Collagen Structure in Solution. II. Analysis of Refolding Kinetics in Terms of Nucleation and Growth Processes*

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ABSTRACT: The kinetics of intramolecular chain folding of two random-coil, single-chain, collagen-type polypeptides have been investigated over a wider temperature range than reported heretofore by employing a solvent consisting of ethylene glycol–water (1:1, v/v). Results indicate that the renaturation rate of RCM-*Ascaris* (reduced-carboxymethylated *Ascaris* cuticle collagen) passes through a maximum about 46° below the thermal transition temperature (T_m) of the native protein. The presence of a maximum in the rate–temperature profile as well as the variation of reaction order in van't Hoff plots suggest the presence of subordinate reactions of opposing temperature dependence. Analysis of the overall mutarotation curves as a sum of parallel first-order processes reveals that two major reactions are responsible for the temperature behavior; (1) a component reaction which nucleates the folding process has been identified; this reaction exhibits a marked increase in rate with increasing levels of undercooling below T_m and follows the temperature dependence expected for the Flory–Weaver equation [rate = $B \exp(-A/RT\Delta T)$]; (2) a component reaction with positive temperature dependence interpreted as reflecting addition of random-coil residues to the nucleus. Superimposed on these two major processes is a very slow reaction which is thought to result from the annealing of segments of the chain frozen into less ordered conformation. Annealing accounts for a major fraction of the overall mutarotation changes observed at low temperature. A qualitatively similar kinetic pattern is found in the refolding of α_1-ratskin. The temperature dependence of chain folding in these collagen systems appears to conform to classical nucleated crystallization kinetics in that both the free energy of formation of the nucleus as well as the free energy of addition of residues in the growth process are included in the exponential terms of a modified Arrhenius equation.

In the previous paper of this series (Harrington and Rao 1970) initial rates of renaturation, $d[\alpha]/dt$, of single, noncross-linked polypeptide chains derived from various collagens have been examined as a function of protein concentration, pyrrolidine content, and temperature. These studies provide evidence that the collagen-type conformational pattern generated in cooled, α-type gelatin chains may develop along two distinctly different pathways depending on the protein concentration. At low protein concentration a stable, collagen-type structure is formed in a unimolecular reaction through interaction of intramolecular segments, probably through reverse folding of the chain. At high concentration, for low levels of undercooling (ΔT) below the helix → coil transition temperature (T_m) of the parent collagen, three separate chains associate in a concentration-dependent, multimolecular reaction to form the parallel-type, triple helix characteristic of tropocollagen.

Evidence for intramolecular folding among the noncross-linked gelatin chains is most convincing for RCM-*Ascaris* (reduced, carboxymethylated *Ascaris* cuticle collagen) and α_1-ratskin where the invariance of the molecular weight in the folded and unfolded state, as well as the concentration independence of the rate of renaturation over a wide span of protein concentration, provides strong support for the view that these polypeptide chains form the characteristic triple helix of collagen through reverse folding of the chain: (1) this type of folding pattern was first proposed by Drake and Veis (1964) and appears to be the stable conformational state of the subunit chains in native *Ascaris* cuticle collagen (McBride and Harrington, 1967a); (2) we find essentially similar kinetic behavior in the other single-chain gelatin systems which were investigated (α_1-ichthyocol and α_2-ratskin) suggesting that this is the preferred folding pattern for all single-chain collagen-type species at low protein concentration. Analysis of the folding kinetics in these single-chain species may not only provide information on the mechanism of formation of the collagen-fold structure, but could also lead to the development of simplified models for the formation of tertiary structure in noncollagenous, globular systems as well.

The present study was prompted by the finding (Harrington and Rao, 1970) that the initial rate *vs.* temperature plot of RCM-*Ascaris*, although exhibiting the characteristic negative temperature dependence of other gelatin systems, shows a leveling-off and slight downward curvature below about 3° suggesting that the rate of renaturation may pass through a maximum. In view of the possible significance of such behavior in developing a kinetic scheme for chain folding, we have reinvestigated the temperature dependence of renaturation of this polypeptide chain as well as the vertebrate single-chain molecule α_1-ratskin in a solvent consisting of ethylene glycol–water (1:1, v/v). Addition of ethylene glycol raises the helix → coil transition temperature (T_m) of collagen by about 6° and, more importantly, lowers the freezing point of water, thus permitting renaturation experiments over a temperature span of about 50°.

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Materials and Methods

All chemicals were reagent grade. Water was glass distilled. RCM-*Ascaris* collagen was prepared according to a method previously reported (McBride and Harrington, 1967a) and α_1-ratskin collagen according to the procedure of Piez *et al.* (1960). (We thank Dr. O. W. McBride for preparation of these samples.) The two gelatins were stored as concentrated stock solutions at 4° in a buffer system consisting of 0.30 M NaCl–0.02 M acetate, pH 4.6.

Protein concentrations were determined by the microbiuret method of Zamenhof (1957) assuming a color factor of 1.12 ml/mg [McBride and Harrington (1967a); Hauschka (1969)] in the equation

$$[\text{protein}] = (OD_{310} - OD_{390})/\text{color factor}$$

Collagen solutions in 50% ethylene glycol–water (1:1, v/v) were prepared by gravimetric dilution of aqueous buffered protein solutions with anhydrous ethylene glycol assuming the density of 50% glycol–water to be 1.0619 (Schwers, 1908).

Optical rotation measurements were made with a Cary 60 spectropolarimeter using either 1- or 0.1-cm jacketed cells (Opticell) for all studies except the melting curves in which case a 10-cm jacketed cell was employed. Temperature was controlled by circulation of water or 1-propanol from a thermostated bath through the jacket. The cell temperature was assumed to be the average of inflowing and outflowing circulent. Temperature of the circulent was controlled to within ±0.02° for runs above 0 and ±0.05° in the temperature range −20 to 0°.

The optical rotatory dispersion spectrum of RCM-*Ascaris* in ethylene glycol–water (1:1, v/v) was corrected for the refractive index of the solvent by applying the factor $3/(n^2 + 2)$ where n is the refractive index of the solvent. Refractive indices in the deep ultraviolet region were estimated from the Sellmeier equation (Fasman, 1963).

