Veis, A., and Drake, M. P. (1963), *J. Biol. Chem. 238*, 2003.
von Hippel, P. H. (1967), *in* Treatise on Collagen, Vol. 1, Ramachandran, G. N., Ed., New York, N. Y., Academic, p 253.
von Hippel, P. H., and Harrington, W. F. (1960), *Brookhaven Symp. Biol. 13*, 213.
von Hippel, P. H., and Wong, K.-Y. (1963a), *Biochemistry 2*, 1387.
von Hippel, P. H., and Wong, K.-Y. (1963b), *Biochemistry 2*, 1399.
Watson, M. R., and Silvester, N. R. (1959), *Biochem. J. 71*, 578.
Wieme, R. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 366.
Woodlock, A. F., and Harrap, B. S. (1968), *Aust. J. Biol. Sci. 21*, 821.
Zamenhof, A. (1957), *Methods Enzymol. 3*, 696.

Collagen Structure in Solution. IV. Conformational Properties of Refolded Cross-Linked Chains*

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ABSTRACT: Cross-linked ichthyocol refolded at various temperatures to the same extent (followed by optical rotation) showed increasing cooperativity ($\sigma^{-1/2}$) and thermal stability as the undercooling ($\Delta T = T_m - T_{refolding}$) decreased. At large ΔT, much of the mutarotation, presumed to indicate collagen-fold structure, is associated with the formation of structure possessing negligible cooperativity. Refolding kinetics were measured in the absence of artefacts due to "freezing-in" of noncooperative structure. The observed negative temperature dependence of *initial* rates and positive (normal) temperature dependence of later mutarotation provide evidence for the participation of separate nucleation and growth processes. Temperature fluctuation during the refolding of cross-linked ichthyocol and native *Ascaris* collagen shows that "frozen-in" regions of poorly ordered collagen-fold structure can be annealed. Formation of each nucleus of collagen structure during the refolding of cross-linked ichthyocol at 1° involves the ordering of 10–20 residues; about 10–20 nuclei are formed in each molecule (300,000 daltons). Correlation between the average nucleus size and $100/\Delta T$ reveals the operation of classical nucleation processes.

The correlation also suggests that at a constant value of ΔT the nucleation step in all gelatins must involve the same number of residues per nucleus, regardless of the composition or degree of cross-linking.

The structure of renatured collagen molecules is known to be severely dependent on the conditions surrounding their formation. The most significant variables in the refolding process are temperature, pH, protein concentration, and the degree of cross-linking between gelatin chains. Studies have shown that the re-formed structure can vary from a loosely cooperative single backfolded gelatin chain in the collagen conformation (Harrington and Rao, 1970), to a highly cooperative triple-helical molecule which is capable of forming specific aggregates (segment long spacing) characteristic of native collagen (Beier and Engel, 1966). At low protein concentrations ($c < 0.1$ mg/ml) the backfolded structure is favored over the entire temperature range below T_m, as indicated by the observation that mutarotation rate is independent of protein concentration in this region. At higher protein concentrations, interchain stabilization of the collagen fold becomes likely, and multichained structures are formed in a reaction which is between second and third order with respect to protein concentration (Harrington and Rao, 1970). If the refolding at high concentration (2–5 mg/ml) is done at temperatures not far below the T_m of the native collagen, then the reformed collagen is highly ordered and triple stranded. At the same concentrations, low-temperature ($<\sim 15°$) renaturation results in a heterogeneous mixture of stabilized collagen-like molecules containing a widely variable number of gelatin chains (Beier and Engel, 1966).

Kinetics of collagen renaturation are usually monitored by optical rotation or viscosity, but interpretation is difficult when several types of structures may be contributing to the observed changes. Optical rotation is clearly sensitive only to local configurational ordering, with other measurements required for estimation of long-range order. Beier and Engel (1966) distinquished between mutarotation leading to nonspecific aggregates and recovery of true native collagen by sedimentation velocity measurements after pepsin digestion of randomly coiled chain segments. They found that mutarotation was a valid indicator of native collagen re-formation *only* at high temperatures ($T_m - T_{refolding} < 15°$) and high protein concentrations.

Cross-linked collagen can be renatured under conditions

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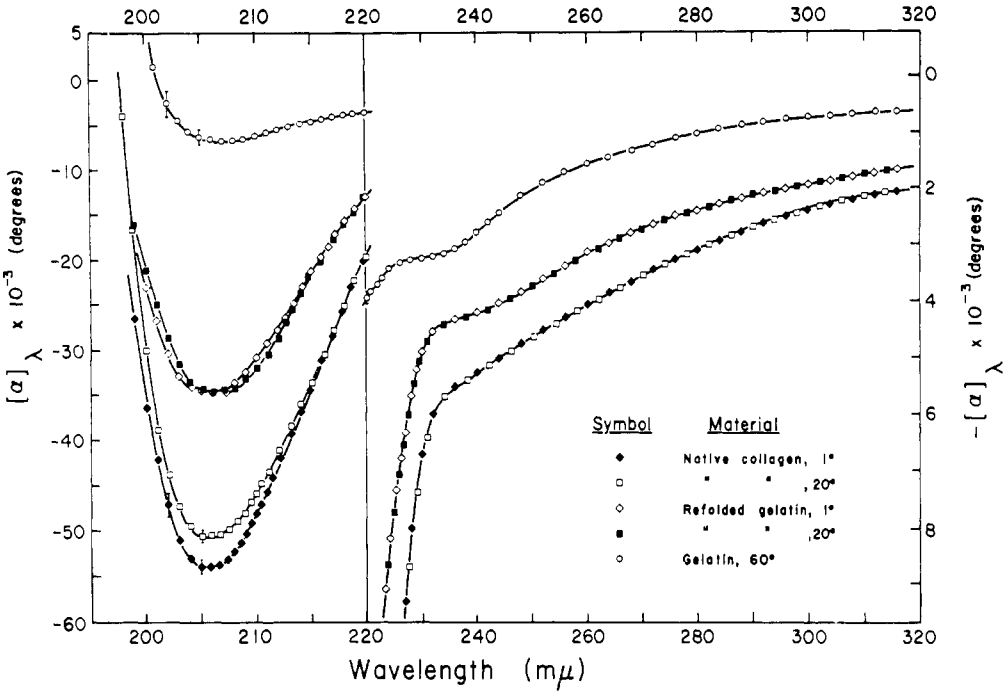

FIGURE 1: Optical rotatory dispersion of cross-linked ichthyocol: solvent, 0.1 M NaCl–0.009 M citrate, pH 5.93; native collagen at 1° (◆); native collagen at 20° (□); gelatin at 60° (○); gelatin refolded at 1° for 1100 min after denaturation at 60° for 10 min (◇); gelatin refolded at 20° for 1350 min after denaturation at 60° for 10 min (■).

