ = 0.74 dl./g. at 20°. The disparity between the two values is largely attributable to the hydrolytic degradation resulting from exposure of the sodium chloride solutions to a temperature of 80° for 2 hr.

Optical rotation measurements on the transformed collagen solutions yielded $[\alpha]_5^{40°} = -131°$, in close agreement with −135° found for transformed calf-skin collagen by Doty and Nishihara.[11]

Transformation of Native Collagen. Preliminary Observations.—Reduced viscosities of dilute native collagen solutions in pure water were determined as the temperature was raised at the rate of 1°/8 min. Typical results are shown in Fig. 1. Optical rotations, measured while the temperature was raised at the same rate, are shown in Fig. 2. The initial reduced viscosities (Fig. 1) greatly exceed even the high limiting value (~90; see above) found by extrapolation of measurements on native collagen at high dilution in pure water. The normal increase in η_{sp}/c with

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

(15) M. B. M'Ewen and M. I. Pratt, in "The Nature and Structure of Collagen," Ed. by J. T. Randall, Academic Press, Inc., New York, N. Y., 1953, p. 158.

(16) R. E. Burge and R. D. Hynes, *J. Mol. Biol.*, **1**, 155 (1959).

(17) C. Cohen, *J. Biophys. Biochem. Cytol.*, **1**, 203 (1955).

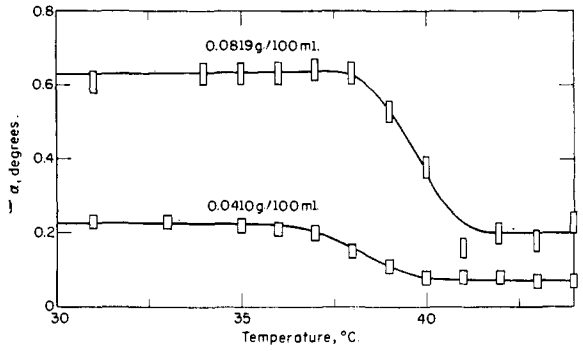

Fig. 2.—Rotation (α) *versus* temperature for native collagen in water at the concentration indicated.

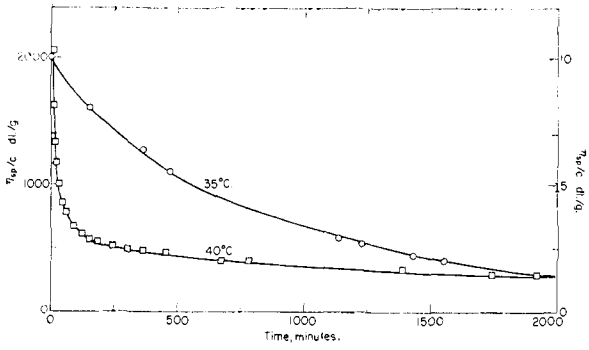

Fig. 3.—Isothermal transformation of native collagen in water at 35° (left hand ordinate scale, O) and at 40° (right hand ordinate scale, □). Concentration 0.0586 g./100 ml. in each experiment.

concentration would account only in small part for the values of η_{sp}/c observed at $c>0.01$ g./100 ml. Aggregation with increase in concentration may be responsible for the very large viscosities observed.

At intermediate and higher concentrations (Fig. 1), the reduced viscosity increases somewhat as the temperature of transformation is approached. Thereafter, it decreases markedly. Similar abrupt decreases in viscosity have been reported previously for dilute solutions of native collagen.[3,11] The temperature of completion of the decrease has been correlated with the melting point observed by other methods at higher concentrations.[3]

The cessation of the abrupt decrease in reduced viscosity (Fig. 1), and the concurrent changes in optical rotation (Fig. 2), occur at 40 ±1°, independent of concentration over the ranges investigated. This temperature may be considered to *approximate* the transformation temperature T_m, the process being regarded as a phase change. It is to be borne in mind, however, that these preliminary results were obtained by raising the temperature continuously, and hence that they may depend to some extent on the rate of heating. The temperature at which the transformation reaches completion under these conditions should not, therefore, be identified precisely with the equilibrium melting temperature T_m. Rather, they set an upper limit for T_m. Coincidence of the results of optical rotation measurements and those of viscometry lends assurance nevertheless that the process observed in each case is the same, that a profound change in configuration takes place and, further, that this change proceeds independently of the concentration in the dilute range.