Nonequilibrium melting curves of native *Ascaris* collagen in aqueous solvent (0.15 M NaCl–0.01 M acetate, pH 4.6) and in the glycol buffer system were obtained by transferring the cold sample into a precooled polarimeter tube and then raising the temperature incrementally. Rotations were measured at a wavelength of 313 mμ. Measurements were taken 30 min after reaching each temperature.

Because of the difficulty in estimating $[\alpha]_{313}^{T}$ for the completely denatured *Ascaris* gelatin we have taken the rotation as a function of temperature in 5 M guanidine·HCl as the true base line of the unfolded polypeptide chains for calculation of the transition temperature, T_m.

In the renaturation studies, samples in 0.30 M NaCl–0.02 M acetate, pH 4.6, were diluted gravimetrically either with distilled water or with anhydrous ethylene glycol immediately before each experiment. A protein concentration of 0.38 mg/ml was employed for all renaturation experiments of RCM-*Ascaris*. No significant differences in initial rate were observed (over the temperature range 8–20°) when the protein concentration was lowered to 0.05 mg/ml, in agreement with the concentration independence of the rate in aqueous systems (McBride and Harrington, 1967b; Harrington and Rao, 1970). A protein concentration of 0.05 mg/ml was employed in the α_1-ratskin experiments since we wished to follow the temperature dependence of the intramolecular folding process in this species. The initial rate of renaturation of α_1-ratskin has been found to be independent of protein concentration in aqueous systems below 0.1 mg/ml. All samples were heated 10 min (RCM-*Ascaris*, 60°; α_1-ratskin, 50°) at a temperature above the helix-coil transition temperature, T_m, of the parent collagen, cooled in the thermostated bath for 20–180 sec and transferred to a preequilibrated polarimeter cell. Before starting a renaturation experiment, solvent was carried through the same heating and cooling schedule and solvent values were recorded for about 1 hr to establish an accurate base line. The recorded solvent values were subtracted from the mutarotation curve at 100-sec intervals. Mutarotation was followed for 10,000–320,000 sec depending on temperature, utilizing the synchronous chart drive motor of the instrument to obtain the time axis of the reaction.

Kinetic Analysis. Raw data $(\alpha_t - \alpha_0)$ from the polarimeter were plotted on a large sheet (40 × 50 cm) of graph paper and the best smooth curve was drawn with the aid of a flexible spline. Results were analyzed as a sum of independent first-order reactions by the method of Prony (Hildebrand, 1956) with the help of an IBM 7094 digital computer according to eq 1 (see Segal and Harrington, 1967). The Prony curve-

$$\alpha_t - \alpha_0 = \sum_{n=1}^{n=i} (\alpha_{\infty,i} - \alpha_0) e^{-k_i t} \qquad (1)$$

fitting technique requires a value for the overall change in rotation at infinite time $[\Sigma_i(\alpha_{\infty,i} - \alpha_0)]$, where $(\alpha_{\infty,i} - \alpha_0)$ is the total rotatory change of the ith reaction. This value was estimated by plotting the logarithm of the observed rate $\log [d(\alpha_t - \alpha_0)]/dt$ *vs.* t for the total reaction. At sufficiently long times the plot becomes linear since only the slowest reaction contributes to the mutarotation process. The rate constant of this reaction, k_s, was estimated from the slope, and its rotatory contribution $(\alpha_{\infty,s} - \alpha_0)$ was determined from the zero time intercept $-k_s(\alpha_{\infty,s} - \alpha_0)$. Since the slowest reaction differs in rate by a factor of at least 10 from the other subordinate reactions, we may assume that at a time corresponding to $t_{1/2}$ of the slow reaction the other component reactions have run to completion. Therefore it is possible to estimate the total rotatory change at infinite time, $\Sigma_i(\alpha_{\infty,i} - \alpha_0)$, by addition of $0.5(\alpha_{\infty,s} - \alpha_0)$ to the observed *overall* rotation, $\alpha_t - \alpha_0$, at $t = t_{1/2}$ of the slow reaction. The method presupposes large differences in rate constants of the individual reactions. Decomposition of the experimental mutarotation curve into three or less subordinate reactions was invariably found.

Results

Before initiating the renaturation experiments in aqueous glycol mixtures it was necessary to demonstrate that the conformational state of RCM-*Ascaris* regenerated at low temperature in this solvent system is indistinguishable from that in the dilute salt–water medium. Optical rotatory dispersion spectra of RCM-*Ascaris* were measured in the two solvent systems. Both solutions were maintained at 5° for 4 days to permit regeneration of structure following a preliminary heating step (60°, 10 min). The two spectra, when corrected for the index of refraction of solvent, are virtually identical over the

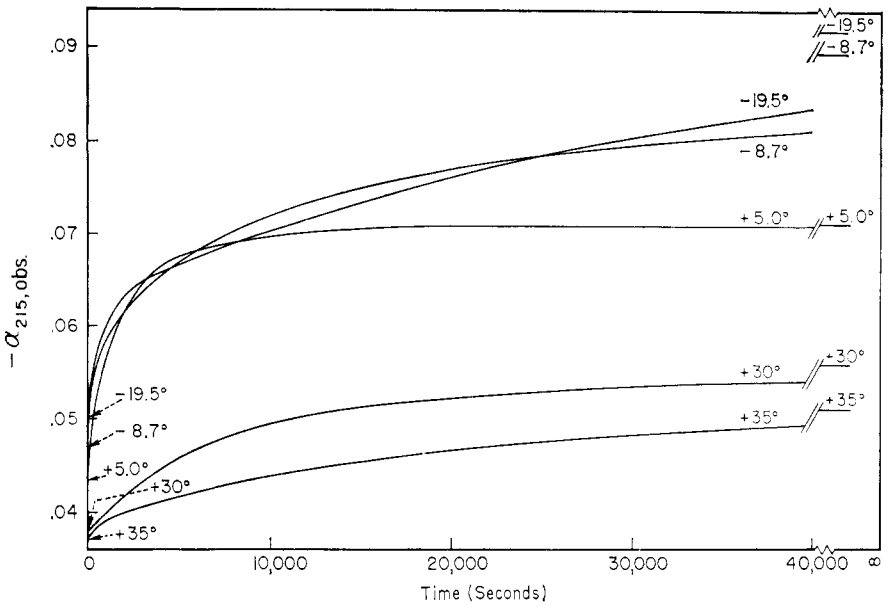

FIGURE 1: Isothermal mutarotation of RCM-*Ascaris* gelatin at various temperatures following a preliminary heating step (60°, 10 min). Solvent is 0.15 M NaCl–0.01 M acetate–50% ethylene glycol. Measured rotations have been corrected for the solvent "blank." Protein concentration is 0.38 mg/ml.