of low protein concentration where intermolecular aggregates are prevented. Work in this laboratory has shown that there is no concentration dependence of the mutarotation rate at concentrations as high as 0.85 mg/ml. Veis and Drake (1963) showed that renaturation of cross-linked ichthyocol could take place without change in molecular weight (M_w 3×10^5) at concentrations of several milligrams per milliliter. While the renaturation process for cross-linked collagen is more rapid and complete than for single-chain gelatins, it has been observed that imperfections in the re-formed structure are, nevertheless, present. Our purpose has been to examine the mechanism of refolding of cross-linked collagens by optical rotation. Because of the possible non-linear relationship between mutarotation and recovery of true native collagen, it has been important to examine the characteristics of the re-formed structure (by thermal stability) in conjunction with the kinetics of re-formation. Measurements have been extended to very early times in order to clarify the role of "nucleation" and "growth" reactions in the overall refolding process. In addition, annealing of cross-linked ichthyocol and native *Ascaris* collagen during the early and late stages of refolding has been examined in a quantitative fashion.

Materials and Methods

Cross-linked ichthyocol and native *Ascaris* collagen were prepared and handled as in the previous paper (Hauschka and Harrington, 1970a). Optical rotatory data were also gathered and calculated as before; no refractive index corrections were applied to the dispersion measurements.

Those kinetic measurements of refolding which involved assaying for perfect collagen-fold (PCF) structure, defined by eq 2, were carried out by the following procedure. Native cross-linked ichthyocol (c = 0.080 mg/ml) in 0.1 M citrate buffer, pH 5.93, was denatured at 60° for 10 min in the jacketed 10-cm polarimeter cell. The sample was rapidly quenched to the renaturation temperature, where it was maintained for anywhere from 10 to 10,000 sec. After the desired renaturation period, the PCF assay was commenced by rapid switching of the jacket temperature to 34.2°, a temperature slightly higher than the T_m of the native structure; recording of levorotation was continued until there was no further melting of the partially refolded protein. Solvent base lines, measured under identical temperature schedules, were subtracted from each run. Because of limitations in the rate at which the sample temperature could be changed, the data for short renaturation times can only be analyzed qualitatively. As an example, the sample renatured at "1° for 120 sec" was at 1° for only about 60 sec, the remaining time being required to cool down to 1° and then to heat up to 34.2° for the melting-out assay. Similarly, the sample renatured at "1° for 10 sec" never reached 1°; rather, the temperature dropped to about 5–10° before the onset of melting. Fresh samples were used for each measurement.

Results

When cross-linked ichthyocol is denatured by heating to 60°, the structural transition may be quantitated by optical rotatory dispersion, as shown in Figure 1. The dispersion curve of the gelatin shows a marked decrease in optical rotation compared with native collagen, although the trough is not shifted from 207 mμ. After the gelatin is quenched and refolding is allowed to proceed, the negative

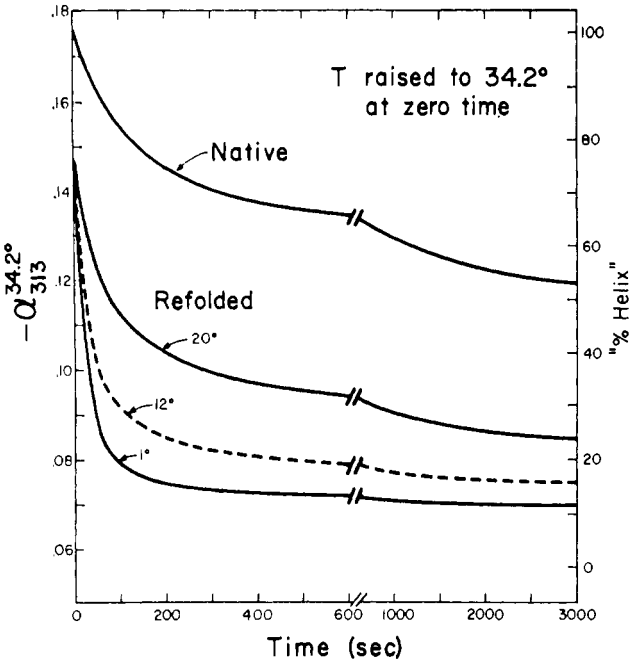

FIGURE 2: Melting rates of native and refolded cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml. Refolded samples containing 75% helix were prepared from gelatin (10 min at 60°) by quenching to 1° for 5500 sec, 12° for 2720 sec, or 20° for 5400 sec.

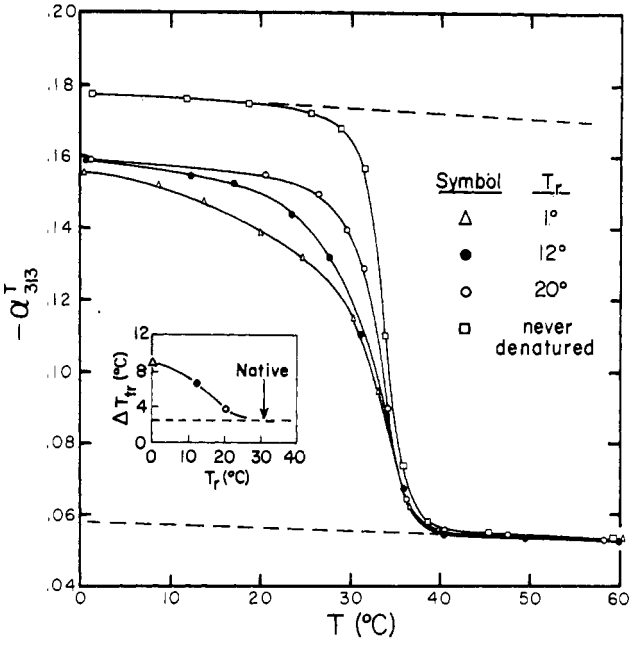

FIGURE 3: Equilibrium melting curves for native and refolded cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml; native collagen (□); gelatin (10 min at 60°) refolded at 1° (△), 12° (●) and 20° (○). Inset: width of melting transition (ΔT_t) as a function of the temperature of refolding (T_r).

levorotation is gradually regained. No dispersion differences are observed between collagen refolded at 1 or 20° over the wavelength range 200–315 mμ (Figure 1). In contrast to the above similarity of products formed at different temperatures, these materials show very different melting behavior. Figure 2 shows the change in optical rotation when native and renatured cross-linked ichthyocol are rapidly raised to 34.2°. All three renatured samples contained the same "per cent helix" at 1° (75%), although they were refolded at different temperatures (1, 12, and 20°). The extreme difference in melting rates suggests a real difference in the stability of the various samples. As the renaturation temperature increases, the stability of the renatured collagen appears to increase. In addition, the extent of melting at 34.2° decreases as the renaturation temperature increases.