Isothermal Rates of Transformation.—In order to assess the rate of transformation and its dependence on temperature more definitively, solutions of native collagen previously stored at 5° were filtered into viscometers and brought to fixed temperatures covering the range of 35° to 40 in increments of 1°. The viscosity was measured immediately after reaching the chosen temperature, and it was re-determined at frequent intervals thereafter. Typical results are shown in Fig. 3. The curves comprise two portions, the first being characterized by a steep slope which diminishes

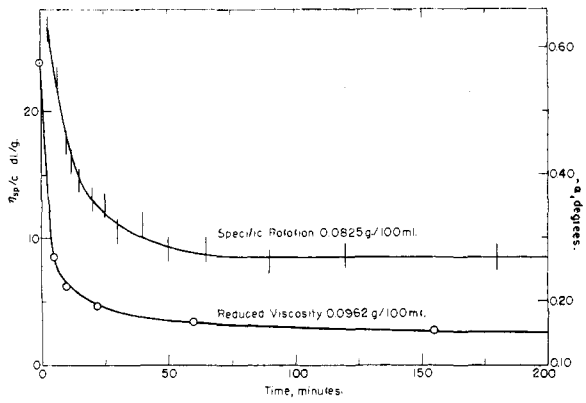

Fig. 4.—Isothermal transformation at 40° as observed polarimetrically (upper plot) and by viscometry (lower plot).

with time, and the second by a very small slope which persists over a much longer period. The former is extremely sensitive to temperature; the latter, being a manifestation of hydrolysis of the transformed collagen chains, is comparatively insensitive to temperature.

The time of completion of the first measurement was taken arbitrarily as zero despite the fact that as much as 2 hr. was required for the first determination on the more viscous solutions. The first measurement is represented as the initial reduced viscosity $(\eta_{sp}/c)_0$ given in the third column of Table I-A. The final reduced viscosity $(\eta_{sp}/c)_\infty$ was obtained by extrapolating the final portion of the curve, representing hydrolytic degradation, to $t = 0$. The interpolated times at which the reduced viscosity reached a value midway between these extremes are recorded in the last column in Table I-A.

TABLE I

TRANSFORMATION OF NATIVE COLLAGEN IN AQUEOUS SOLUTION

A. Viscometric results

Temp., °C.	Conc., g./100 ml.	$(\eta_{sp}/c)_0$ dl./g.	$(\eta_{sp}/c)_\infty$ dl./g.	$t_{1/2}$, min.
35	0.0586	2000	42	600
35	.0962	1770	53	700
35	.1758	1400	14	650
36	.0962	1520	20	350
36	.1758	1850	20	800
37	.0962	1080	10	130
37	.1758	1060	10	250
38	.0962	600	3.0	55
38	.1758	550	5.0	80
39	.0962	100	3.0	10
39	.1758	115	2.0	20
40	.0586	62	0.8	3
40	.0768	30	2.8	7
40	.0962	24	2.0	3
40	.1758	44	2.0	7

B. Polarimetric results

Temp., °C.	Conc., g./100 ml.	α_0, degrees	α_∞, degrees	$t_{1/2}$, min.
38	0.0825	−0.59	−0.25	100
40	.0825	−0.59	− .25	13
40	.1650	−1.08	− .47	20

Results obtained similarly using polarimetry instead of viscometry are presented in Fig. 4 and in Table I-B. Viscometric results obtained at approximately the same concentration are shown for comparison in Fig. 4. These observations are similar to those of Doty and Nishihara[11] on calf skin collagen in citrate buffer. They did not, however, pursue the decreases in viscosity and optical rotation to their ultimate values at each temperature.

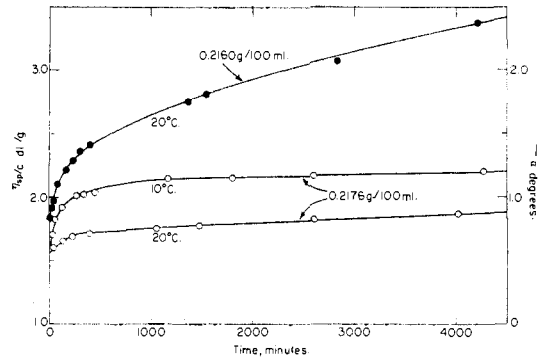

Fig. 5.—Reversion of collagen solutions transformed by heating 0.5 hr. at 80°. Reduced viscosity, ●; optical rotation, O.

The half-times $t_{1/2}$ derived by the viscosity method cannot, unfortunately, be accepted as proper measures of the time for half-conversion. In the first place, appreciable transformation often occurred before the first measurement could be completed. Secondly, for values of η_{sp}/c so far removed from the limiting value $[\eta]$ the contribution of the residual native collagen to the specific viscosity is by no means proportional to its concentration. (The values of $(\eta_{sp}/c)_\infty$ also are subject to a considerable uncertainty; however, they are of little consequence insofar as $t_{1/2}$ is concerned inasmuch as they are generally negligible compared with $(\eta_{sp}/c)_0$.)