spectral range 200–300 mμ with minima in the Cotton trough at 210 mμ and with reduced, mean residue rotation, $[m']_{210} = -19{,}300°$. This finding is supported by the published circular dichroism spectrum of guinea pig skin collagen in 2:1 (v/v) ethylene glycol–water solution which agrees closely with the spectrum of this collagen in water (Brown *et al.*, 1969; Carver and Blout, 1967).

The effect of ethylene glycol on the melting curve of native *Ascaris* was investigated, and the characteristic two-stage melting process (Hauschka and Harrington, 1970a) was observed in both aqueous and mixed aqueous–glycol solvents. A relatively sharp helix → coil transition ($\Delta T_t = 9°$ in water, $\Delta T_t = 6°$ in water–glycol) accounts for about 80% of the rotatory change observed with increasing temperature. This is followed by a broader melt curve above 60° which is thought to reflect the unlocking of noncooperative residual structure stabilized by the disulfide cross-linkages within the molecule (Hauschka and Harrington, 1970a). Addition of glycol (1:1, v/v) elevates the apparent midpoint of the collagen → gelatin cooperative transition temperature, T_m, about 6° (from 51 to 57°) but the general shape of the melting curve remains unaltered. Flory and Garrett (1958) have reported an increase in T_m of about 11° on transferring bovine achilles tendon collagen and rat-tail-tendon collagen from water into *anhydrous* ethylene glycol. Thus, although the thermal stability of the collagen structure is increased in the presence of ethylene glycol, there is no evidence from optical rotatory dispersion and thermal transition experiments for changes in the conformational pattern.

Kinetics of Renaturation of RCM-Ascaris in Ethylene Glycol–Water. Figure 1 shows typical, isothermal, time-dependent changes in optical rotation (λ 215 mμ) of RCM-*Ascaris* in ethylene glycol–water at various temperatures from −20 to +35°. Following a preliminary heating step at 60° (10 min), solutions were quenched to the desired renaturation temperature and transferred to a preequilibrated, jacketed, polarimeter cell. The broad temperature range accessible for renaturation studies in the glycol–water system brings out several significant features in the kinetics of reversion. In the high temperature range (10–35°) the levorotation increases in a seemingly monotonic fashion in agreement with previous studies of predominantly single-chain vertebrate and invertebrate systems in dilute salt–water solvents. Initial rates of reaction show a strong negative temperature dependence and the extent of isothermal renaturation after prolonged incubation is dependent on the temperature. The situation is more complex in the low temperature range (−20 to 0°) where the α *vs.* time profiles show extremely rapid mutarotation over a short time interval then drop abruptly to a relatively low rate; crossing-over of the kinetic curves is observed.

Earlier studies of the kinetics of coil → helix regeneration in a number of gelatin systems have been confined primarily to the high temperature range with levels of undercooling below T_m of the respective collagens varying between 5 and 30°. These studies have consistently shown renaturation to follow apparent second-order dependence with respect to the concentration of chain elements in the unfolded form [Smith (1919); Harrington and von Hippel (1961); Drake and Veis (1964); McBride and Harrington (1967b); von Hippel (1967); Harrington and Rao (1970)]. Oriel and Blout (1966) have also reported second-order dependence in the renaturation of synthetic poly(Gly-Pro-Gly) at 25° in 0.7 M acetic acid. Poly(Gly-Pro-Gly) exhibits a broad collagen-like thermal transition with $T_m = 67°$ in 1.4 M acetic acid and is thought to form a poly-L-proline II or polyglycine II type conformational pattern at low temperature in these solvent systems.

It seems clear from the anomalous behavior of the α *vs.* time profiles at low temperature displayed in Figure 1 that no simple, integral order will account for the kinetics of renaturation of RCM-*Ascaris* over the entire temperature range. van't Hoff plots of log rate *vs.* log $(\alpha_\infty - \alpha_t)$ presented in Figure 2 confirm this interpretation. A linear van't Hoff plot is obtained at the highest temperature of renaturation examined (35°) with slope, $n = 1$. Within the intermediate

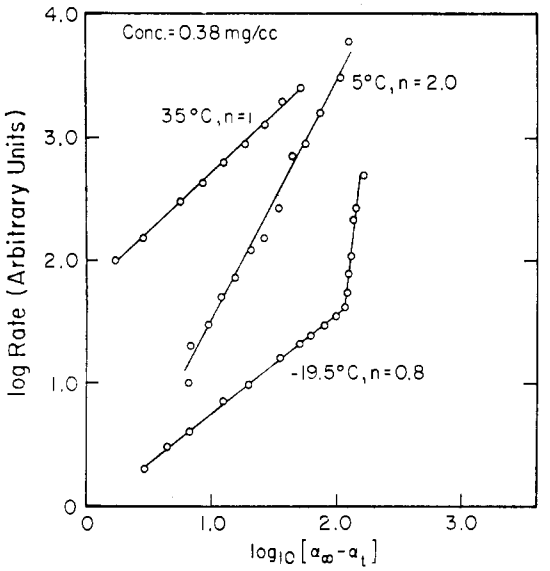

FIGURE 2: van't Hoff plots of isothermal RCM-*Ascaris* gelatin mutarotation at various temperatures. Data were obtained from Figure 1. Plots have been shifted along the ordinate to avoid confusion.

