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Collagen Structure in Solution. V. Kinetic Mechanism of Refolding of Cross-Linked Chains*

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ABSTRACT: Kinetic analysis of the renaturation reaction of cross-linked ichthyocol and native *Ascaris* collagen over a wide range of temperatures discloses three subordinate first-order processes (nucleation, growth, and annealing), whose sum is equal to the total reaction. The apparent second-order kinetics of mutarotation observed over a *limited* temperature range ($10° < \Delta T < 30°$, where $\Delta T = T_m - T_{refolding}$) are an artefact resulting from the relative rates and sizes of the three-component reactions. For native *Ascaris* collagen, the nucleation and growth processes increase in rate ($T\Delta T$ constant) as the isoelectric pH is approached from below; hence both nucleation and growth involve interaction of several polypeptide chain segments. The nucleation reaction fits the Flory–Weaver relationship $k' = B \exp(-A/RT\Delta T)$ with values of A = 14,300 cal-deg/mole for cross-linked ichthyocol, pH 5.93, and A = 8400 cal-deg/mole for native *Ascaris* collagen, pH 2.58. The three- to tenfold larger values of A for single-chain gelatins account for their greatly reduced rates of refolding compared with cross-linked chains. Positive temperature dependence of the growth reaction conforms to the Arrhenius relationship with an activation energy (ΔF^*) of about 7400 cal/mole for all species of gelatin. The maximum overall refolding rates [occurring at T_{opt}, where $\phi \equiv T_{opt}/T_m$ (T in °K)] are closer to T_m for the cross-linked collagens (ϕ = 0.93 to 0.94) than for the single-chain gelatins studied by Harrington and Karr (*Biochemistry 9*, 3725 (1970)), where ϕ = 0.85–0.86. This is in agreement with predictions from classical crystallization theory relating ϕ to the dimensionless ratio $T_m\Delta F^*/A$.

In two previous papers (Hauschka and Harrington, 1970a,b) we have examined some of the refolding properties of cross-linked collagens. The data suggest certain similarities to single-chain gelatin refolding. Negative temperature dependence of the initial refolding rate, slow annealing in the late stages of the reaction, and first-order dependence on protein concentration in dilute solution have all been observed previously for single-chain gelatins. Some controversy has existed over the temperature dependence of initial refolding rate in cross-linked systems. Drake and Veis (1964) found increasing rates with decreasing temperature for cross-linked ichthyocol between 15 and 20°. We have confirmed this observation and extended the temperature range from 34 (T_m) to 0° (Hauschka and Harrington, 1970a). However, a *positive* dependence of initial rate on temperature was reported for the extensively cross-linked native *Ascaris* collagen (Josse and Harrington, 1964; McBride and Harrington, 1967). Clearly such a discrepancy would obviate a unified refolding mechanism for all types of collagen.

This paper presents detailed examination of the temperature dependence of refolding in the cross-linked ichthyocol and native *Ascaris* collagen systems. The seemingly aberrant earlier results for native *Ascaris* were found to be a consequence of insufficiently fast measuring techniques, and negative temperature dependence of all initial refolding rates for cross-linked collagens has been established. Analysis of the refolding kinetics in terms of nucleation, growth, and annealing processes corroborates the single-chain gelatin studies of Harrington and Karr (1970).

Materials and Methods

Collagen solutions were prepared and handled as described in a previous paper (Hauschka and Harrington, 1970a).

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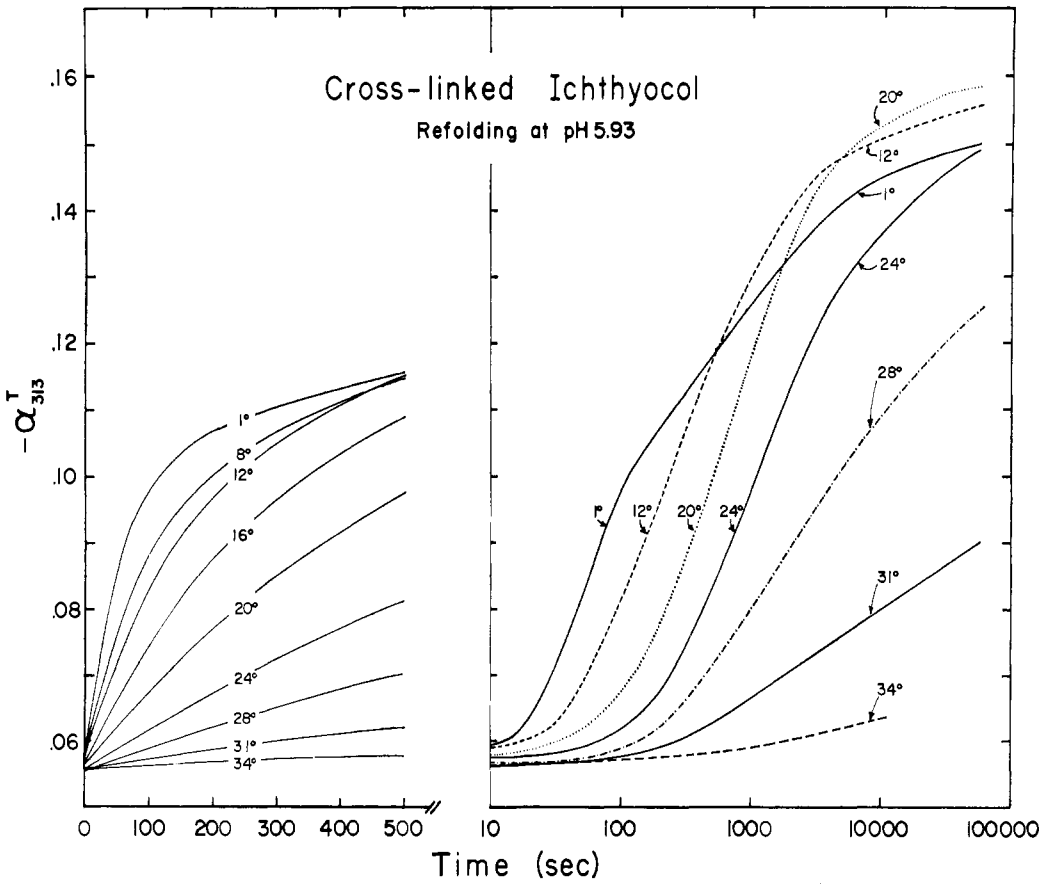

FIGURE 1: Refolding kinetics of cross-linked ichthyocol at various temperatures; protein concentration, 0.080 mg/ml in 0.1 M citrate, pH 5.93. Left: early kinetics plotted on linear time axis. Right: overall kinetics plotted on logarithmic time axis.