The impact of these vitiating circumstances becomes increasingly severe as the concentration is increased. Hence, the viscometric half-times are of limited significance in answer to the question of the dependence of the rate of transformation on concentration; positive confirmation of the unimolecularity of the transformation (*i.e.*, half-times independent of concentration) suggested by the results cited in the preceding section consequently is not to be found in those given here.

Optical rotation afforded a direct measure of the transformation. Accuracy was seriously limited, however, by the turbidity of the native collagen solutions. The polarimetric half-times presented in Table I-B are somewhat greater than those obtained from viscometry at the same temperature. This difference occurs in the direction to be expected from the fact that the specific viscosity should vary with a power of concentration of untransformed collagen which exceeds unity. Thus, the decrease in the specific viscosity to one-half its initial value occurs prior to the point of half-conversion. In other respects, the polarimetric results support those obtained by viscometry.

In spite of the limitations discussed above, several significant conclusions may be drawn from the results given in Figs. 3 and 4 and in Table I. They demonstrate that the rate of transformation is finite, although the temperature range within which it is measurably so is small. The temperature coefficient is very great, the rate increasing about 130-fold in 5°. Doty and Nishihara[11] found a similar temperature coefficient for the rate of transformation of the soluble fraction of calf-skin collagen in citrate buffer at *p*H 3.7.[18]

The values of the reduced specific viscosities $(\eta_{sp}/c)_\infty$ representing the asymptotic limit of the transformation process and given in the fourth column in Table I-A appear to depend on the temperature of transformation. Although they are negligible by comparison with the initial values, for temperatures less than 39° they are nevertheless substantially greater than the reduced viscosity for transformed collagen (*ca.* 2.0) in pure water at the same concentration. The discrepancy is greater the lower the temperature. These observations suggest that transformation does not go to completion in the range 35 to 38°; a very small fraction—too small to be detected polarimetrically—may remain untransformed.

(18) The change in viscosity after 30 minutes[10] is not an appropriate measure of the rate of transformation. As is apparent from the results given in Figs. 3 and 4 and in Table I, assessment of the rates in this manner can be quite misleading and "activation energies" thus derived[10] are of no significance. Burge and Hynes[16] take the intrinsic viscosities and optical rotations of collagen solutions after 30 minutes at the chosen temperature as measures of the state of equilibrium between helix and coil and proceed to calculate enthalpies of transformation (which they inconsistently designate as activation energies). Our results show that this brief time interval is inadequate for establishment of equilibrium for any temperatures where the fraction of the native form persisting at equilibrium is appreciable.

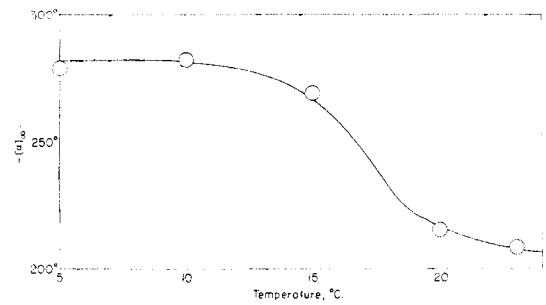

Fig. 6.—Ultimate values of the specific rotation of collagen in water plotted against the temperature at which reversion occurred.

A state of equilibrium between transformed collagen and residual native material suggests itself as a possible explanation. As we shall point out later, however, the temperature coefficient for the transformation equilibrium is certainly much too large to permit a range of even as much as several degrees over which the two distinct forms (protofibrillar particles of native collagen and component chains wholly in random coil configurations) may co-exist in detectable amounts.

Hydrolysis of Transformed Collagen.—That the sustained slow decrease in viscosity after conclusion of the helix-coil transition is due to hydrolytic degradation of the random coil molecules was demonstrated by further studies, which it suffices merely to summarize here. Dilute aqueous solutions of collagen were heated for various lengths of time at 59, 77.5 and 93°. They were quickly cooled to 40° and their viscosities measured immediately. The extent of chain scission was computed from the decrease in the reduced viscosity. The process was first order, with an activation energy of about 25 kcal./mole, in good agreement with results of Boedtker and Doty[10] and of Scatchard, Oncley, Williams and Brown[19] for the hydrolysis of ichthyocol and of gelatin, respectively. The rates computed by extrapolation to 35–40° are compatible with the slow decreases in the reduced viscosity which continue after apparent cessation of the transformation process in the experiments discussed above.

Rates of Reversion.—For the purpose of investigating rates of reversion of solutions of transformed collagen in pure water, dilute solutions of the native material were heated at 80° for 30 minutes, then filtered at once into either a viscometer or a polarimeter tube. The sample was brought to the desired temperature in the range 5 to 23° and measurements were commenced at once, the temperature being maintained constant. Since the solutions were devoid of the turbidity characteristic of native collagen solutions, optical rotation measurements could be performed with satisfactory accuracy.