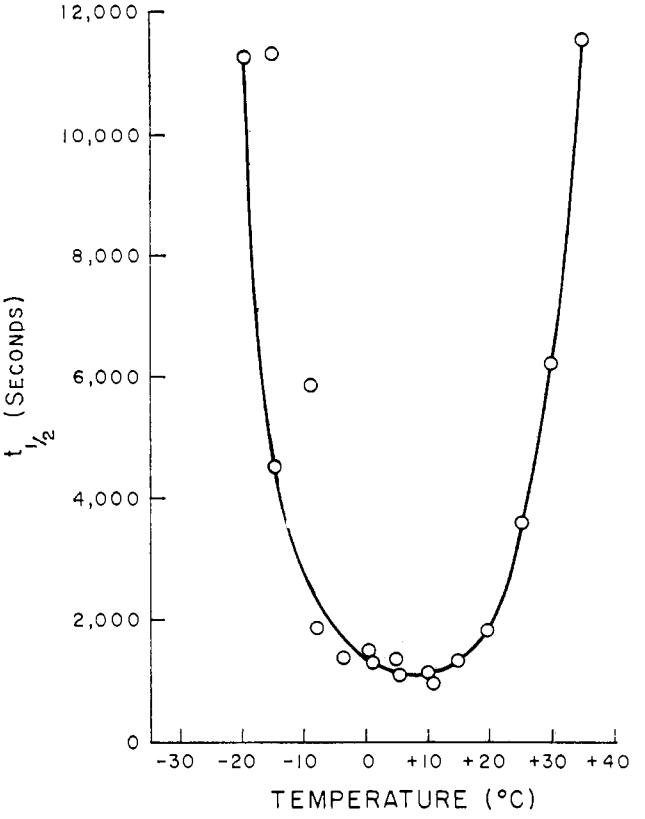

FIGURE 3: Half-time of the overall isothermal mutarotation of RCM-*Ascaris* in 0.15 M NaCl–0.01 M acetate–50% ethylene glycol as a function of temperature. Protein concentration = 0.38 mg/ml.

temperature range slopes corresponding to an order of reaction, $n = 2.0 \pm 0.2$ are seen in agreement with previously reported kinetic behavior. Striking discontinuities in the plots are observed at low temperatures, however, leading to the suspicion that the overall mutarotation reaction may be a sum of several processes with widely differing velocity constants. If the postulated first-order subsidiary reactions in the coil–helix reversion of RCM-*Ascaris* were of differing temperature dependence, apparent second-order behavior could occur over a restricted region of the temperature scale.

The possible existence of component reactions of differing temperature dependence is brought into sharp focus when the half-time of the overall mutarotation reaction is plotted against temperature (Figure 3). A well-defined maximum in the renaturation rate (minimum in $t_{1/2}$ *vs.* temperature) occurs near 10°, thus the suspected maximum rate at 3° in water (Harrington and Rao, 1970) is confirmed. The rate maximum appears at virtually the same level of undercooling (ΔT) below T_m of *Ascaris* cuticle collagen in the two solvent systems. Thus the maximum rate occurs ~47° below T_m of *Ascaris* collagen in ethylene glycol–water (1:1, v/v) and ~48° below T_m in dilute salt–water.

The presence of a maximum in rate–temperature studies of the three-dimensional crystallization of a variety of homopolymers is well documented (Mandelkern, 1964) and has been interpreted in terms of the unique temperature dependence of nucleation and growth processes (Becker, 1938; Turnbull and Fisher, 1949). Similar behavior has been observed in the kinetics of formation of ordered helical structures from the random-coil form of nucleic acids. Ross and Sturtevant (1962) found the rate of formation of the poly (A + U) double-stranded helical complex from random-coil polyriboadenylic acid and polyribouridylic acid to pass through a maximum about 40° below the helix melting temperature, T_m. The temperature dependence of this reaction was explained in terms of essentially equivalent theories utilizing either steady-state kinetics (Saunders and Ross, 1960) or a one-dimensional, biased random walk [the "gambler's ruin" problem (Flory, 1961)], both of which predict that at some temperature below T_m, the apparent second-order rate constant of helix formation should pass through a maximum.

In keeping with the qualitative similarity between the regeneration rate–temperature dependence of the kinetics of homogeneous, nucleated crystallization processes, the rate maximum of RCM-*Ascaris* renaturation displayed in Figure 3 occurs at 0.86 T_m when temperature is expressed in degrees Kelvin. A rate maximum between 0.8 and 0.9 T_m is characteristic of bulk crystallization reactions of linear polymers (Mandelkern, 1964).

The existence of a maximum at 10° in the rate–temperature profile for the refolding of the RCM-*Ascaris* chain is not in accord with expectations from the Flory–Weaver theory (1960) of helix regeneration in gelatin systems. Moreover, helix regeneration is first order with respect to concentration in single-chain gelatin polypeptides and exhibits a negative temperature coefficient which depends more strongly on temperature than the predictions of the two theories cited above (Flory, 1961). To account for the apparent contradiction of a first-order process leading to the formation of a three-stranded, triple-helical structure, Flory and Weaver (1960) postulated the formation of a unimolecular transitory intermediate, possibly a segment of the chain in polyproline II conformation, as the rate-determining step of the