Measurement of refolding kinetics by optical rotation involved the same procedures as before.

Analysis of renaturation reactions in terms of a sum of parallel first-order reactions was carried out by hand calculation in a somewhat different fashion than the computer method of Harrington and Karr (1970). The method of analysis was such that meaningful information would result only if rates of the composite reactions differed by more than a factor of 5–10. This being the case, then at sufficiently long times only the *slowest* of the parallel first-order reactions was contributing to the overall observed reaction. By plotting ln ($[\alpha]_{obsd,t} - [\alpha]_\infty$) *vs.* t, the slowest reaction was found to emerge from the total reaction at large t as a straight line with slope $-k_s$. From the y intercept of this straight line, the amount of "reactant" participating in the slowest reaction, $[\alpha]_{s,o}$, was determined. In this case, the "reactant" was measured in degrees of specific rotation. The slow reaction, now completely characterized by eq 1, was subtracted from

$$[\alpha]_{s,t} = [\alpha]_{s,o}e^{-k_s t} \quad (1)$$

the overall reaction and a second plot was made of ln ($[\alpha]_{obsd,t} - [\alpha]_{s,t}$) *vs.* t. By repetition of the above sequence of steps, it was possible to finally write an expression for the observed reaction in terms of separate parallel first-order reactions (eq 2). The subscripts s, i, and f refer to "slow," "intermediate," and "fast" reactions, respectively. Equation 2 is written with *three* first-order terms because it was found that exactly three such reactions were both necessary and sufficient to completely describe the total renaturation process of the cross-linked collagens at all temperatures which were examined. When the observed mutarotation data were

$$-[\alpha]_{obsd,t} = -[\alpha]_\infty - [\alpha]_{s,o}e^{-k_s t} - [\alpha]_{i,o}e^{-k_i t} - [\alpha]_{f,o}e^{-k_f t} \quad (2$$

plotted together with the calculated reaction (eq 2), the curves were superimposable to better than ±1% over the entire time course of the reaction. This analysis was applied to a number of separate renaturation curves measured at different temperatures, and it became desirable to trace the temperature dependence of the three subordinate reactions. One reasonable assumption was required: variations in size and rate of all reactions occurs in a *continuous* fashion as the temperature is changed. With sufficient data, there was no difficulty in tracing the course of each separate reaction, especially since the rate constants usually differed by a factor of 5–10.

Results

Cross-Linked Ichthyocol. REFOLDING AT VARIOUS T. Cross-linked ichthyocol (c = 0.080 mg/ml) in 0.1 M citrate, pH 5.93, was melted at 60° for 10 min, and then quenched to temperatures between 1 and 34°. A pH of 5.93 was chosen

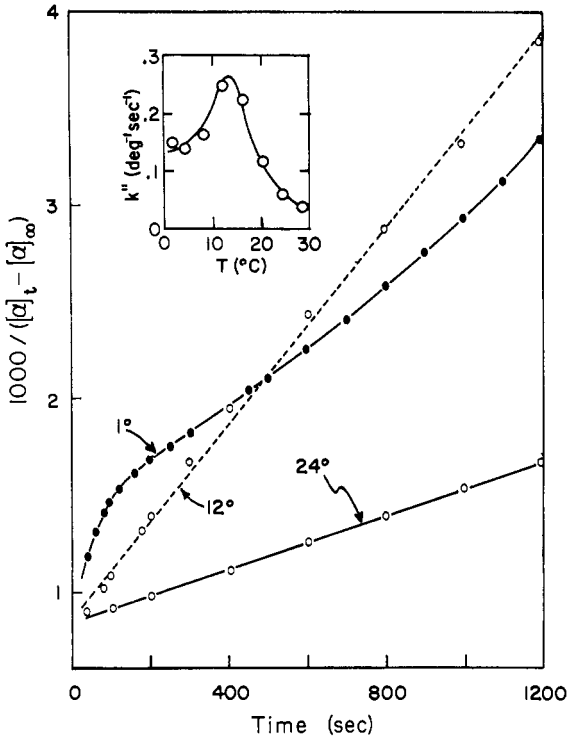

FIGURE 2: Second-order kinetic analysis of cross-linked ichthyocol refolding. Conditions were those of Figure 1. Inset: dependence of second-order rate constant (k'') on temperature of refolding.

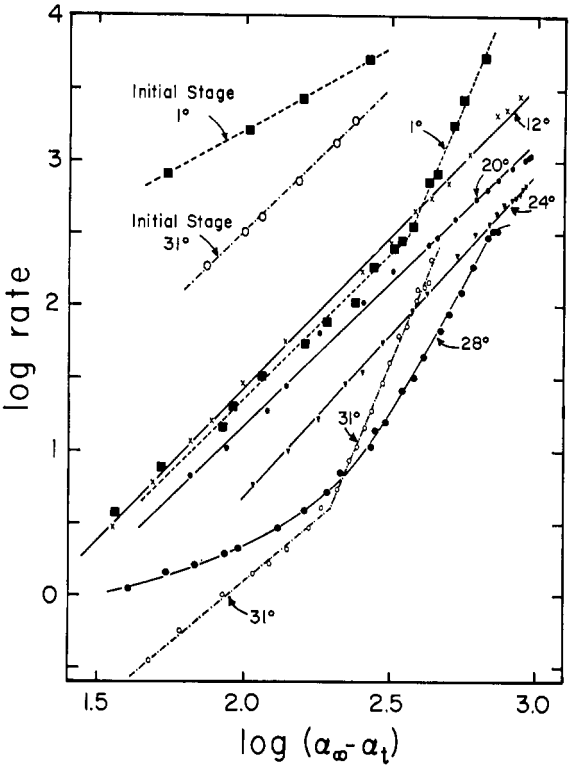

FIGURE 3: van't Hoff analysis of kinetic reaction order for cross-linked ichthyocol refolding. Conditions were those of Figure 1. At 12, 20, 24, and the initial stage at 31° (see text) the slope is $n = 2.0 \pm 0.1$. At 1° the initial reaction slope is $n = 1.1 \pm 0.1$. Initial stage at 31° has been raised one unit on the ordinate.

to maximize the available range of ΔT by increasing T_m (within the limits of the buffering capacity of citrate). Results are presented in several ways so that all phases of the reactions can be examined. In Figure 1, $-\alpha_{313}^{T}$ is plotted against log time to show the overall reactions at various temperatures. The sigmoidal shape of the curves is an artifact of the abscissa, for when $-\alpha_{313}^{T}$ *vs.* time is plotted, a set of normal monotonic functions with downward curvature is generated (Figure 1, left).