Typical results at 20° are shown in Fig. 5. Whereas the negative rotation appears to proceed smoothly to an upper limit, the viscosity continues to increase long after the rotation has become constant.

Ultimate values of the specific rotation are shown in Fig. 6 as a function of the temperature; they were independent of concentration within experimental error. According to these results, from 40 to 75% of the native configuration is recovered, the value depending upon the degree of supercooling. The extent of reversion is much greater than that manifested at higher concentrations in the work of Flory and Garrett.[3,4] The viscosity, on the other hand, increases to only a very small fraction of its original value. Evidently the asymmetry of the native collagen protofibril is not re-established in the process of reversion. Segmented helices, comprising helical (native form) sections joined by disordered intervening regions may instead be regenerated.

(19) G. Scatchard, J. L. Oncley, J. W. Williams and A. Brown, THIS JOURNAL, **66**, 1980 (1944).

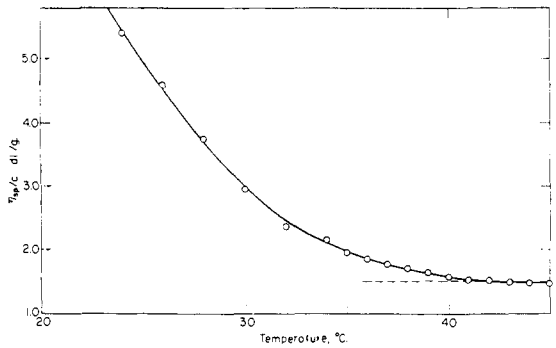

Fig. 7.—Reduced viscosity *versus* temperature upon warming dilute reverted collagen solution at the rate of 1°/8 min.; c = 0.4097 g./100 ml.

Being less extended in length, such particles would be less effective in raising the viscosity. Moreover, these regenerated helices may be less prone to aggregation, which we have suggested as being responsible for the extremely high reduced viscosities of native collagen solutions. The increase in reduced viscosity which continues beyond cessation of the change in optical rotation may represent gradual improvement in the structural organization of the regenerated helices, without appreciable change in the amount of the helical form; or it may be brought about by gradual aggregation.

Results of the reversion measurements are summarized in Table II. Initial rotations were calculated from the

TABLE II

REVERSION OF TRANSFORMED COLLAGEN HEATED ONE-HALF HOUR AT 80°, FOLLOWED POLARIMETRICALLY

Conc., g./100 ml.	Temp., °C.	$t_{1/2}$, min.	$t_{1/2}$, ave., min.
0.3301	5	20	
.2854	5	17	
.1981	5	30	25
.1320	5	30	
.0660	5	30	
.2854	10	70	
.2176	10	85	79
.1088	10	80	
.4097	15	190	
.3301	15	160	209
.2176	15	220	
.1088	15	260	
.3301	20	800	
.2176	20	930	
.1981	20	940	803
.1650	20	780	
.1088	20	540	
.0544	20	830	
.4097	23	2000	
.2176	23	5000	3360
.1088	23	3100	

experimental specific rotations $[\alpha]^{40}D$ previously given for transformed collagen, a possible very small effect of temperature being disregarded. The half-times recorded in the third column of Table II have been obtained by interpolation to the value of the rotation which is the mean between the calculated initial value and the observed asymptote for long times. The half-times show no consistent dependence on concentration. Specifically, they do not decrease with increasing concentration as would be expected if three separate molecules are joined to regenerate a particle having the native configuration. The average values given in the last column display a striking dependence on the degree of supercooling; the change in rate exceeds 100-fold over the temperature range of only 18°. These two observations are deemed to be of foremost significance.

Finally, we cite the results shown in Fig. 7 on the transformation of reverted collagen. As in experiments previously described on native collagen, the temperature was raised at the rate of 1/8° per minute. Transformation as indicated by the viscosity measurements appears to proceed over a range of temperature. It reaches completion, however, at about 41°, in good agreement with the similarly determined transition temperature of native collagen. These results are in accord with those of Flory and Garrett[3] on the collagen–glycol system. They confirm the essential equivalence of the basic structure of reverted collagen to that of the native material.