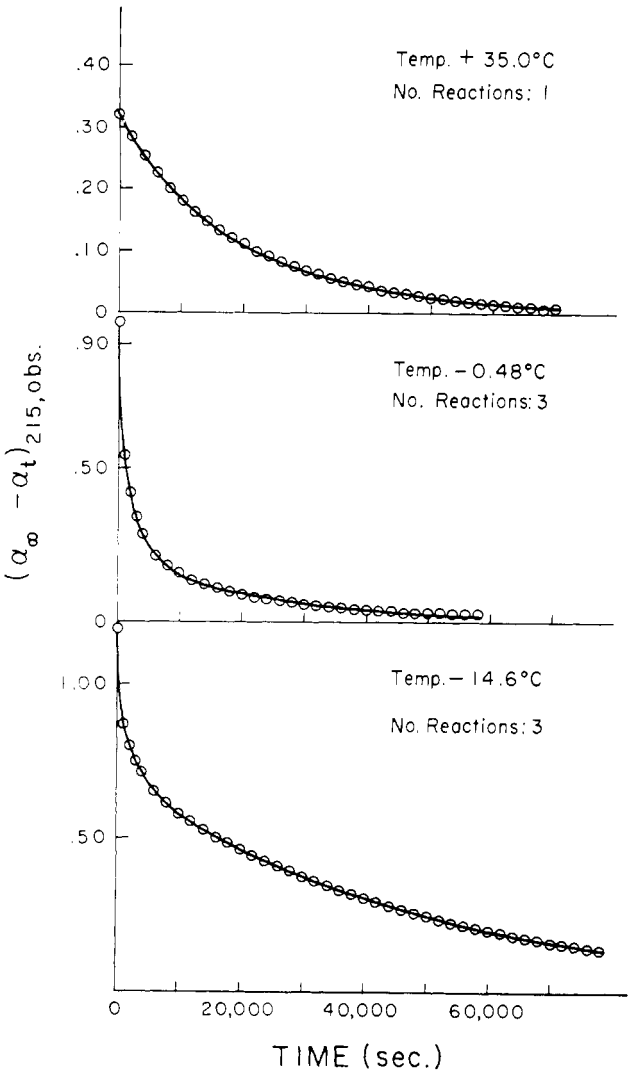

FIGURE 4: Analysis of three isothermal mutarotation curves of RCM-*Ascaris* in 0.15 M NaCl–0.01 M acetate–50% ethylene glycol. Points are taken from experimental α *vs.* time curves. Solid curves are reconstructed renaturation reactions obtained by summing contributions of first-order subordinate reactions according to eq 1.

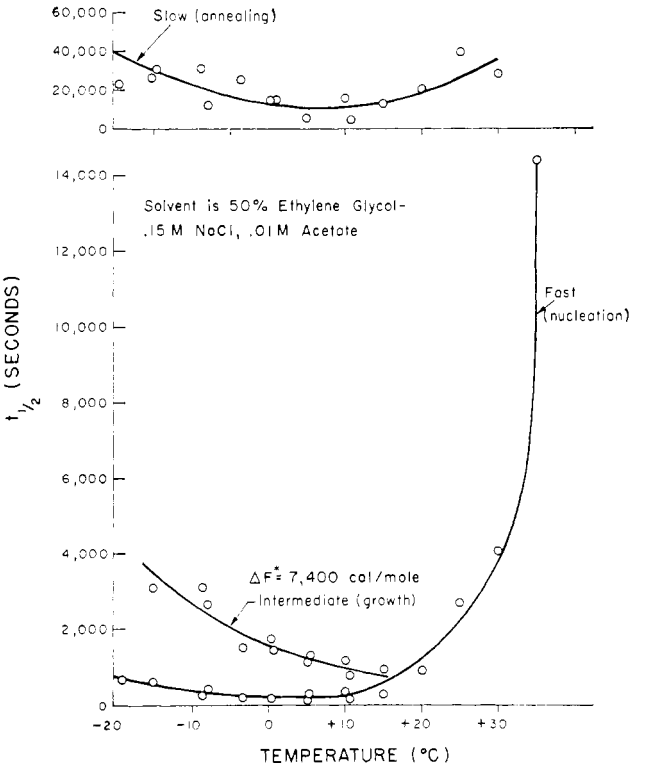

FIGURE 5: Half-time *vs.* temperature dependence of postulated nucleation, growth, and annealing reactions derived from isothermal mutarotation curves of RCM-*Ascaris* in 0.15 M NaCl–0.01 M acetate–50% ethylene glycol; protein concentration = 0.38 mg/ml.

reversion process. The theory leads to an equation for the apparent rate constant, k, in the form

$$k = Be^{-\frac{A}{RT\Delta T}} \qquad (2)$$

where A and B are constants and ΔT is the degree of undercooling ($T_m - T$). On the basis of the results of the previous paper (Harrington and Rao, 1970) we have proposed that the rate-limiting step is the formation of a hydrogen-bonded, triple-helical nucleus. This mechanism leads to a rate-temperature dependence identical with that of the Flory–Weaver formulation but is first order only at very low protein concentration. At high protein concentration ($\gg$2 mg/ml) we expect the rate-limiting step to become third order as a consequence of the association of three α-type chains to form the prototypic collagen nucleus. Equation 2 predicts a maximum in the rate of renaturation to lie somewhere between 0 °K and T_m, but it should occur far below the freezing point of water (at ~160 °K, if nucleation remains rate limiting). Conformity of experimental results to eq 2 over a wide range of protein concentrations is now well documented (Flory and Weaver, 1960; Drake and Veis, 1964; von Hippel, 1967; Harrington and Rao, 1970; Russell and Cooper, 1969). Reasoning by analogy with bulk crystallization processes, it seems possible that at temperatures far removed from T_m the Flory–Weaver rate expression becomes dominated by the temperature dependence of the growth reaction, which is expected to have a positive temperature coefficient. With these ideas in mind the overall renaturation process was interpreted tentatively as a sum of parallel first-order reactions of opposing temperature coefficients, this being the simplest kinetic scheme compatible with the data presented in Figures 1, 2, and 3.