Further differences between these collagens which have been renatured to the same apparent per cent helix (83% at 1°) may be seen in the equilibrium melt curves of Figure 3. As the renaturation temperature increases, the melting profile increasingly resembles that of the native collagen. While there is a slight artificial sharpening of the melting curves of the renatured collagens because of annealing during the equilibrium melting measurements, it is possible to assess the relative structural perfection or "cooperativity" of the structures by the width of the melting transitions. From the analysis of Flory (1961) and Harrington and Rao (1967), the average value of the cooperative unit size, $\sigma^{-1/2}$, may be calculated (see also Hauschka and Harrington, 1970a). Table I summarizes the data pertaining to the cooperative unit size of ichthyocol and native *Ascaris* collagen. The large decrease in the cooperative unit size with decreasing refolding temperature is a measure of the decreasing amount of long-range order in collagen renatured at low temperatures.

When cross-linked ichthyocol is denatured and then refolded at 1 or 20°, melting curves of the refolded samples show a cooperative transition in the region of the native

TABLE I: Helix—Coil Transition Parameters.

Material	T_m (°C)	ΔT_t (°C)	$\sigma^{-1/2}$ (Residues)[a]
Native ichthyocol, pH 5.95	32.2	2.5	126
Native cross-linked ichthyocol, pH 5.93	33.6	2.6	122
Cross-linked ichthyocol, refolded at 20°	33.3	3.8	83
Cross-linked ichthyocol, refolded at 12°	32.4	6.5	48
Cross-linked ichthyocol, refolded at 1°	31.8	8.9	35
Native *Ascaris* collagen, pH 7.15 (total)	52.7	6.9	58[b]
Native *Ascaris* collagen, pH 7.15 (cooperative)	52.2	4.1	97[b]

[a] Calculated by eq 1 of Hauschka and Harrington (1970a) using ΔH_{res} = 1180 cal/mole for ichthyocol and 1060 cal/mole for native *Ascaris* as given by Harrington and Rao (1967). Solvents: 0.1 M citrate (ichthyocol); 0.2 M NaCl–0.009 M citrate (native *Ascaris* collagen). [b] From Hauschka and Harrington (1970a).

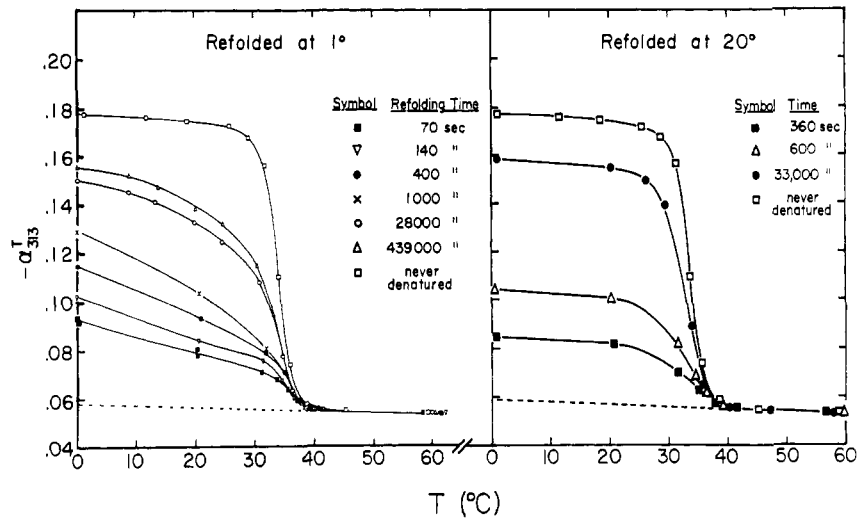

FIGURE 4: Equilibrium melting curves of native and refolded cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml. Gelatin refolded at 1° (left) or 20° (right) for various times after melting for 10 min at 60°. For the shortest refolding times at both temperatures some additional refolding, analogous to Figures 8 and 10, occurred during measurement of the melting curves. This was corrected for by extrapolating the refolding kinetics back to the time at which the temperature was raised.

cross-linked ichthyocol T_m at the earliest measureable time (Figure 4). Superimposed on the cooperative transition is a large noncooperative decrease in levorotation, occurring primarily between 1 and 30°. This noncooperative change is significantly larger for the 1° refolded ichthyocol than for the material refolded at 20°, and indicates an increasing degree of imperfection in renatured collagen as the refolding temperature is decreased. It is apparent that kinetic measurements of collagen renaturation by optical rotation will be subject to uncertainties stemming from the variable amount of noncooperative structure formed at each temperature. This problem has been eliminated in the following experiments which measure only the formation of truly native collagen structure. While renaturation was carried out at various temperatures, the *assay* for collagen structure was in all cases the same—measurement of the equilibrium value of $-\alpha^{34.2°}_{313}$. At a temperature of 34.2°, slightly above the equilibrium T_m of 33.6° for native cross-linked ichthyocol, only about one-half of the reformed cooperative structure is stable. However, there is essentially *no* contribution to the rotation by the noncooperative structure (*i.e.*, that structure which melts out between 1 and 30° without an inflection point for α *vs.* T). The equilibrium value of $-\alpha^{34.2°}_{313}$ may be related to the amount of perfect collagen-fold (PCF) structure by the definition

$$\text{PCF} = \alpha^{34.2°}\,(\text{gelatin}) + 2[\alpha^{34.2°}\,(\text{equilibrium}) - \alpha^{34.2°}\,(\text{gelatin})] \quad (1)$$

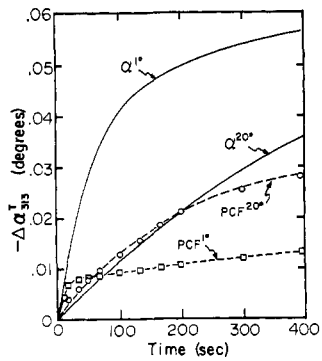

FIGURE 5: Comparison of mutarotation kinetics (α) with the kinetics of "perfect collagen-fold" (PCF) formation for cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml. The gelatin rotation has been subtracted from both α^T curves. PCF was calculated by eq 1 from the data of Figure 6.