Discussion

The transformation of collagen being largely reversible, we direct attention first to the formulation of conditions of equilibrium. Assuming the process to be

$$\text{Three-strand helix} \rightleftarrows 3 \text{ Random coil molecules} \quad (1)$$

and assuming further that the solutions concerned are sufficiently dilute to permit assumption of ideal behavior, the relevant equilibrium relationship may be written

$$K = c_0^2\alpha^3/(1 - \alpha) \quad (2)$$

α being the degree of conversion and c_0 the total concentration. Adoption of the ideality condition is not as severe as might be expected from consideration of the large deviations which are characteristic of solutions of long-chain molecules. It may be shown by extension of the theory of solutions of rod-like molecules[20] to mixtures of rods and random coils that the two solute species are subject to similar deviations from ideality. The deviation from ideality for each species depends, in first approximation, on the total concentration of both. Hence, assuming dissociation into three molecules, eq. 2 should apply up to concentrations considerably in excess of that at which deviation from ideality for each solute species becomes large.

Characterization of the transformation as a phase transition is reconcilable with the mass action expression eq. 1 in consequence of the very large temperature coefficient of K.[21] This temperature coefficient depends of course on the enthalpy change for the transformation, which lies in the range 1.0 to 1.5 kcal. per mole of peptide unit,[3,6] or in excess of 3000 kcal. per mole of native collagen particles. It follows that K must change by 10^6 to 10^9 fold per degree. This extreme dependence on temperature overshadows the concentration dependence. The use of equilibrium studies for establishing the number of random coil species released in the transformation of a native collagen particle is therefore less promising than would at first appear to be the case.

The values of $(\eta_{sp}/c)_\infty$ given in Table I-A indicate incomplete transformation at equilibrium within the temperature interval 35–38°. The breadth of this range is much greater than would be predicted from the enthalpy change cited above. This observation immediately calls to mind the recent theories[22,23] of helix-coil transitions, which em-

(20) P. J. Flory, unpublished.

(21) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg Ser. chim.*, **29**, 230 (1955); *J. Phys. Chem.*, **62**, 1485 (1958).

(22) B. H. Zimm and J. K. Bragg, *J. Chem. Phys.*, **28**, 1246 (1958), **31**, 526 (1959).

(23) J. H. Gibbs and E. A. DiMarzio, *ibid.*, **28**, 1247 (1958).

phasize the importance of intermediate, partially disordered species in broadening the transition range. These intermediate species may be formed through: (i) partial unwinding of the helix from its ends and (ii) destruction of order within mid-portions of the helix. Owing to the multiplicity of hydrogen bonds between turns of polypeptide helices, initiation of destruction of the ordered configuration within the helix requires a considerable sacrifice of energy before the increase in entropy associated with disorientation commences to be realized.[18-20] In a compound helix of the type proposed for collagen,[12] consisting of three polypeptide chains joined laterally by hydrogen bonds, initiation of disorientation according to (ii) would necessitate sacrifice of an inordinately large number of hydrogen bonds. Even if this particular model is rejected, the measured width of the collagen protofibrillar unit nevertheless bespeaks a fairly complex structure, initiation of disruption of which must involve a rather large number of peptide units. It should be sufficient therefore to confine attention to unravelling of the native helical particle from its ends according to (i).

Calculations carried out on this basis indicate that the finite breadth of the transition, assumed to proceed by stepwise unravelling of the compound helix from its ends, cannot exceed approximately one degree. The quantities required for this calculation are the equilibrium transformation temperature T_m (*ca.* 308° *K*), and the heat of transformation ΔH_u (1400 cal./mole of peptide units[5,6]). The greater breadth of the transition observed suggests small, though significant, variations in the constitution of the native collagen particles. Slight differences in stabilities of different particles would readily account for the observations. The insensitivity of these limiting values to the concentration is explicable on the same basis: the extreme sensitivity of the transformation to temperature causes the effect of dilution to be obscured by the dominating influence of variation in intrinsic stability.

Prominent features of the reversion process are the apparent first order dependence of the rate on concentration, as manifested by half-times independent of the concentration and the marked negative temperature coefficient of the reversion rate. The former observation offers substantial grounds for questioning the three-chain helix model; the latter places severe limitations on the types of processes which may be invoked to account for the facile reversion to the native form. While the proportionality of the rate of reversion to the first power of the concentration virtually demands unimolecularity for the rate-controlling step, this observation does not necessarily contravene the accumulated evidence which has been offered in support of the three-chain model. Critical evaluation of this evidence will not be attempted here. Rather, we shall present a mechanism for the reversion process which is consistent with the unexpected kinetic observations and not incompatible with the currently accepted model.

Before entering upon discussion of the reversion mechanism, it is necessary to emphasize that the structure of the reverted collagen duplicates that of the native material—at least at the morphological level of arrangement of peptide units in relation to one another. Evidence for this equivalence is furnished by the similarity of the optical rotations for the reverted and the native collagens and by the virtual coincidence of the melting points (taken as the temperature for completion of the transformation, as is generally appropriate for polymer systems). If native collagen consists of a triple strand compound helix, we therefore are led to conclude that reverted collagen is similarly constituted.