Kinetic parameters were derived using a straightforward computer analysis (see Methods), and when these parameters were employed to reconstruct the mutarotation curve the fit was found to be good at each temperature over at least 90% of the transformation process. The fit of the summed reactions (eq 1) with the experimental α *vs.* time profiles for three typical renaturation experiments is shown in Figure 4. At 35° the overall change in optical rotation follows a single first-order process with respect to the amount of the chain in random-coil form with a van't Hoff slope of 1 as estimated in Figure 2. Two first-order reactions are required to fit the α *vs.* time plots in the temperature interval between 15 and 30°, and, at all temperatures below 15°, three uni-

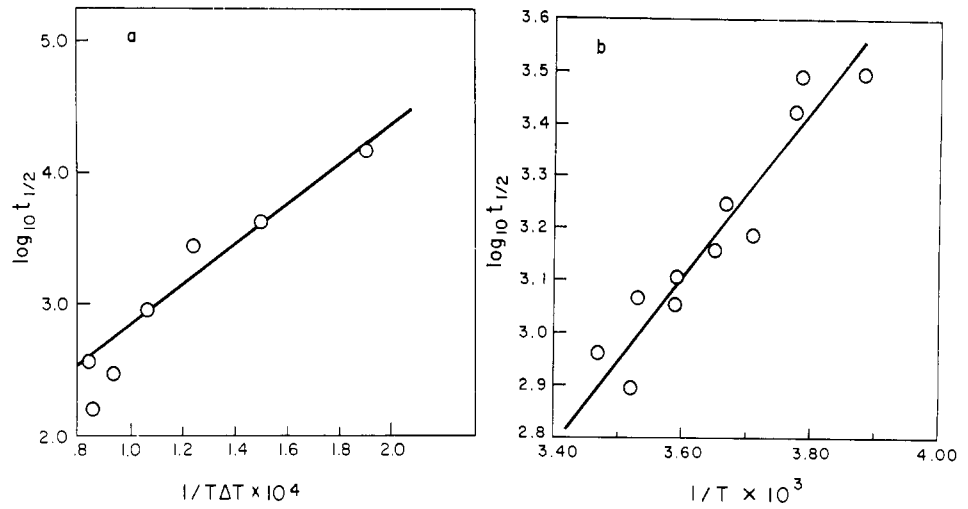

FIGURE 6: RCM-*Ascaris*. (a) Flory–Weaver plot (eq 2) of the nucleation reaction shown in Figure 5: A = 64,000 cal-deg-mole^{-1}; T_m = 52°. (b) Arrhenius plot of the growth reaction shown in Figure 5. ΔF^* = 7400 cal/mole.

molecular reactions are obtained from kinetic analysis of the mutarotation curves. Since we expected that each subordinate reaction would change in a continuous fashion with temperature, it was not difficult to identify and follow a specific reaction from inspection of the temperature variation of kinetic parameters (rate constant and size) derived from the computer analyses.

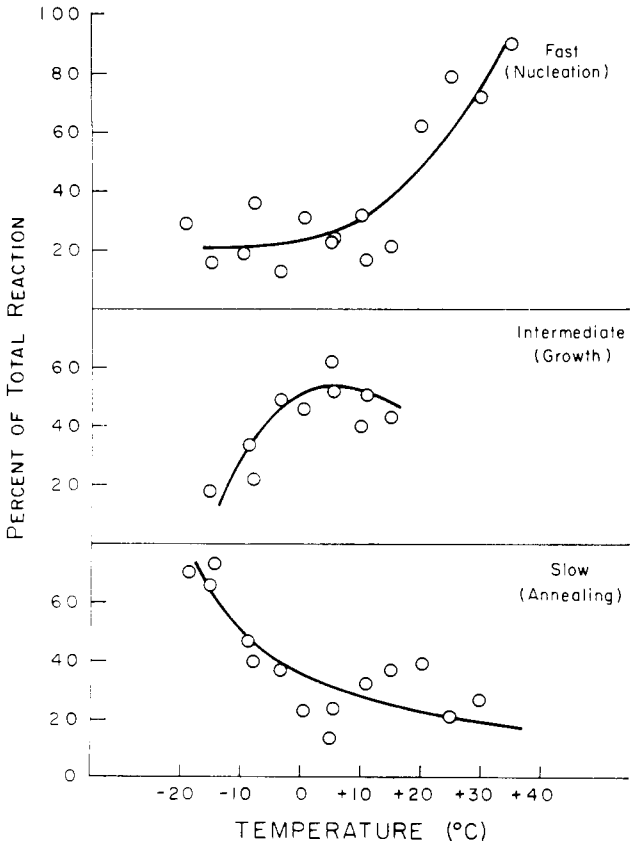

FIGURE 7: RCM-*Ascaris*. Plot of fractional contribution of each subordinate reaction as a function of temperature.

The rate–temperature dependence of each component reaction is shown in Figure 5. As the temperature is lowered below 35° it will be seen that one of the first-order processes, that of the right-hand limb of Figure 5, increases rapidly in rate ($t_{1/2}$ decreases) in accordance with the behavior expected for a nucleated crystallization reaction. A plot of log $t_{1/2}$ *vs.* $1/RT\Delta T$ according to the Flory–Weaver equation is sensibly linear over the temperature range 5–35° with slope A = 64,000 cal-deg-mole^{-1} (see Figure 6a). Most emphasis is placed on relatively low levels of undercooling, ΔT, since determination of accurate rates for this reaction becomes increasingly difficult at lower temperatures as a consequence of the presence of subordinate reactions with similar rates. At very low temperatures the extremely rapid rate of this reaction obviates accurate analysis.

The presence of a reaction exhibiting positive temperature dependence is detected at temperatures below 15° (Figure 5), and it is the experimental decline in rate of this process with decreasing temperature which appears to be responsible for the deep trough in the $t_{1/2}$–temperature profile of Figure 3. An Arrhenius plot of this process is presented in Figure 6b yielding an activation free energy ΔF^* = −7400 cal/mole. Superimposed on the two processes of opposing temperature dependence described above is a much slower, temperature-dependent reaction which contributes to the mutarotation curves over the temperature range −20 to +30°. A rather wide scatter in estimated rates is seen as a result of the extended times required for completion of this reaction and the consequent difficulty of determining the infinite-time value of α. Results suggest a minimum in the $t_{1/2}$ temperature profile near 10°.