A very interesting phenomenon is noted for the reactions carried out at the lower temperatures (1–8°). After an initial burst of mutarotation, rapid decrease in the rate causes crossing-over of the kinetic curves. The higher temperature isotherms (12–24°) definitely provide a more complete recovery of levorotation at longer times. Depending on the time at which levorotation values of refolded gelatins are compared, the renaturation of cross-linked ichthyocol can appear to have either a negative temperature dependence or a complex dependence (positive below the optmum refolding temperature and negative above this temperature). The apparent ambiguity of this situation arises from the impossibility of viewing the renaturation process as one reaction.

It is clear that the initial rate is not the only meaningful kinetic parameter of the renaturation reaction because of the crossing of the mutarotation curves. In order to obtain a rate parameter characteristic of the overall reaction, kinetic curves were analyzed according to a second-order reaction of the randomly coiled chain segments; this order had been found in numerous previous studies on the refolding of various gelatins (Smith, 1919; Ferry, 1948; von Hippel and Harrington, 1959; Piez and Carrillo, 1964; Harrington and Rao, 1970). Some results of this second-order analysis are presented in Figure 2. Cursory inspection would suggest that the data conform fairly well to second-order kinetics, especially if the first 100 sec of the 1° reaction is overlooked as it has been in previous studies. Slopes of these plots show a very interesting behavior of the second-order rate constant, k''. As the renaturation temperature decreases to about 12–14°, k'' increases, in keeping with the negative temperature dependence of the reaction; below 12°, the magnitude of k'' decreases (Figure 2, inset). The temperature optimum of ~14° occurs at (0.93) (T_m °K). Harrington and Karr (1970) have observed optimum mutarotation temperatures for RCM-*Ascaris* and α_1-ratskin gelatin chains in 50% ethylene glycol. This has been viewed as a consequence of the opposing temperature dependence of the nucleation and growth processes. Later in the present study, similar data and interpretations will be discussed for native *Ascaris* collagen.

Second-order behavior is not always exhibited by the refolding kinetics of cross-linked ichthyocol. At low temperatures (1–8°) the initial stages of the process show marked curvature (Figure 2), and at 28° or more the second-order plots are slightly curved over the entire range of time plotted. Because of the failure of a second-order rate law to fit *all* the data, an examination of reaction order was carried out by the method of van't Hoff. Plots of log rate *vs.* log ($\alpha_\infty - \alpha_t$) are shown in Figure 3. In keeping with their apparent

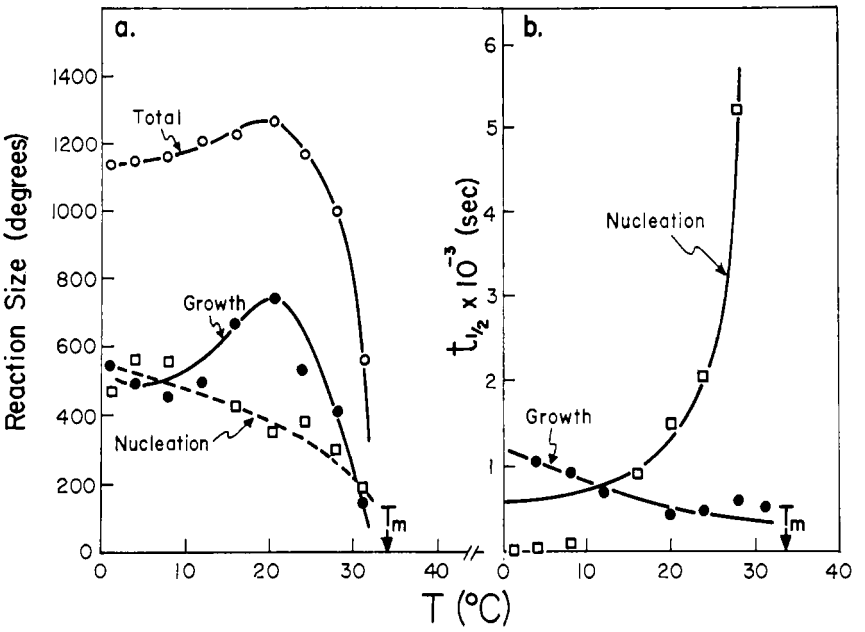

FIGURE 4: Component first-order reactions for the refolding of cross-linked ichthyocol. Conditions were those of Figure 1. (a) Reaction size as a function of temperature; (b) observed half-times of nucleation (□) and growth (●) reactions. Solid line for growth reaction drawn according to the Arrhenius relationship using $\Delta F^* = 7400$ cal/mole. Solid line for nucleation reaction fits the Flory–Weaver relationship with $A = 14{,}300$ cal-deg/mole. At low temperature, "freezing in" of noncooperative structure causes abnormally small observed half-times for the nucleation reaction. The third component reaction, annealing, amounted to only ~10% of the total reaction and is not included in this figure.

second-order kinetics from Figure 2, the reactions between 12 and 24° give perfectly linear van't Hoff plots over a 100- to 1000-fold change in rate with a slope (reaction order) of $n = 2.0 \pm 0.1$. Anomalous behavior is observed outside of this temperature range. Both the 1 and 31° plots, for example, show distinct breaks where the slopes change from 2 to 5. Such biphasic curves cannot be directly interpreted, but reanalysis is possible by choosing new α_∞ values equal to the observed rotation at the time where the break in the van't Hoff curve occurred. Subsequent plots (Figure 3) revealed the kinetic order of the early stages of these anomalous reactions. At 31°, the initial portion continued to be second order ($n = 2.0$), while at 1° (also at 4 and 8° which are not shown) the early process was first order ($n = 1.1$).

To further investigate this complex situation, the renaturation reactions were analyzed as the sum of parallel first-order reactions. Each observed renaturation curve could be accurately described by three first-order reactions (slow, intermediate, and fast), and could be written in the form of eq 2. Results of this analysis are presented in Figure 4, and it can now be surmised why the van't Hoff plots behaved as they did. At 1 and 4°, the fast reactions are 17–27 times faster than the intermediate reactions, while at other temperatures the ratio is not nearly so large (between 4 and 9). Such a separation in rates allows the fast reaction to reach 90% completion at low temperatures before there is more than a 10–15% contribution to the observed rotational change by the intermediate reaction. For this reason, the fast reaction is separable by the van't Hoff analysis as shown in Figure 3. The 8° reaction shows the shift from a clearly separable initial first-order reaction to an overlapping case. At 12° and above there is sufficient overlap between the fast and intermediate first-order reactions that they *appear* as one second-order process. Since van't Hoff analysis revealed second-order kinetics for both limbs of the 31° reaction, it is believed that the break was caused by a slower second-order process taking over after completion of a slightly faster one. Transition between normal second-order behavior at 24° and the compound kinetics at 31° is shown by the 28° curve which defies analysis (Figure 3).