The main features of the reversion kinetics find ready explanation in the postulation of an intermediate formed by unimolecular rearrangement of a single random coil molecule. If this step is rate controlling and if the concentration of the intermediate is always very small compared to that of the "reactant" random coil molecules, the first order kinetics follow at once.

The postulated intermediate might, for example, consist of a helix of the poly-L-proline II type, this being the form of arrangement considered to represent the individual chains of the native collagen compound helix.[13] The entire polypeptide molecule may not necessarily be so arranged; it would suffice for a portion of the polypeptide chain to adopt the required conformation (*e.g.*, the poly-L-proline II helix), adjoining portions of the same molecule remaining in the random coil form. The number of consecutive units so arranged must be assumed to be fairly large, however. Whatever the nature of the intermediate may be, its conversion to the native form is considered to be sufficiently rapid to have no effect on the over-all rate.

If, for definiteness, the native form is assumed to be a three-chain (compound) helix, the processes involved in reversion (left to right) and in transformation (right to left) may be represented as

$$\mathrm{C} \underset{k_1}{\overset{k_1'}{\rightleftharpoons}} \mathrm{I} \underset{k_2}{\overset{k_2'}{\rightleftharpoons}} (1/3)\,\mathrm{H} \qquad (3)$$

where C, I and H represent respectively the random coil, the intermediate and the native helix. According to the assumptions introduced above, the rate R' of the reversion process is given by

$$R' = R_1' = k_1' C$$

where the concentration of the random coil C is represented by the symbol C. Since the concentration of the intermediate is postulated to be very small compared to C, the latter may be identified with the concentration of unreverted material, hence the process is necessarily first order. That the second step should be both comparatively rapid and easily reversible is not implausible inasmuch as this step involves little more than lateral aggregation of the postulated intermediate species, the angle of twist being small.

Von Hippel and Harrington[12] have reported a deviation from first order kinetics in the course of the collagenase-catalyzed proteolysis of gelatin at temperatures below T_m. At temperatures $T > T_m$ the process is strictly first_order; at $T < T_m$ how-

ever an anomalously high rate is observed in the initial stages of the process. They attribute the observed deviation from first order kinetics to "*a local configurational change ...*", presumably consisting in the locking "of proline residues into the poly-L-proline II configuration". Yet, the postulated conformational change was not revealed in the optical rotation. Inasmuch as the anomaly appears to set in sharply within a degree or two of T_m, it must be cooperative in origin and hence involve many consecutive peptide units. Such a profound change in conformation, involving a substantial fraction of the total protein, should surely affect the optical rotation, contrary to observation. Whatever may be the explanation of their results, an intermediate such as they have postulated to account for a feature of the enzyme catalyzed proteolysis fails to meet the requisites of our unstable intermediate I, hence distinction of one from the other is important. Theirs, since it is claimed to exist in considerable quantity relative to C, must be accorded stability comparable to that of C, even at temperatures only a few degrees below T_m.

It remains to consider the energetics of the processes involved in the scheme (3). The stipulation that the concentration of I is invariably very small requires that the standard state free energy change for the first reaction be positive and large; in symbols, $n\,\Delta F_1' >> 0$ where $\Delta F_1'$ denotes the standard state free energy change per peptide unit for the process C→I and n is the number of units involved.[24] (Note that $\Delta F' = -\Delta F$ for the overall process C→$^1/_3$ H is necessarily very small in the neighborhood of T_m. Hence, I must be unstable relative to both C and H throughout the temperature range of interest on either side of T_m.) Presuming I to consist of an ordered conformation (e.g., single strand helix), its entropy should be substantially less than that of a random coil; hence, it is to be expected that $\Delta S_1' < 0$. The overall enthalpy change for C→H is appreciably negative (*ca.* 1.4 kcal. per peptide unit). It is reasonable to suppose that a portion of this decrease in enthalpy is realized in the formation of the intermediate; *i.e.*, that $\Delta H_1' < 0$. These circumstances are implicit in the postulation of an unstable intermediate of ordered conformation. They lead to the unusual situation of a *negative* heat (enthalpy) of activation and a large positive free energy of activation. The negative temperature coefficient for the reversion process is thus qualitatively explained.