In Figure 5, the kinetics of PCF formation at 1 and 20° are compared with the mutarotation kinetics. A striking difference is observed between these two temperatures. When refolding is carried out at 1°, the large increases in levorotation ($-\alpha^{1°}_{313}$) are relatively unrelated to the slow production of PCF. At 20°, however, changes in $-\alpha^{20°}_{313}$ and PCF are very nearly equal. Beier and Engel (1966) have found similar relationships for calfskin collagen renaturation at 10 and 26°. In their work, perfect collagen structure was assayed by areas of schlieren peaks from sedimentation runs on pepsin-treated renatured collagen. There is clear evidence in these results of the nonlinearity between mutarotation and re-formation of true native collagen at low temperatures.

Extension of the PCF assay method to other temperatures and longer times yields the renaturation kinetics shown in Figure 6. During the first 100 sec, the formation of cooperative collagen-fold structure is more than ten times faster at 1° than at 20°, with an intermediate rate shown at 12°. The situation is completely reversed from 100 sec to about 10,000 sec, during which time the growth at 20° is much faster than at either 1 or 12°. Renaturation at 34° proceeds very slowly, compared with the other temperatures. These data may be simply examined in terms of a nucleation and growth mechanism for collagen-fold formation (see Harrington and Karr, 1970; Hauschka and Harrington, 1970b). The negative

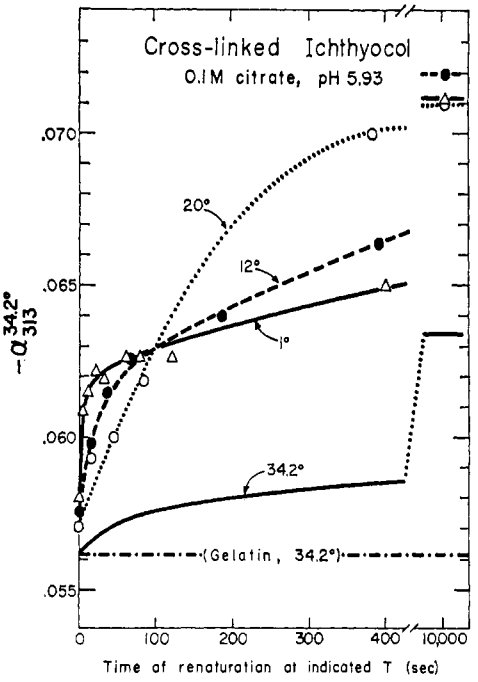

FIGURE 6: Renaturation kinetics of cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml. Equilibrium values of $-\alpha_{313}^{34.2°}$ were determined as described in Methods.

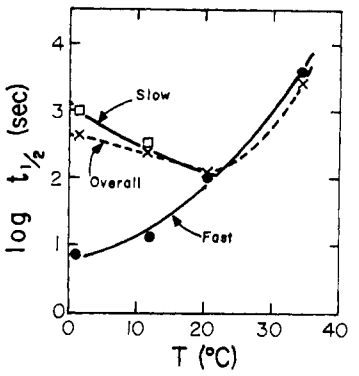

FIGURE 7: Rates of component first-order reactions for the cross-linked ichthyocol renaturation kinetics of Figure 6.

temperature dependence of the reaction rate observed before 100 sec can only be the result of a nucleation process.

An examination of the later stages of the refolding ($t >$ 100 sec) in Figure 6 reveals the switch from negative temperature dependence to a strict positive temperature dependence of the rate. The reactions of Figure 6 may be subjected to kinetic analysis according to a sum of parallel first-order reactions by the method outlined in the following paper (Hauschka and Harrington, 1970b). At low temperatures (1 and 12°) there are two distinct reactions with opposite temperature dependence. The fast reaction apparently represents a nucleation process because of its strong negative temperature dependence, while the slow reaction has the positive temperature dependence of a growth process (see Harrington and Karr, 1970; Hauschka and Harrington, 1970b). At 20 and 34°, the entire refolding reaction of Figure 6 is fit by a single first-order reaction. Because the half-times of these reactions are in accord with a nucleation process (that is, they increase with increasing temperature) it would appear that nucleation is rate limiting at these temperatures. At 12 and 1°, however, nucleation is very rapid so that the overall reaction rate is determined primarily by the growth reaction rate. Figure 7 shows the half-times of the apparent component reactions compared with the overall reaction of Figure 6. The similarity between Figure 7 of this paper and Figure 4b for cross-linked ichthyocol, (Hauschka and Harrington, 1970b), and Figure 5 of Harrington and Karr (1970) for RCM-*Ascaris* collagen renaturation argues for a common chain-folding mechanism for both single-chain and cross-linked gelatins.

A classical method for direct examination of the role of nuclei in crystallization reactions has been to isolate the nuclei by physical or kinetic means and determine their effect upon addition to a nonnucleated system (see, for example, Waugh, 1957). Because "crystallization" of cross-linked ichthyocol and native *Ascaris* is intramolecular under the present conditions of low protein concentrations, mixing solutions containing varying amounts of nuclei would be fruitless. Piez and Carrillo (1964) found that the mutarotation rate of equal mixtures of α_1 and α_2 ratskin gelatins was exactly equal to the average of the individual rates which, under their conditions, differed by 15-fold. The intrinsic difference in rates for the two chains is attributed to the pyrrolidine contents (226 residues/1000 for α_1, 196 residues/1000 for α_2; Piez *et al.*, 1963). The chains are expected to have different contents of nuclei (Josse and Harrington, 1964; Piez and Carrillo, 1964; Harrington and Rao, 1970) yet because of intramolecularity of the folding reaction, there is no alteration in rate upon mixing at low protein concentration.

Opposing temperature dependence of the rates of the nucleation and growth reactions (Figure 7; and Hauschka and Harrington, 1970b) points to the possibility of kinetic isolation of the nuclei. For instance, immediately after quenching the gelatin to 1° the content of nuclei should increase very rapidly, yet growth on these nuclei should be slow. Switching to high temperatures (but still below T_m) should slow nucleation, but growth of collagen-fold structure on the preformed nuclei should begin at a rate directly proportional to the concentration of nuclei in the molecule. In the following studies, temperatures of fluctuation (T_f) were both higher and lower than the refolding temperature (T_r), and the effects were examined for early and late stages of the total renaturation process.