Figure 7 illustrates how each of the subordinate reactions, plotted as a percentage of the total regeneration reaction, varies with temperature. The contribution of the fast (nucleation-controlled) reaction decreases on lowering the temperature, levelling off at about 20% in the low temperature range. Above 15° the rate of the intermediate reaction,

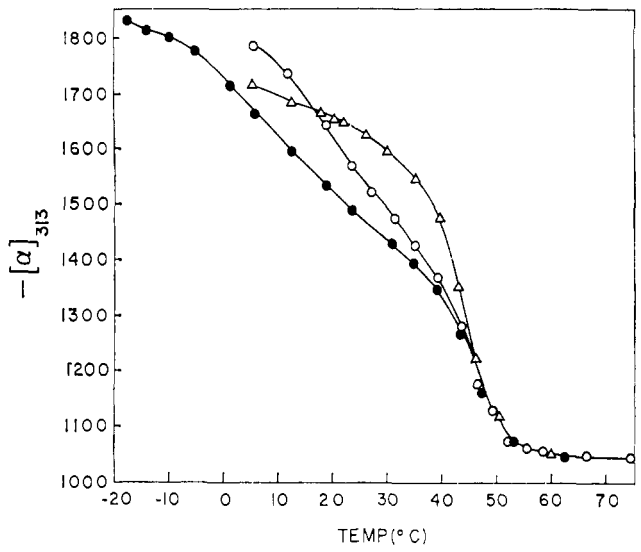

FIGURE 8: Melting curves of RCM-*Ascaris* gelatin refolded at various temperatures. Points represent 30-min incubation times at each temperature. Solvent is 0.15 M NaCl–0.01 M acetate–50% ethylene glycol: (●) renatured at −18° (3.5 days); (○) renatured at +5° (3.5 days); (△) renatured at +20° (12 days). Specific rotation is not corrected for index of refraction; protein concentration = 0.38 mg/ml.

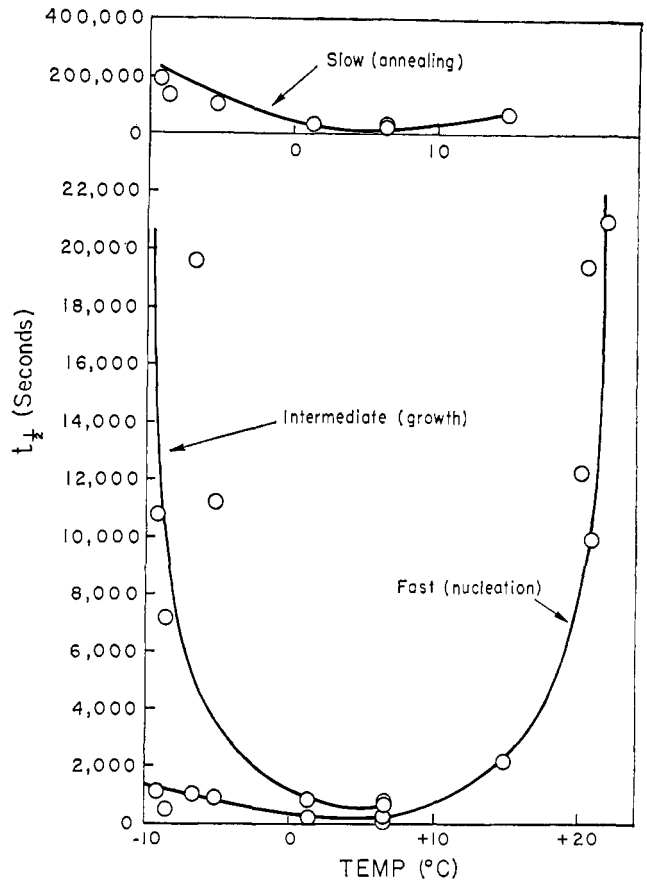

FIGURE 9: Half-time *vs.* temperature dependence of postulated nucleation, growth, and annealing reactions derived from isothermal mutarotation curves of α_1 ratskin in 0.15 M NaCl—0.01 M acetate–50% ethylene glycol; protein concentration = 0.05 mg/ml.

provisionally identified as a growth process, exceeds the rate of the nucleation-controlled reaction, and the measured optical rotatory change will thus include a contribution from both processes. The growth reaction declines in relative amount below 0°, whereas the fraction of the total mutarotation attributable to the slowest time-dependent process (Figure 7, bottom) increases with decreasing temperature over this range until it dominates the overall reaction. At −20° this process accounts for greater than 70% of the rotatory change observed during renaturation. It seems reasonable to postulate that this reaction reflects the annealing of large segments of the chain which have been "frozen" into disordered conformational patterns by the extremely rapid nucleation rate at these low temperatures. [Further insight into the nature of this reaction will be provided in a later paper (Hauschka and Harrington, 1970b).] In the high temperature range the contribution of the "annealing" process declines to a small fraction of the total reaction (Figure 7). Following this line of reasoning it would be expected that a more highly ordered structure would be regenerated in the high temperature range, and this conclusion is supported by the studies of Beier and Engel (1966) who have reported that the melting profiles of renatured calfskin collagen exhibit increasing cooperativity, as judged by a decrease in width of the helix → coil transition profile and elevation in T_m, as the temperature of isothermal renaturation is increased.

Melting profiles of RCM-*Ascaris* obtained after isothermal renaturation in 50% glycol at various temperatures below T_m indicate similar behavior (Figure 8). The sharpness of the helix → coil transition and the fraction of the chain undergoing cooperative melting increases as ΔT, the level of undercooling employed for renaturation, decreases. The virtual coincidence of the melting points (52°), taken as the temperature for completion of the transformation, indicates that the three-dimensional pattern of the cooperative structure in all of these refolded systems is the same.