Parallel first-order reaction analysis reveals other interesting facets of the cross-linked ichthyocol renaturation. Figure 4 shows sizes and half-times of the two largest subordinate first-order reactions. The very slowest reaction (~10% of the total) is omitted from this discussion. The total observed reaction increases slightly with temperature to a maximum size of 1270° at 20°C which is 86% of the 1475° difference between $[\alpha]_{313}^{20°}$ (native collagen) and $[\alpha]_{313}^{20°}$ (gelatin). When the melting temperature of 33.6° is approached, the total renaturation which is observed sensibly undergoes a sharp decline. The two major subordinate reactions at each temperature have been assigned to two separate processes, under the assumption that these processes will show monotonic changes in rate with temperature. The two processes thereby observed have strikingly different temperature dependence (Figure 4b). Following the treatment of Harrington and Karr (1970) the two reactions have been provisionally identified as nucleation and growth processes. The nucleation reaction, analyzed according to the Flory–Weaver equation (eq 2 in Hauschka and Harrington, 1970a), gives a good fit with $A = 14{,}300$ cal-deg/mole. The T_m used in this calculation was 36°, corresponding to the most stable regions of structure in cross-linked ichthyocol. It might be noted that melting curves of partially refolded cross-linked ichthyocol show inflection points at $T_m = 36°$ (Figure 4 of Hauschka and Harrington, 1970b); presumably

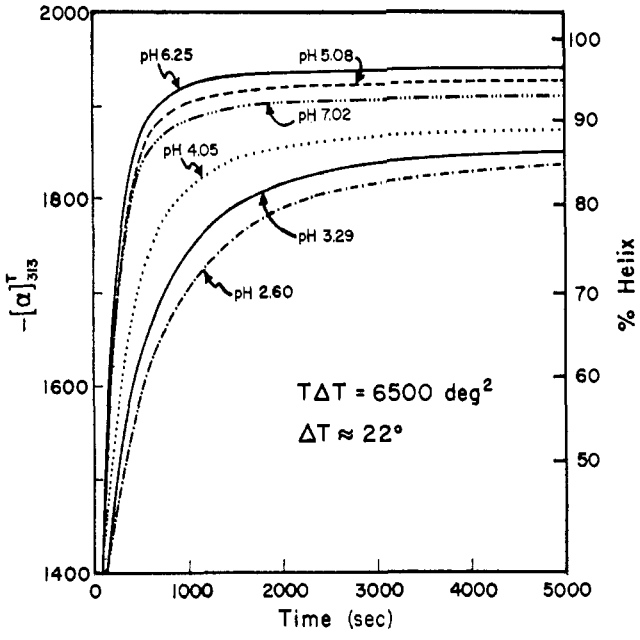

FIGURE 5: Refolding of native *Ascaris* collagen at constant $T\Delta T$; protein concentration, 0.107 mg/ml in 0.2 M NaCl–0.009 M citrate. Collagen was converted into gelatin (10 min at 80°) before refolding. Approximate per cent helix is indicated on right ordinate.

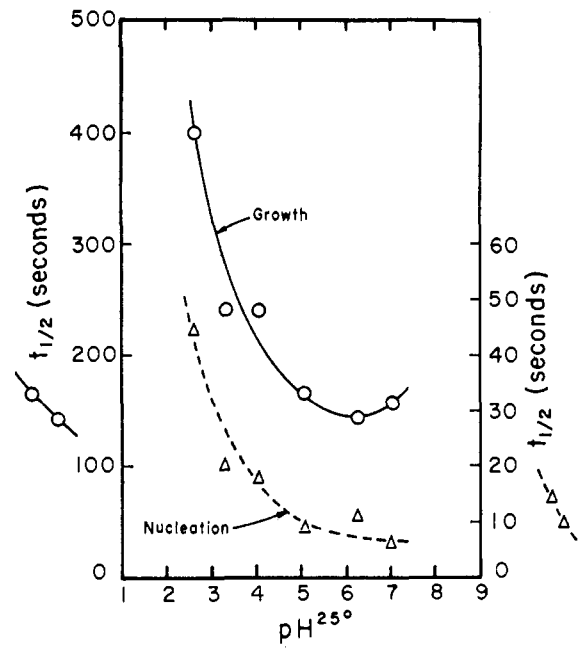

FIGURE 6: pH dependence of nucleation and growth reaction half-times for native *Ascaris* collagen refolding. Conditions were those of Figure 5. Left ordinate: growth reaction. Right ordinate: nucleation reaction.

these most stable regions of structure are the first to form during renaturation.

The growth reaction of Figure 4b was analyzed according to the normal Arrhenius relationship, yielding an activation energy ΔF^* = 7400 cal/mole. In Figure 4b the observed half-times of the subordinate first-order reactions are plotted along with the theoretical nucleation and growth reactions calculated from A and ΔF^*. Agreement between observations and theory is good except in those regions where the subordinate reaction is *not* rate limiting. This is partially caused by the inability of the kinetic analysis to completely resolve the faster component reactions. In addition, at low temperatures there is a very rapid "freezing-in" of marginally stable collagen-fold structure which does not correspond to the true nucleation process (Hauschka and Harrington, 1970b). This explains the differences between the calculated and observed nucleation reaction at low temperatures in Figure 4b.

Native Ascaris Collagen. REFOLDING AT VARIOUS pH ($T\Delta T$ = CONSTANT). With a knowledge of the dependence of T_m on pH for native *Ascaris* collagen, it was feasible to examine the overall refolding kinetics as a function of pH at constant $T\Delta T$. The initial rate data from this study were presented earlier (Hauschka and Harrington, 1970a). Figure 5 shows the overall renaturation process at $T\Delta T$ = 6500 deg². Analysis of these curves in terms of parallel first-order reactions discloses the large pH dependence of the component nucleation and growth reactions (Figure 6). Both processes are slowed considerably by protonation of the native *Ascaris* collagen below its isoelectric point. This observation provides firm support for the multichain segment nucleation mechanism proposed by Harrington and Rao (1970). If nucleation first proceeded within an extended single-chain segment (Flory and Weaver, 1960; Veis, 1964; von Hippel and Harrington, 1960) by development of a poly-L-proline II conformation, then protonation of polar residues should accelerate such a process by facilitating extension of the chain, since poly-L-proline II is very extended compared with the random chain. The opposite result is observed. Hence both nucleation and growth are believed to involve simultaneous interaction of several (probably three) chain segments. As pointed out by Harrington and Rao (1970) the protein concentration will determine whether the chain folding is intramolecular or intermolecular.