Case I. PREINCUBATION AT TEMPERATURES BELOW THE REFOLDING TEMPERATURE ($T_f < T_r$). Quenching of melted cross-linked ichthyocol to 1° (T_f) for periods of 70–400 sec was followed by incubation at 20° (T_r). As in all cases, solvent base lines measured under identical conditions were subtracted to obtain continuous traces of $-\alpha_{313}^{T}$ *vs.* time (Figure 8). Over a period of about 100 sec after switching to 20°, 45–60% of the levorotation regained at 1° is lost by melting. Superimposed on the melting process is the 20° refolding reaction which, after 100 sec, is the only contributing factor to the observed changes in $-\alpha_{313}^{20°}$. Instead of the predicted stimulation of the 20° growth rate by preincubation at 1°, there is, if anything, a slight decrease in this rate. Differences are also seen in the amount of structure

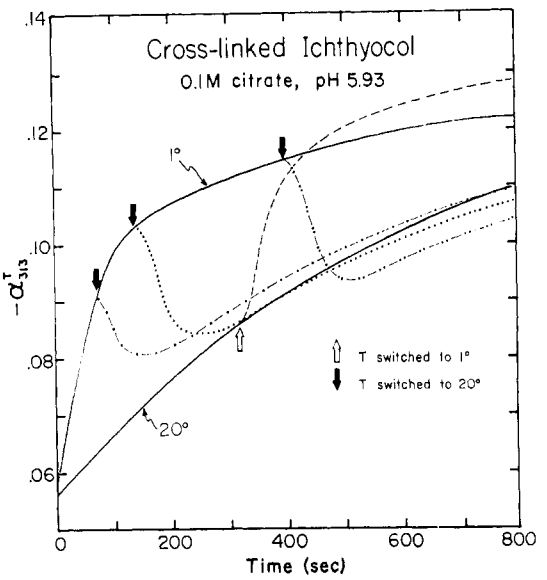

FIGURE 8: Temperature fluctuation at early times during the refolding of cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml. Samples were melted for 10 min at 60° before quenching. Corrected for solvent perturbation by subtraction of identical runs on solvent alone.

present in the various samples, as judged by $-\alpha^{20°}_{313}$. While the 70-sec preincubation at 1° seems to have increased the amount of structure relative to the 20° control sample, this difference has disappeared by 700 sec. The 400-sec pretreatment at 1° has definitely decreased the amount of structure present at 700 sec. After 800 sec (not shown in Figure 8), the control sample which had experienced no 1° treatment was more levorotatory and refolding faster than all of the preincubated samples. This would suggest that the collagen-fold structure formed rapidly at 1° is not entirely suitable for participation in the 20° growth reaction.

A similar experiment with native *Ascaris* collagen is shown in Figure 9, where data have been plotted in terms of per cent helix to eliminate confusion caused by simple temperature dependence of optical rotation [d $[\alpha]_{313}/dT = -0.12\%$ per degree for native *Ascaris* collagen and cross-linked ichthyocol below the thermal transition region (Hauschka, 1969)]. A melted sample which had been refolded for 320 sec at 2° was switched to 43°. There was a rapid loss of about 15% of the newly acquired levorotation which was soon replaced by the mutarotation occurring at 43°. This mutarotation proceeded with an initial rate approximately 50% greater than the normal 43° refolding (compared at the same per cent helix levels), yet there was no difference in the final value of the helical content. The increased rate presumably reflects an increase in the number of growth centers (nuclei) caused by the low temperature preincubation. Coincidence of the 2° fluctuated sample and its 43° control at times greater than 3000 sec indicates that the nuclei will eventually form at either temperature.

Case II. PREINCUBATION AT TEMPERATURES GREATER THAN THE REFOLDING TEMPERATURE ($T_f > T_r$). Preincubation of cross-linked ichthyocol gelatin at 20° (T_f) for 320 sec was followed by refolding at 1° (Figure 8). This procedure caused a marked increase in both the growth rate and the amount of mutarotation at 1°. Even after 20,000 sec, the preincubated sample possessed about 5% more collagen-fold structure than the 1° control. However, the 20° control sample was the most levorotatory of all at times greater than 10,000 sec, indicating that isothermal growth at small undercooling is more efficient for a cross-linked system than fluctuated temperature growth at large undercooling.

Samples of native *Ascaris* collagen (Figure 9) which were refolding at 43° were switched to 2° after 120, 320, and 520 sec. These fluctuated samples remained well ahead of the 2° control in acquisition of helical content even at 10,000 sec. The time required to reach 90% helix ($t_{0.9}$) was decreased by a factor of 2 to 4 by the 43° preincubation (Figure 9, inset). Presumably the structure formed at high temperatures is more perfectly ordered than the 2° structure and may therefore serve as a better template for further growth. As in case I, no long-term benefits in per cent helix are gained from the 43° preincubation, since the fluctuated samples converge with the 2° control sample at about 15,000 sec.

Case III. $T_f < T_r$, LATE STAGE. After almost complete refolding (85% helix) of cross-linked ichthyocol at 20° (T_r), switching to 1° had no permanent effect on the amount of structure (Figure 10a). While there was an apparent increase in collagen-fold content at 1° to 89% (corrected for temperature dependence of α), this increment was completely unstable at 20°. Similar effects were observed for native *Ascaris*. Switching to low temperature (2°) after refolding at 43°, the helical content exhibits a rapid increase of about 4% and levels off in exact coincidence with the 2° control. This increase is immediately lost upon returning to 43°. It appears that these small increments of helix content are the consequence of imperfect collagen-fold formation by chain segments which are unable to form stable structure at the higher temperature (T_r).

Case IV. $T_f > T_r$, LATE STAGE. Cross-linked ichthyocol which had been refolded for 55,000 sec at 1° (77% helix) was fluctuated between 20 and 1° (Figure 10b). The first 20° pulse destroyed 22% of the regained levorotation (or 15% helix, after temperature correction). Subsequent refolding during the first 20° pulse proceeded at only one-third the rate of the normal 20° mutarotation (from relative slopes of tangents at $-\alpha_{313}$ 0.132°). Upon returning the temperature to 1°, it was evident that helical content was increasing. Through several cycles, about 4% helix was added to the 1° structure. A similar increase in helix content of calfskin collagen was obtained by Kühn *et al.* (1964) using temperature oscillation between 4 (T_r) and 22° (T_f).

In both experiments, the 20° (or 22°) pulse releases some of the imperfectly folded regions of the 1° (or 4°) structure to a state where these chain segments are able to refold into a more stable conformation. The relatively slow rate of this refolding is caused by the fact that growth at 20° involves residue addition to the poorly crystalline helical regions formed at the lower temperature (1°). We have already seen from Figure 8 that 1° nuclei do not grow as rapidly at 20° as do 20° nuclei.