Examination of the results presented in Table II reveals that the fractional change in rate with temperature, *i.e.*, $-\mathrm{d}\ln(t_{1/2})/\mathrm{d}T$, increases as T_m is approached. An Arrhenius plot of log $t_{1/2}$ against $1/T$ is therefore nonlinear; in other words, the magnitude of the (negative) activation energy computed in the conventional manner increases with temperature. These observations are suggestive of the temperature coefficient of nucleated crystallizations[25] as observed in other polymer systems.[26] The form of the dependence of the rate of reversion on temperature finds explanation in concepts relating to the conditions for stability of a polymer crystallite.[27,28]

Let the postulated intermediate be assumed to consist of a helical segment comprising n consecutive units of the protein chain. Three of these segments are considered to combine to form a compound helix of the same length. Imperfections in the secondary bonding and arrangement of the chains at the juncture between helical and random coil regions require assignment of an excess free energy σ for each traversal of the "interface" by a polypeptide chain at the ends of the protofibril. The free energy of formation of the protofibrillar segment consisting of 3 n peptide units may therefore be written

$$(\Delta F')_{3n} = 3(n\,\Delta F' + 2\sigma) \qquad (5)$$

where $\Delta F' = \Delta S'\Delta T = -\Delta S\Delta T, \Delta S = -\Delta S'$ being the overall entropy change per peptide unit; $\Delta T = T_m - T$ represents the degree of undercooling.

If $(\Delta F')_{3n} < 0$, the compound helical segment constructed by joining three primary helix intermediates I of length n will be stable relative to C. The minimum sequence length n^* for stability, obtained by setting eq. 5 equal to zero, is given by

$$n^* = 2\sigma/\Delta S\Delta T \qquad (6)$$

In order to meet this condition, either the lengths n of the intermediate helices (I) must equal or exceed n^*, or the incipient protofibril H_{3n} must grow by accretion of adjoining units from the pendent polypeptide chains subsequent to its generation. Postponing consideration of the second alternative, we tentatively adopt the former, namely, that $n \geqq n^*$. In view of the mounting difficulties confronting attainment of molecular cooperation throughout a longer sequence of units, the actual length (n) of the sequence generated in the first step is unlikely to exceed n^* appreciably. Accordingly, the error committed in adopting n^* as the actual length should be inconsequential. The free energy change for the first, and rate determining, step may therefore be written (neglecting terminal effects, which in this instance should be unimportant relative to other magnitudes involved)

$$n^*[(\Delta F_1')_{T_m} - \Delta S_1\Delta T] = (2\sigma/\Delta S)\,[(\Delta F_1')_{T_m}/\Delta T - \Delta S_1] \qquad (7)$$

where $(\Delta F_1')_{T_m}$ represents the standard free energy change per peptide unit for the first step at the temperature T_m. The foregoing expression for the free energy of activation of the rate-determining step may be simplified to $A/\Delta T - B$, where A and B are constants. Hence, the rate constant for the reversion can be written

$$k' = \text{Const.}\,\exp(-A/kT\Delta T) \qquad (8)$$

That the results given in Table II are in accord with this relationship is demonstrated in Fig. 8.

If an embryonic protofibril is formed with $n<n^*$, its length must nevertheless be sufficient to pre-

(24) For simplicity, the conventional superscript ° denoting the standard state is omitted from $\Delta F_1'$, and related symbols.

(25) R. Becker and W. Döring, *Ann. Phys.*, [5] **24**, 719 (1935); M. Volmer, "Kinetik der Phasenbildung," Dresden, 1939.

(26) L. Mandelkern, F. A. Quinn, Jr., and P. J. Flory, *J. Applied Phys.*, **25**, 830 (1954); L. Mandelkern, *Chem. Revs.*, **56**, 903 (1956).

(27) P. J. Flory, *J. Chem. Phys.*, **17**, 223 (1949); P. J. Flory and A. D. McIntyre, *J. Polymer Sci.*, **18**, 592 (1955).

(28) F. P. Price, *J. Chem. Phys.*, **31**, 1679 (1959); J. I. Lauritzen, Jr., and D. Hoffman, *ibid.*, **31**, 1680 (1959).

clude the likelihood of reversal to the random coil form; otherwise its formation will be of no consequence.[29] Without resort to a detailed analysis, it is apparent in light of the considerations presented above that the minimum size of the embryo which offers reasonable assurance of subsequent growth to stable size will increase as T_m is approached (*i.e.*, as $\Delta T \rightarrow 0$). Moreover, the form of the dependence of this minimum size on ΔT may be expected to duplicate eq. 6, and this leads directly to (8).

In order to apply the foregoing scheme to the transformation process (which occurs at temperatures $T > T_m$), we assume that I remains in equilibrium with H. Then

$$I = K_2 H^{1/3} \qquad (9)$$

where $K_2 = k_2/k_2'$ and H and I are concentrations of H and I, respectively. Since the rate R of transformation must be governed by step 1

$$R = R_1 = k_1 I = k_1 K_2 H^{1/3} \qquad (10)$$

The equilibrium constant K for reaction 1 is related to the constants for the scheme (3) as

$$K^{1/3} = K_1 K_2 = k_1 K_2 / k_1'$$

Hence

$$R = K^{1/3} k_1' H^{1/3} \qquad (11)$$

The temperature coefficient of the equilibrium constant K is positive and very large as previously noted. The observed temperature coefficient for k_1' is negative but smaller than that of $K^{1/3}$. Hence, the temperature coefficient of the latter dominates that of the former, with the result that the temperature coefficient of R is predicted to be positive and large in accordance with observation. The transformation should be one-third order according to eq. 11. Experimental results of this investigation are indecisive on this point (see above).