Native Ascaris Collagen. REFOLDING AT VARIOUS TEMPERATURES. Renaturation kinetics of native *Ascaris* were studied as a function of temperature at pH values of 6.25 and 2.60 in the solvent 0.2 M NaCl–0.009 M citrate. This choice of pH values was for the purpose of examining the process at its maximum rate, pH ~6, and at a much lower rate, pH ~2.6. Because of the 12° shift in T_m over this pH range, it was hoped that any relationships between the kinetic parameters and T_m might be clarified by deliberately varying the latter quantity.

In Figure 7 are the observed kinetics of renaturation at pH 6.25 for the early and overall stages of the reaction. To indicate the salient features of the temperature dependence without confusion, only a fraction of the data have been plotted here. The renaturation goes essentially to completion (88–97%), and the final values of $-[\alpha]^T_{313}$ (*i.e.*, $-[\alpha]^T_{313,\infty}$) are very nearly coincident with the equilibrium melting curve for native *Ascaris* collagen in the same solvent.

The complexity of the refolding process under examination is readily apparent from the crossing-over of the mutarotation curves in Figure 7. During the first minute or so after quenching there is a strict negative temperature dependence—that is, both the rate and extent of the mutarotation increase with decreasing temperature. Later on, however, the steepest curves at low temperatures suddenly break and exhibit

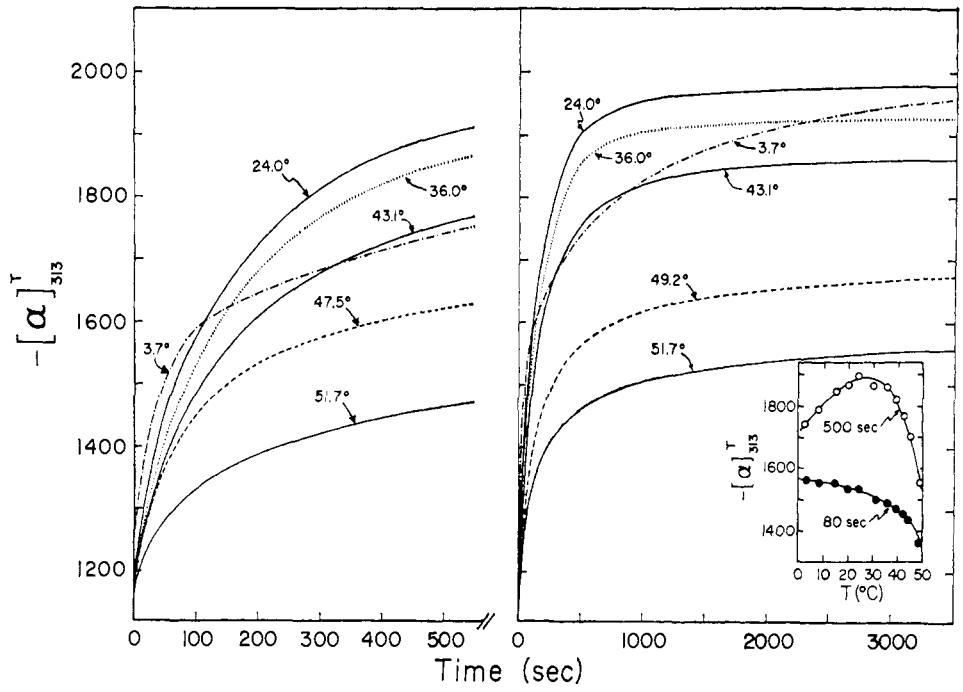

FIGURE 7: Refolding kinetics of native *Ascaris* collagen at pH 6.25; protein concentration, 0.107 mg/ml in 0.2 M NaCl–0.009 M citrate. Collagen was melted at 80° for 10 min prior to quenching. Left: early kinetics. Right: overall kinetics. Inset: specific rotations reached after 80 and 500 sec of refolding at various temperatures.

a much lower rate and degree of completion than the curves at higher temperatures. This effect is graphically demonstrated in Figure 7 (inset) where the extent of the pH 6.25 mutarotation at early (80 sec) and late (500 sec) times is plotted *vs.* temperature. The shapes of these isochrones is obviously dependent on the chosen time. At early times there is a monotonic negative temperature dependence while at late times the complex parabolic temperature dependence suggests an optimum temperature for the refolding process. Confirmation of an optimum temperature is obtained by plotting the overall rate parameter $10^4/t_{0.9}$ *vs.* temperature (Figure 8), where $t_{0.9}$ is the time required for 90% completion of the reaction. The maximum of this curve for the pH 2.60 process occurs at about 24° which is 0.94 (T_m, °K); at pH 6.25 the maximum overall "rate" is at 30° or 0.93 (T_m, °K). In this sense, native *Ascaris* renaturation is identical with cross-linked ichthyocol.

The initial negative temperature dependence for native *Ascaris* refolding was an unexpected result, in view of the positive temperature dependence reported for this process by Josse and Harrington (1964) and McBride and Harrington (1967). However, it is clear that the methods used in these earlier studies were not sufficiently fast to yield data during the first minute after quenching. Comparison of the appropriate curves from Figure 7 of the present study with Figure 5 of Josse and Harrington (1964) shows an identity of results at times greater than 2 min.

Initially, the empirical order of the native *Ascaris* collagen renaturation reaction may be examined by the method of van't Hoff. As for cross-linked ichthyocol (Figure 3) in the intermediate temperature range, straight line plots are obtained, yet the observed order is now $n = 1.3 \pm 0.2$, significantly different from $n = 2.0 \pm 0.1$ for cross-linked ichthyocol. At very large and very small ΔT, the van't Hoff plots show a sharp break from a slope of ~1.3 to ~3.6. These initial portions may be reanalyzed by choosing new α_∞ values, with the result that the initial stages of the reactions are approximately first order (Hauschka, 1969).