With native *Ascaris* collagen which has been refolded at 2°, only about 8% of the structure is found to be unstable at a T_f of 43°. As in case III, this loose structure is rapidly regained upon return to 2°. Control runs on native *Ascaris* collagen which had never been melted indicate that similar

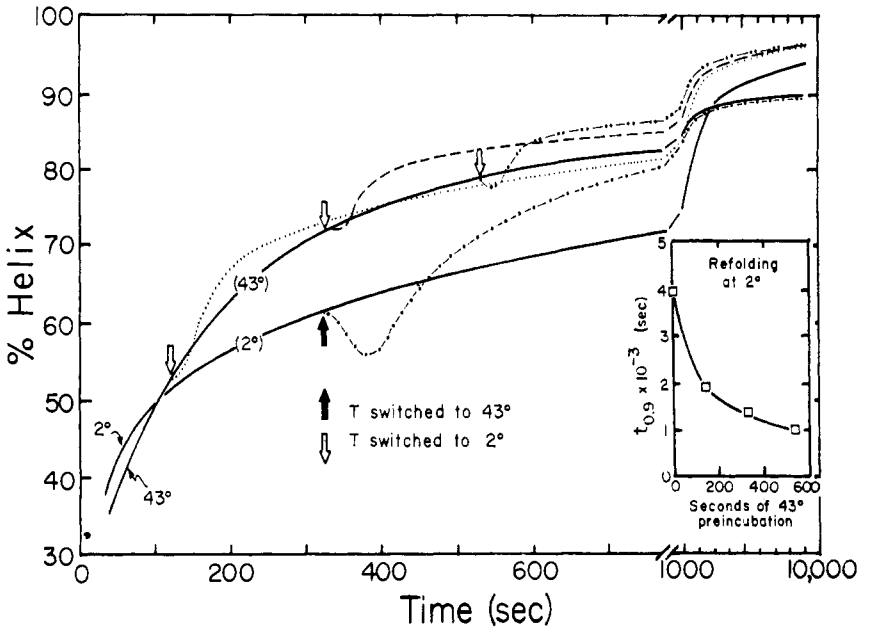

FIGURE 9: Temperature fluctuation during native *Ascaris* collagen refolding: solvent, 0.2 M NaCl–0.009 M citrate, pH 6.25; c = 0.107 mg/ml. Samples were melted for 10 min at 80° before quenching. Per cent helix was calculated as defined in Hauschka and Harrington (1970a). All curves were corrected for solvent base line behavior. Inset: time to reach 90% helix ($t_{0.9}$) as a function of preincubation at 43°.

temperature fluctuations have virtually no effect on the helix content of truly native material. Refolding of the "released" chain segments was not observed at 43° in contrast to the early-stage behavior of native *Ascaris* (case I, Figure 9) and the 20° refolding for cross-linked ichthyocol (case IV, Figure 10b). The high content of cross-links in native *Ascaris* collagen may be inhibitory to the refolding of released chain segments, especially in the late stages of the reaction (case IV) where the large helix content makes it likely that the released segments are isolated from each other by stiff helical segments.

In summary, these fluctuation studies show that increases in helical content can only be achieved when the fluctuation temperature is greater than the isothermal temperature of refolding.

Discussion

The two major factors controlling the energetics of nucleus formation are surface free energy and free energy of fusion. Surface free energy (ΔF_s) is usually positive (unfavorable) and is a consequence of the interface between the ordered and disordered chain segments. Free energy of fusion (ΔF_v) includes all terms within the nucleus, such as the enthalpy due to hydrogen-bond formation and the entropy due to ordering the polymer chain segments and/or disordering bound solvent molecules. Following the treatment of Flory and Weaver (1960), the free energy of formation of a collagen-like nucleus of n residues is given by

$$\Delta F_{nuc} = n\Delta F_{v,res} + \Delta F_s \qquad (2)$$

where ΔF_s represents the surface free energy contribution of both ends of the nucleus. Because of the opposite sign of ΔF_v and ΔF_s, the minimum number of residues (n^*) required for a stable nucleus is found by setting eq 2 equal to zero

$$n^* = \frac{\Delta F_s}{\Delta F_v} \qquad (3)$$

From the relationship $\Delta F_v = \Delta H_v - T\Delta S_v$ and the fact that $\Delta F_v = 0$ at $T = T_m$, the approximation $\Delta F_v = \Delta H_v \Delta T/T_m$ may be derived, where $\Delta T = T_m - T$. Substituting into eq 3 one obtains

$$n^* = \frac{\Delta F_s T_m}{\Delta H_v \Delta T} = C/\Delta T \qquad (4)$$

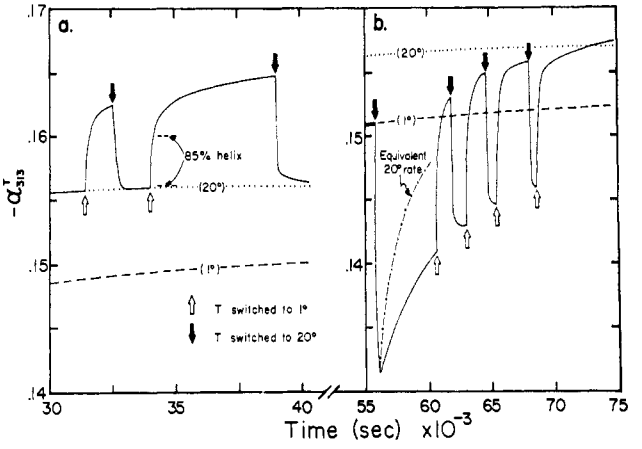

FIGURE 10: Temperature fluctuation at late times during the refolding of cross-linked ichthyocol: solvent, 0.1 M citrate, pH 5.93; c = 0.080 mg/ml. Samples were melted for 10 min at 60° before quenching. (a) Fluctuation to a temperature less than the refolding temperature; 85% helix indicated at 1° (α = −0.160°) and at 20° (α = −0.156°); (b) fluctuation to a temperature above the refolding temperature.

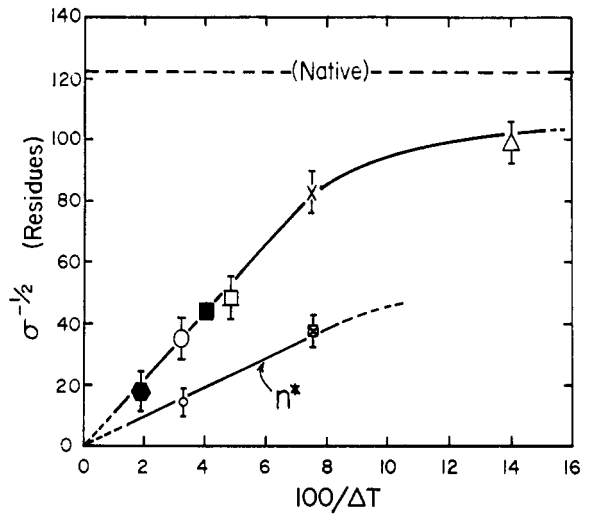

FIGURE 11: Average cooperative unit size of native and refolded collagens. $\sigma^{-1/2}$ calculated by using appropriate values for ΔH_{res} as given by Harrington and Rao (1967). The undercooling is defined by $\Delta T = T_m - T_{refolding}$. Upper curves: cross-linked ichthyocol (0.1 M citrate, pH 5.93; c = 0.080 mg/ml) refolded at 1° (○), 12° (□), and 20° (×); native cross-linked ichthyocol (----). Untreated ichthyocol (0.025 M $CaCl_2$, pH 7; $c \sim 1.0$ mg/ml) refolded at 5° (■), from von Hippel and Wong (1963). Native *Ascaris* collagen assumed to have folded *in vivo* at ΔT = 7° (△). Poly(Pro-Gly-Pro) refolded at 4° (●); data of Engel *et al.* (1966). Lower curve: initial values for cross-linked ichthyocol refolded at 1° (○) and 20° (⊠).