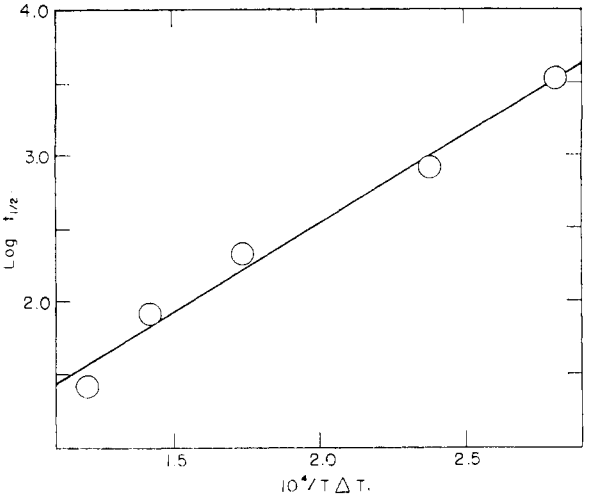

Fig. 8.—Logarithm of the half-time for reversion *versus* $1/T\Delta T$, where $\Delta T = T_m - T = 308 - T$ in °K.

Thus, the main features of the kinetics of the reversion and transformation processes can be satisfactorily explained by the foregoing scheme involving an intermediate, the formation of which is considered to be rate determining. The interpretation given is compatible with a model for the collagen protofibril consisting of a plurality of polypeptide chains which separate on transformation.

Acknowledgment.—Partial support of this investigation by the National Science Foundation Grant No. NSF G-2625 is gratefully acknowledged. One of us (E. S. W.) is indebted to the Procter and Gamble Company for a graduate fellowship during the academic year 1957–1958.

(29) Regeneration of native structures by first joining three random coil polypeptide chains in suitable juxtaposition followed by stepwise helical coiling of successive units of the member chains has been considered. The kinetics become third order; although the temperature coefficient is negative, the rate is linear in ΔT rather than exponential in $-(T\Delta T)^{-1}$ as prescribed by eq. 8.

[CONTRIBUTION FROM THE ESSO RESEARCH AND ENGINEERING COMPANY, LINDEN, NEW JERSEY]

The Radiation-induced Chain Alkylation of Ethylene with Propane

BY W. BARTOK AND P. J. LUCCHESI

RECEIVED NOVEMBER 10, 1959

The gas phase reaction of propane with ethylene has been studied in static experiments. Experiments performed using the mixed neutron-gamma radiation available from a nuclear reactor were also done thermally, without radiation. Propane-rich systems containing 4.5–10 mole % ethylene were investigated in the range of temperatures of 240–454°, at initial total pressures of 9 to 20 atm. Irradiations were done at two levels of intensity corresponding to energy absorption rates of 17×10^6 and 48×10^6 rad./hr. G values exceeding 100 molecules of ethylene reacted per 100 ev. establish the chain nature of the reaction. At the conditions of this work, radiation only accelerates the thermal process, since the products of both the thermal and the radiation-induced reactions were very similar at the same conversions of ethylene. Changing the surface/volume ratio by packing the reactor with stainless steel wire has practically no effect. The equivalence between the radiation-promoted and thermal reactions suggests that ions and other species peculiar to radiation-initiation are not an important factor at the conditions of this work. This also means that the radiation technique can be used to gain valuable insight concerning the mechanism of the ordinary thermal reaction and indicates the chain nature of alkylation.

Introduction

The question whether the chain reactions of hydrocarbons induced by ionizing radiation occur *via* free radical or other mechanisms remains unanswered to date. In the case of vapor phase alkylation reactions, the experimental evidence reported previously is overwhelming in favor of a chain mechanism[1–4] for the alkylation reactions of al-

(1) P. J. Lucchesi, B. L. Tarmy, R. B. Long, D. L. Baeder and J. P. Longwell, *Ind. Eng. Chem.*, **50**, 879 (1958).

(2) P. J. Lucchesi and C. E. Heath, THIS JOURNAL, **81**, 4770 (1959).

(3) F. W. Lampe, "The Radiation Chemistry of Light Hydrocarbons," Paper presented at Fifth World Petroleum Congress, New York, N. Y., June, 1959.

(4) W. Bartok and P. J. Lucchesi, THIS JOURNAL, **81**, 5918 (1959).