Results of kinetic analysis in terms of parallel first-order reactions are summarized in Figure 9. Similarity of the

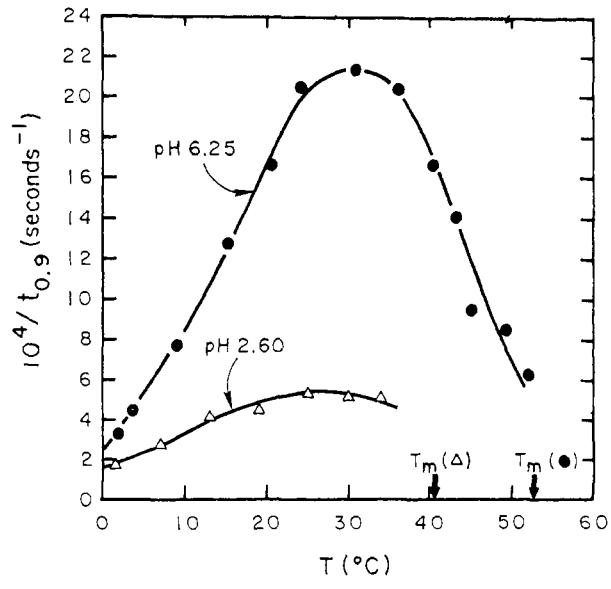

FIGURE 8: Temperature dependence of the overall rate of refolding of native *Ascaris* collagen. Conditions were those of Figure 7. $t_{0.9}$ is the time required for 90% completion of the observed refolding (mutarotation) reaction.

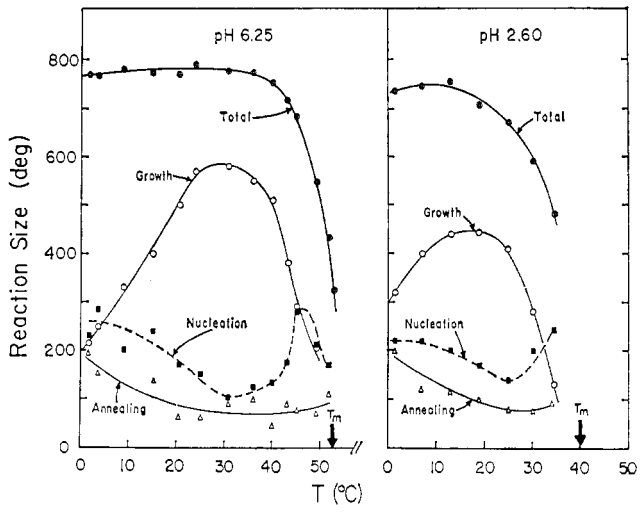

FIGURE 9: Component first-order reaction size for the refolding of native *Ascaris* collagen. Conditions were those of Figure 7. Left: refolding at pH 6.25. Right: refolding at pH 2.60.

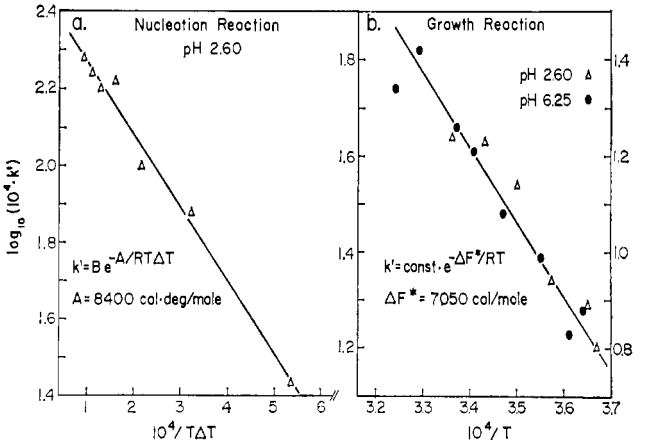

FIGURE 10: Component first-order reactions for the refolding of native *Ascaris* collagen. (a) Nucleation reaction (Flory–Weaver equation); (b) growth reaction (Arrhenius equation).

curves to Figure 4a for cross-linked ichthyocol is obvious. The growth reaction is maximum in size near the optimum refolding temperature, and the nucleation reaction increases in size as the undercooling becomes very large. At low undercooling the nucleation reaction is probably artefactually large because it contains some contribution from the much faster growth reaction. Accretion of ordered residues at the ends of helical nuclei (growth) should proceed immediately once nuclei of minimum stable size (n^*) have been generated. A third very slow subordinate reaction also increases in size at large undercooling. This probably represents the annealing process (Harrington and Karr, 1970; Hauschka and Harrington, 1970b); it is expected that the amount of "reactant" for annealing would follow the observed size-temperature profile because of "freezing-in" of poorly ordered collagen-fold structure at very low refolding temperatures.

Arrhenius and Flory-Weaver plots of the growth and nucleation reaction for native *Ascaris* collagen refolding are shown in Figure 10. A Flory–Weaver constant A = 8400 cal-deg/mole was obtained for the pH 2.60 nucleation process (Figure 10a). Nucleation at pH 6.25 was faster ($t_{1/2}$ < 30 sec) than could be accurately measured, except at very small undercooling, where a value of A = 13,000 ± 4000 cal-deg/mole was in agreement with the existing data. In Figure 10b the Arrhenius activation energy for the growth reaction at both pH values is the same ΔF^* = 7050 cal/mole.

Discussion

Despite the numerous observations of second-order kinetics for polypeptide chain folding in collagen systems, no satisfactory mechanism has been proposed to account for these kinetics. Furthermore, studies of the refolding process over an extremely wide temperature range have now shown that the apparent kinetic order is temperature dependent for both single-chain gelatins (Harrington and Karr, 1970) and cross-linked gelatins (Figure 3; see also Hauschka, 1969). While second-order kinetics are found in the intermediate temperature range for RCM-*Ascaris* and cross-linked ichthyocol, the low and high temperature extremes show complex kinetic order by van't Hoff analysis. Regardless of the particular collagen system, in most cases where complex van't Hoff plots have been found, the initial stage of the reaction is first order. Native *Ascaris* collagen refolding exhibits nonintegral kinetic order (n = 1.3) even in the intermediate temperature range. Because of the many serious exceptions; second-order kinetics are not generally applicable to the refolding process. Under certain conditions of temperature, there is clear evidence for the participation of a rapid first-order subordinate reaction (for example, the 1° refolding in Figure 3). The success of completely describing the refolding kinetics at all temperatures by the sum of first-order nucleation, growth, and annealing processes, suggests that apparent second-order kinetics is merely an artefact of the relative rates and sizes of the subordinate first-order processes. The nonintegral kinetic order of native *Ascaris* collagen refolding corroborates this hypothesis.

Earlier studies in this laboratory have shown that the *overall* refolding rates of single-chain gelatins conform to an expression of the type

$$\text{rate} = \text{const} \times e^{-\left(\frac{A/\Delta T + \Delta F^*}{RT}\right)} \qquad (3)$$

where A is the Flory–Weaver constant for the nucleation rate, and ΔF^* is the Arrhenius activation energy for the growth reaction rate. Observations in the present paper of refolding rates of cross-linked collagens are also described by eq 3. This equation was first derived by Becker (1938) to account for the odd temperature dependence of mixed crystal formation in platinum–gold mixtures.