where C is a constant. Inverse dependence of the critical nucleus size n^* on the undercooling ΔT (eq 4) is a very important consequence of the thermodynamics of nucleation. Further development of these relationships leads to the Flory–Weaver expression for the temperature dependence of the nucleus formation.

It is likely that the parameter $\sigma^{-1/2}$ representing the average size of the cooperative unit, is directly proportional to the minimum nucleus size n^*. The proportionality constant would reflect the increase in cooperative unit size caused by ordering of residues at the ends of the nucleus. A plot of $\sigma^{-1/2}$ *vs.* $C/\Delta T$ should be a straight line at large ΔT if proportionality exists between n^* and $\sigma^{-1/2}$. Figure 11 confirms the above hypothesis using the data of Table I for cross-linked ichthyocol. The straight line is drawn on the basis of the three points for samples of cross-linked ichthyocol renatured at 1, 12, and 20° whose melting curves are shown in Figure 3. In accord with the theory expressed in eq 4, this line extrapolates to $\sigma^{-1/2} \sim 0$ as $T \rightarrow 0$ °K. At small ΔT, the cooperativity of native cross-linked ichthyocol ($\sigma^{-1/2}$ = 122 residues) is approached by the refolded gelatin. *Ascaris* collagen also fits this theory. Table I lists a value of $\sigma^{-1/2}$ = 58 residues for native *Ascaris* collagen. In part, this low apparent degree of cooperativity for a native collagen is caused by the broad melting profile of the *noncooperative* portions of the structure. When this is subtracted, the cooperative portion of native *Ascaris* collagen has a maximum average helical length of 97 residues (Hauschka and Harrington, 1970a). The cooperativity for *Ascaris* collagen is still lower than for ichthyocol. Two factors contribute to the difference. First, the even distribution of polar residues in *Ascaris* causes more frequent disruption of the helical regions (Hauschka and Harrington, 1970a). Second the *Ascaris* collagen is probably formed at large ΔT *in vivo*. Adult *Ascaris* worms live at a temperature of about 37° in the hog intestine, yet the T_m of their cuticle collagen at physiological pH is 53°, a contradiction to the commonly observed identity between body temperature and soluble tropocollagen T_m. The unusually high thermal stability of native *Ascaris* collagen is caused in part by the disulfide cross-links which form after the collagen-fold conformation has been achieved (McBride and Harrington, 1967). Therefore, in order to estimate the ΔT(undercooling) at which native *Ascaris* collagen was formed, the noncross-linked T_m of 44° (for RCM-*Ascaris;* Harrington and Karr, 1970) must be used; this gives a ΔT value of about 7°. As shown in Figure 11, the observed point for native *Ascaris* collagen is in agreement with the expected asymptotic approach to the cooperativity of native collagen.

The generality of the relationship plotted in Figure 11 is further emphasized by the agreement of the poly(Pro-Gly-Pro) melting behavior (Engel *et al.*, 1966). The very broad melting profile of this synthetic collagen-type polypeptide, although due in part to the low molecular weight, was very likely caused by the large undercooling at which the collagen-fold structure was formed ($\Delta T \sim$ 40–70°). The point for poly(Pro-Gly-Pro) in Figure 11 has been calculated from the data in Figure 3 of Engel *et al.* (1966) following the method of Harrington and Rao (1967).

The concept of annealing or tempering has been applied by several groups to the slow structural changes exhibited by imperfectly refolded collagen molecules. These changes may occur during temperature cycling (Kühn *et al.*, 1964; and Figure 10) or under isothermal conditions (Beier and Engel, 1966). Isothermal annealing is observable by the very slow continuous increase in levorotation after the initial stage of refolding has been completed. In other studies this levorotatory change has been quantitated by analysis of refolding kinetics into fast, intermediate, and slow component reactions (Harrington and Karr, 1970; Hauschka and Harrington, 1970b). The slow reaction is believed to represent annealing because its size increases with decreasing temperature. At very low quenching temperatures, rapid "freezing-in" of residues into poorly ordered collagen-like conformation is known to accompany refolding of gelatin chains (see Figure 4). Because annealing is defined as the recrystallization of poorly ordered structure, it is apparent that more residues will be candidates for such a reaction at the lowest quenching temperatures, yet, in principle, the annealing process is in operation at all temperatures.

Analysis of the melt curves of refolded cross-linked ichthyocol gives further insight into the annealing process. The average size of the cooperative unit ($\sigma^{-1/2}$) increases with the time of refolding as shown in Figure 12. In agreement with Figure 11, the cooperativity is greatest at the smallest ΔT for refolding. This continuous perfecting of the refolded structure is viewed as an annealing process because it is accompanied by relatively small optical rotatory changes. For example, at 1° there are only slight changes in cooperativity during the first 1000 sec of folding by which time 60% of the mutarotation is complete. After 1000 sec, the cooperativity more than doubles, while there is only an additional 20% increase in optical rotation. Furthermore, the rate of increase of $\sigma^{-1/2}$ is more than twice as large at 20° as

it is at 1°. It is expected that an annealing reaction would have positive temperature dependence, because the two fundamental aspects of its mechanism as proposed by Veis (1964) [(1) melting of poorly structured regions, and (2) repositioning of chain segments] are both facilitated by increased temperature. Extrapolation of the curves in Figure 12 to early time is useful in that it provides an estimate of the cooperative unit size (n^*, eq 3) of the nucleus before growth and annealing have enlarged it. These initial sizes are about 30–40 residues for 20° refolding and about 10–20 residues for 1° refolding. As shown in the lower curve of Figure 11, the relative cooperative unit sizes are in agreement with the prediction of eq 4. Enlargement of the cooperative units during refolding is caused both by growth (ordering of previously uncommitted random residues) and annealing.