Kinetics of Renaturation of α_1-Ratskin Gelatin. The kinetic scheme advanced to interpret the mechanism of formation of the collagen fold in RCM-*Ascaris* also applies to renaturation of the vertebrate single-chain gelatin α_1-ratskin. Half-time temperature studies of α_1-ratskin in ethylene glycol–water (1:1, v/v) at a protein concentration within the concentration-independent range (0.05 mg/ml) reveal a well-defined maximum in rate near 4°. Resolution of the mutarotation reaction into component subordinate reactions according to the procedure adopted for RCM-*Ascaris* again suggests that the minimum observed in the half-time temperature plot results from opposing temperature dependencies of nucleation and growth processes (Figure 9) Although there is no indication of an inversion in the renaturation rate–temperature profile of α_1-ratskin in aqueous solvent systems, indeed, a rate maximum has not been reported for any of the vertebrate gelatins which have been examined, it seems clear that as a consequence of the low T_m of this collagen (36°) the temperature of maximum rate should lie below 0°. Based on an elevation of T_m in 50% glycol comparable to that found for RCM-*Ascaris*, the α_1-ratskin chain would be expected to exhibit a maximum in renaturation rate near −4° in water.

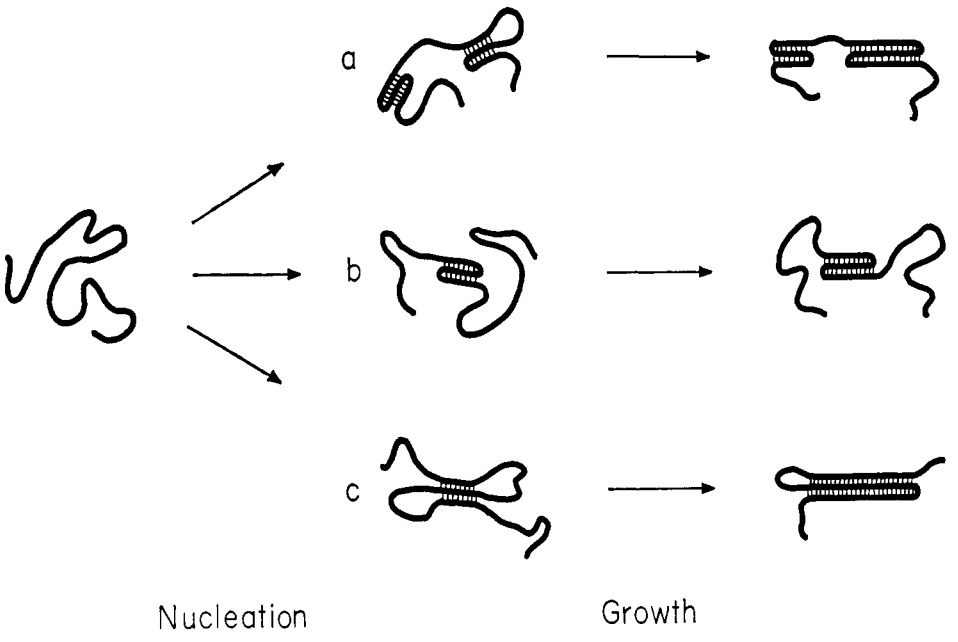

FIGURE 10: Possible arrangements of the polypeptide chain during intramolecular nucleation and growth of collagen-fold structure.

The kinetic studies of α_1-ratskin are less detailed than those of RCM-*Ascaris*, but qualitatively similar behavior is observed in the percentage contribution of the subordinate reactions to the overall mutarotation as a function of temperature. Interpretation of the temperature dependence of each reaction follows the same line of argument as that proposed for renaturation of RCM-*Ascaris*.

Conclusion

The form of the dependence of the rate of renaturation on temperature for RCM-*Ascaris* and α_1-ratskin polypeptide chains observed in the present study finds explanation in ideas which have been developed for nucleation and growth of polymer crystallites. In the temperature region above 15° we assume nucleation to be rate controlling in the RCM-*Ascaris* system (8° in α_1-ratskin). Addition of residues to the nucleus (the growth reaction) will occur so rapidly in the high temperature region near T_m as to have no influence on the rate. Thus the temperature dependence of the rate would conform to the Flory–Weaver formulation (eq 2). The precise arrangement of the chain conformation in the nucleating step cannot be defined unambiquously at the present time, but evidence that renaturation in several types of single-chain gelatin molecules occurs intramolecularly at low concentration in dilute salt–water solvent systems has been provided in earlier work (McBride and Harrington, 1967b; Harrington and Rao, 1970) leading to the proposal that probably all collagen-type, noncross-linked polypeptide chains fold back upon themselves to form a stable triple helix under these conditions. This type of folding pattern has been examined in the recent model building study of Ramachandran *et al.* (1968) who have shown that a stereochemically acceptable, reverse-folded, triple-helical structure can be constructed which will accommodate the sequence Gly-X-Y where X and Y are any amino or imino acid. Thus it seems reasonable to postulate that nucleation in these chains involves interaction of three rather widely separated segments of the chain to form the prototypic triple-helical pattern of collagen through reverse folding. Various possible arrangements of the chain during the nucleation and growth steps are shown in Figure 10. It seems likely that a number of local regions along the chain could act as initiating sites for nucleation so that a variety of reverse-folded conformations with varying amounts of the chain in the triple-helical conformation would result. Certain of the nuclei would be much more favorable to subsequent growth of the triple helix than others. According to the model, growth would occur by accretion of adjoining peptide units at each of the two nucleus–random coil interfaces as the pendant chain segments twist about each other to form the coiled-coil structure. The transformation to the three-dimensional geometry of collagen from nuclei such as those shown in Figure 10a,b would be limited, since growth through a creeping movement of each chain segment entering the nucleus in opposing directions would be expected to be a slow and unfavorable process, requiring the breaking of a large number of hydrogen bonds. Such a process may well be involved, however, in the slow annealing of the chain conformations which are "frozen in" at very low temperature.

Regions of the chain which are rich in pyrrolidine residues are good candidates as initiating sites for nucleation. Local ordering of the chain into the polyproline II type conformation characteristic of the triple helix would require a comparatively small decrease in entropy (per residue) in these regions since the number of conformational states available to an imino acid residue in the random polypeptide chain is significantly lower than for an amino acid residue (Garrett, 1960;[1] Harrington, 1964; Schimmel and Flory, 1968).

[1] Quoted by P. J. Flory.

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