One fundamental feature of linear polymer crystallization reactions is their complex temperature dependence which arises from the interplay of nucleation and growth processes having opposite dependence of rate on temperature. The overall crystallization (refolding) rate passes through a maximum at some temperature (T_{opt}) well below the polymer melting temperature (T_m). In order to characterize various

TABLE I: Refolding Parameters for Single and Cross-Linked Gelatin Chains.

Gelatin	T_m[a] (°K)	T_{opt} (°K)	ϕ (obsd)	$\Delta F^*/A$[b] (pred)	A (cal-deg/mole)	ΔF^* (cal/mole)	$\Delta F^*/A$ (obsd)	ϕ[c] (pred)
RCM-*Ascaris* in 50% glycol (v/v)	330	283	0.86 ± 0.02	0.11	69,000	7400	0.11	0.86
RCM-*Ascaris* in dilute salt, pH 4.6	324	276	0.85 ± 0.03	0.10	44,000	7400	0.17	0.88
α_1-Ratskin, pH 4.6	309	(266)			61,000	(7400)[d]	(0.12)	(0.86)
α_2-Ratskin, pH 4.6	308	(284)			15,000	(7400)[d]	(0.49)	(0.92)
α_1-Ichthyocol, pH 4.6	307	(258)			88,000	(7400)[d]	(0.08)	(0.84)
α_2-Ichthyocol, pH 4.6	305	(268)			43,000	(7400)[d]	(0.17)	(0.88)
Cross-linked ichthyocol, pH 5.93	309	287	0.93 ± 0.01	0.57	14,300	7400	0.52	0.93
Native *Ascaris*, pH 2.60	314	297	0.94 ± 0.02	0.78	8,400	7050	0.84	0.94
Native *Ascaris*, pH 6.25	326	303	0.93 ± 0.01	0.54	13,000[e]	7050	0.54	0.93

[a] T_m from *most-stable* region of melt curve for single-chain gelatins and cross-linked ichthyocol (Harrington and Rao, 1970). T_m by midpoint method for native *Ascaris*. [b] Predicted on the basis of ϕ (obsd) using eq 6. [c] Predicted on the basis of $\Delta F^*/A$ (observed) using eq 6. [d] ΔF^* assumed by analogy to other measured values. ϕ (predicted) and T_{opt} calculated on the basis of the assumed ΔF^*. [e] Based on only four points; possible error is ±4000 cal-deg/mole.

polymer systems, we may define the dimensionless parameter ϕ by eq 4, where the temperatures are in degrees Kelvin.

$$\phi = T_{opt}/T_m \quad (4)$$

Observations of rate maxima for the crystallization of polymers other than proteins are numerous. Natural rubber, polyethylene adipate, and polypropylene show temperatures of maximum crystallization with ϕ = 0.79 to 0.84 (Mandelkern, 1964). Ross and Sturtevant (1962) have demonstrated a turnover (ϕ = 0.89) in the apparent second-order rate constant for the formation of poly (A+U). While the phenomenon of an optimum temperature for protein refolding has not received attention in the literature, it has been observed previously. Kunitz (1948) presented, without comment, rates for soybean trypsin inhibitor renaturation which passed through a maximum at 30°, compared with T_m ~45° (ϕ = 0.95). Because renaturation was measured by precipitation, the interpretation of his results is difficult. Beier and Engel (1966) demonstrated an optimum temperature (24°) for generation of "re-formed collagen" from heat-denatured calfskin collagen (T_m = 36°). Harrington and Karr (1970) have reported a maximum (ϕ = 0.85–0.86) in the refolding rate of single-chain RCM-*Ascaris* collagen, and in this paper the cross-linked collagens have been shown to have ϕ values of 0.93–0.94.

In these refolding processes there is no single parameter which unequivocally describes the *overall* rate. Each parameter for overall rate reflects a bias toward that component reaction (nucleation, growth, or annealing) which happens to be the major contributor to mutarotation at the time of comparison. For instance, the inverse half-times ("rate" = $1/t_{0.5}$) for native *Ascaris* collagen refolding show a very flat "rate"–temperature profile (T_{opt} ~30°) with only a twofold variation in "rate" from 2 to 30°. However, if $1/t_{0.9}$ is used as an index of overall rate ($t_{0.9}$ = time to reach 90% completion), the "rate"–temperature profile shows sevenfold variation in magnitude from 2° to the unshifted T_{opt} of 30° (Figure 8). These differences are caused by domination of the early reaction stage ($t_{0.5}$) by the nucleation process, while relative rates of the growth process are more important at longer times. It is possible to fit the rate–tem-

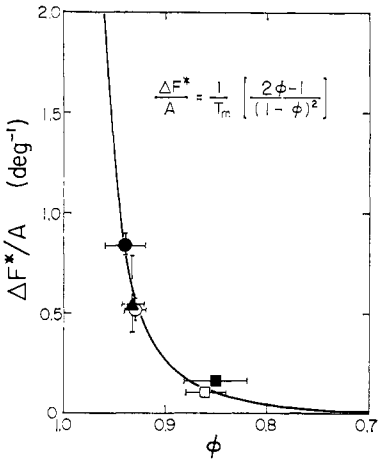

FIGURE 11: Comparison of predicted and observed refolding rate maxima; abscissa, $\phi = T_{opt}/T_m$; ordinate, $\Delta F^*/A$ where ΔF^* is the activation energy for the growth reaction, and A is the Flory–Weaver constant for the nucleation reaction. The solid line is drawn for T_m = 300° K according to eq 6. The points represent the observed values of $\Delta F^*/A$ and ϕ for several collagen systems. Cross-linked collagens: (●) native *Ascaris*, pH 2.60; (▲) native *Ascaris*, pH 6.25; (○) cross-linked ichthyocol, pH 5.93. Single-chain gelatins: (□) RCM-*Ascaris* in 50% glycol; (■) RCM-*Ascaris* in dilute salt, pH 4.6 (data of Harrington and Karr, 1970). Error bars indicate maximum possible error in observed values.