Estimates of nucleus size and the minimum size of a stable collagen-fold structure have appeared previously. From optical rotatory changes in gelatin which accompanied stepwise decreases in temperature below T_m, Veis and Legowik (1963) calculated, in reasonable agreement with the present findings, that about 27 residues must be involved in the nucleation process of α-gelatin at 25°. von Hippel and Wong (1963) have thoroughly investigated the chain-length dependence of ichthyocol gelatin refolding. Digestion with collagenase or trypsin produced gelatin fragments whose ability to form collagen-fold structure was directly related to their weight-average molecular weight ($\bar{M}_w$). Below a limit of 40 to 80 residues/fragment, there was no observable refolding. The reformed structure exhibited a degree of cooperativity ($\sigma^{-1/2} \sim 45$ residues) which was independent of $\bar{M}_w$ and showed only a 2° increase in T_m over the range $\bar{M}_w = 6300$ to $\bar{M}_w = 85{,}900$. The requirement for minimum chain length is presumably a consequence of having to build a nucleus for refolding which contains at least n^* residues. At the particular protein concentration (~1.0 mg/ml) studied by von Hippel and Wong (1963), it is now known that both intramolecular and intermolecular collagen-fold formation could occur (Figure 2 of Harrington and Rao, 1970). Therefore, refolded molecular species formed by $\bar{M}_w = 6300$ chains may have contained anywhere from 65 to 200 residues, and we are able only to place a very rough upper bound of $\sigma^{-1/2} \leq 45$ residues on the number of residues required for nucleation at 5°.

It is interesting that the average cooperative unit size formed by refolding noncross-linked ichthyocol at 5°, calculated from the data of von Hippel and Wong (1963), is in good agreement with the relationships plotted in Figures 11 and 12 for cross-linked ichthyocol. It is apparent from Figure 11 that the cooperativity of the structure formed by various gelatin species is determined primarily by the degree of undercooling (ΔT). Final values of $\sigma^{-1/2}$ for the refolded gelatins plotted in Figure 11 are 2 to 4 times larger than the initial values (presumably n^*). Also, the initial and final values of $\sigma^{-1/2}$ increase in a similar fashion as ΔT decreases. At a constant value of ΔT, therefore, the nucleation step for the various gelatins must involve the same number of residues in each nucleus, regardless of composition or degree of cross-linking. Depending on protein concentration, chain length, and cross-linking, the nucleus may be formed either by intrachain or interchain interaction (Figure 8 of Harrington and Rao, 1970). Differences in refolding rates of various gelatins at the same ΔT have been correlated with pyrrolidine doublet content (Josse and Harrington, 1964; Harrington and Rao, 1970.) Doublets probably govern only the number and not the size of the nuclei which form in each chain. From Figure 6 we may calculate that about 6% of the residues (or ~200 res/molecule) are participating in stable nucleation of cross-linked ichthyocol at low temperature. Using a value for n^* of 10–20 residues at 1° (Figure 12), it is estimated that between 10 and 20 nuclei are formed in each molecule ($\bar{M}_w = 300{,}000$) during the first minute after quenching to 1°. This number of nuclei is probably related by more than coincidence to the 10 methylene cross-links per molecule.

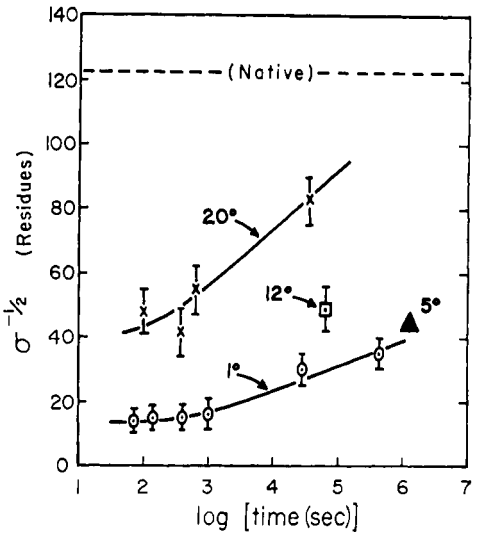

FIGURE 12: Average cooperative unit size during refolding of cross-linked ichthyocol. $\sigma^{-1/2}$ calculated as in Figure 11. Cross-linked ichthyocol (0.1 M citrate, pH 5.93; $c = 0.080$ mg/ml) refolded at 1° (○), 12° (□), and 20° (×); native (----). Untreated ichthyocol (0.025 M $CaCl_2$, pH 7; c ~ 1.0 mg/ml) refolded at 5° (▲), from von Hippel and Wong (1963).

References

Beier, G., and Engel, J. (1966), *Biochemistry 8*, 2744.

Engel, J., Kurtz, J., Katchalski, E., and Berger, A. (1966), *J. Mol. Biol. 17*, 255.

Flory, P. J. (1961), *J. Polym. Sci. 49*, 105.

Flory, P. J., and Weaver, E. S. (1960), *J. Amer. Chem. Soc. 82*, 4518.

Harrington, W. F., and Karr, G. M. (1970), *Biochemistry 9*, 3725.

Harrington, W. F., and Rao, N. V. (1967), *in* Conformation of Biopolymers, Ramachandran, G. N., Ed., London, Academic, p 513.

Harrington, W. F., and Rao, N. V. (1970), *Biochemistry 9*, 3714.

Hauschka, P. V. (1969), Dissertation, The Johns Hopkins University, Baltimore, Md.

Hauschka, P. V., and Harrington, W. F. (1970a), *Biochemistry 9*, 3734.

Hauschka, P. V., and Harrington, W. F. (1970b), *Biochemistry 9*, 3745.

Josse, J., and Harrington, W. F. (1964), *J. Mol. Biol. 9*, 269.

Kühn, K., Engel, J., Zimmerman, B., and Grassman, W. (1964), *Arch. Biochem. Biophys. 105*, 387.
McBride, O. W., and Harrington, W. F. (1967), *Biochemistry 6*, 1499.
Piez, K. A., and Carrillo, A. L. (1964), *Biochemistry 3*, 908.
Piez, K. A., Eigner, E. A., and Lewis, M. S. (1963), *Biochemistry 2*, 58.
Veis, A. (1964), The Macromolecular Chemistry of Gelatin, New York, N. Y., Academic.
Veis, A., and Drake, M. P. (1963), *J. Biol. Chem. 238*, 2003.
Veis, A., and Legowik, J. T. (1963), *in* Structure and Function of Connective and Skeletal Tissue, Fitton-Jackson, S., Harkness, R. D., Partridge, S. M., and Tristram, G. R., Ed., London, Butterworths, p 70.
von Hippel, P. H., and Wong, K.-Y. (1963), *Biochemistry 2*, 1399.
Waugh, D. F. (1957), *J. Cell. Comp. Physiol. 49, Suppl. 1*, 145.

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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