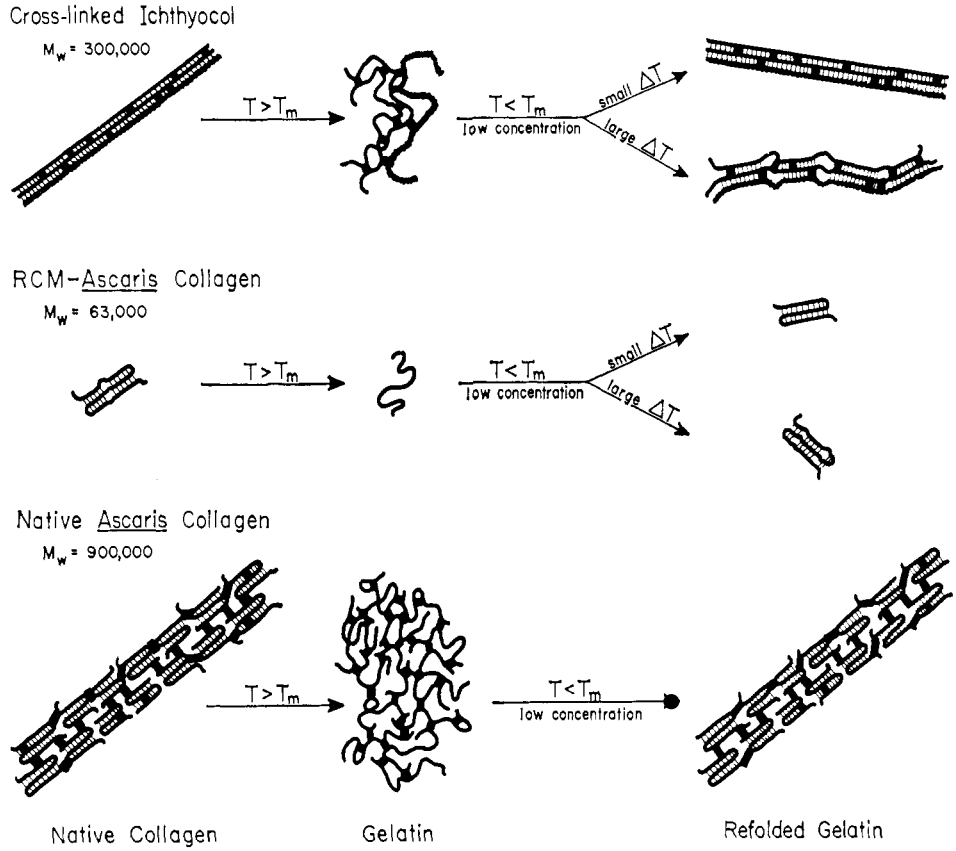

FIGURE 12: Schematic representation of refolding in the various collagen systems. Heavy bars signify dovalent cross-links, of which there are ~10/300,000 daltons in cross-linked ichthyocol, 0 in RCM-*Ascaris*, and ~150/900,000 daltons in native *Ascaris* collagen. Fine cross-hatching indicates triple-helical collagen structure.

perature profile of the collagen refolding process with the Becker equation (eq 3), yet the fit is not unique. Any pair of ΔF^* and A whose *ratio* ($\Delta F^*/A$) gives the proper T_{opt} (see eq 6) will be satisfactory. While the eq 3 curve becomes sharper as ΔF^* and A are increased, it is difficult to choose the proper sharpness, because the sharpness of the *observed* overall rate–temperature profile is somewhat arbitrary as discussed above.[1] Because of these problems, eq 3 was only used to compare values of A and ΔF^* determined from the component first-order nucleation and growth reactions with the observed T_{opt} as shown in Table I.

Examination of the relationship between ϕ and the parameters ΔF^* and A (eq 3) yields eq 5 which holds only at $T = T_{opt}$.

$$\frac{d(\ln \text{rate})}{dT} = 0 = \frac{\Delta F^*}{RT^2} + \frac{A(T_m - 2T)}{RT^2\,\Delta T^2} \qquad (5)$$

[1] Saunders and Ross (1960) have considered the steady-state solution of a crystallization process involving a bimolecular initiation step between chain segments A and B, with rate constant k_i, and many identical "growth" steps with rate constants k_f (forward) and k_0 (reverse). The overall rate is given by

$$\text{rate} = k_i[A][B](1 - k_0/k_f)$$

While an equation of this form predicts a maximum rate somewhat below T_m, it drops off too steeply near T_m to fit the observed data for collagen refolding.

Substituting eq 4 into eq 5 and rearranging we obtain:

$$\frac{\Delta F^*}{A} = \frac{1}{T_m}\left[\frac{2\phi - 1}{(1 - \phi)^2}\right] \qquad (6)$$

Equation 6 states that the optimum temperature of any crystallization or refolding reaction conforming to eq 3 is simply related to the T_m of the polymer and the quantity $\Delta F^*/A$. This function is plotted in Figure 11 for T_m = 300°K, and it is clear that the variation of $\Delta F^*/A$ is large (0.02–1.2) in the region of ϕ = 0.75–0.95 which seems to apply to most polymers. Comparison of polymers of widely different T_m would be more easily done with the dimensionless quotient $T_m\Delta F^*/A$ obtained by rearranging eq 6.

Values of A and ΔF^* for refolding of single and cross-linked gelatin chains were computed from the temperature dependence of the subordinate nucleation and growth reactions. Attention was focused on those temperature regions where the subordinate process of interest was *rate limiting*, since the method of kinetic analysis provided the best resolution of the process under this condition. Table I presents a summary of these rate determining parameters for the refolding of gelatin chains (see Figure 12). Several conclusions may be drawn from these data. First, the activation energy for the growth process, ΔF^*, is constant (~7400 cal/mole) for all gelatins. This suggests that the mechanism by which residues are added to the ends of helical regions

is independent of the gelatin species and its degree of cross-linking. Second, the Flory–Weaver constant, A, is three- to tenfold smaller for cross-linked gelatins than for single-chain gelatins. In this regard, α_2-ratskin appears to have the properties of a cross-linked gelatin. The greatly accelerated refolding in cross-linked chains is solely a consequence of their more rapid nucleation reaction; ΔF^* for the growth reaction is essentially unchanged. Third, because T_{opt} is determined by the ratio $\Delta F^*/A$ (eq 6), the cross-linked chains with their smaller A values show optimum refolding at higher temperatures (ϕ = 0.93–0.94) than the corresponding single chains (ϕ = 0.85–0.86). It is noteworthy that Table I shows no significant differences between observed values of the various parameters and values predicted according to eq 6. This suggests that the Becker relationship (eq 3), from which eq 6 was derived, is well suited for description of collagen-chain refolding rates. In this equation the activation free energies of nucleation ($A/\Delta T$) and growth (ΔF^*) act in concerted fashion to determine the rate of propagation of ordered structure in quenched random chains. Whether this equation is also appropriate for intrachain globular protein renaturation kinetics will have to await measurement of such processes at temperatures outside the region of T_m.

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