Collagen Structure in Solution. I. Kinetics of Helix Regeneration in Single-Chain Gelatins*

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ABSTRACT: Initial rates of helix regeneration, $d[\alpha]/dt$, in various single-chain, collagen-type polypeptides have been investigated as a function of protein concentration, pyrrolidine (proline + hydroxyproline) content, and temperature. At low concentration (<0.1 mg/ml) all of the chains show first-order dependence of the rate with respect to protein, but above this level the order of reaction n increases rapidly with increasing concentration indicating the presence of intermolecular interaction in the initial stages of helix regeneration. Molecular weight measurements of a representative vertebrate chain, α_1-ratskin, show that this chain, like the invertebrate RCM-*Ascaris*, folds into a stable collagen-type structure at low concentration through a completely intramolecular mechanism. The initial rate–concentration and initial rate–temperature dependence of the α_1 chains appear to be markedly greater than their corresponding α_2 counterparts. Initial rates of refolding below the concentration-dependent threshold have been compared for the various chains at a fixed temperature and also at the same level of undercooling below the thermal transition temperature of the respective collagens. Although the rates of intramolecular folding do not follow a simple linear relationship with respect to the fractional content of pyrrolidine residues, there appears to be a correlation with the number of contiguous pyrrolidines based on a statistical distribution of these residues. The temperature dependence of the unimolecular process at low protein concentration conforms to the Flory–Weaver equation [rate = $B \exp(-A/RT\Delta T)$] which is believed to reflect intramolecular formation of a hydrogen-bonded, triple-helical nucleus through reverse folding of the chain.

At high protein concentration ($\gg$2 mg/ml) the nucleation reaction is thought to approach third-order, the nucleus being formed through interaction of segments in three separate chains. Thus, at low levels of undercooling, chain folding may proceed along either of two pathways to form (1) a stable, reverse-folded collagen structure (at low concentration) or (2) a stable, parallel-type collagen structure at high protein concentration.

Investigations of the mechanism of re-formation of collagen structure from random chain gelatin molecules have in general been complicated in the past by the presence of significant amounts of cross-linked chains. Moreover, as is now well documented (Piez *et al.*, 1960; Seifter and Gallop, 1966) these systems are mixtures of at least two types of subunits of differing primary structures, the α_1 and α_2 chains. Altgelt *et al.* (1961), Engel (1962), Drake and Veis (1964), and McBride and Harrington (1967b) have examined the kinetics of renaturation of various cross-linked gelatin species. These studies clearly show that the rates of isothermal helix regeneration in the multichain structures at low temperatures are much more rapid than their corresponding single-chain components. Significant differences have also been found between the rates of reversion of the constituent α_1 and α_2 chains of the single collagen species which have been examined. Helix formation in the α_2 chain of ratskin collagen is very much slower, under comparable conditions of concentration and temperature, than the α_1 component (Piez and Carrillo, 1964). Of special significance was the finding of Piez and Carrillo that the rate of helix formation in both the α_1 and α_2 components, as measured by changes in the specific optical rotation, was dependent on protein concentration. These observations are contrary to the earlier studies of Harrington and von Hippel (1961) on ichthyocol and Flory and Weaver (1960) on rat-tail-tendon gelatin who reported first-order reversion kinetics with respect to protein concentration. Since this feature is fundamental to the alternative mechanisms of chain refolding which were proposed by these authors, we have investigated the concentration and temperature dependence of mutarotation in several purified α-chain systems over a wide range of concentration. Two unfractionated invertebrate chains, reduced-carboxymethylated *Ascaris* cuticle collagen and earthworm cuticle gelatin, are also included in this study to assess the effect of chain composition, particularly the effect of the imino acids proline and hydroxyproline, on the rates of helix formation. Subsequent papers in this series will be devoted to an analysis of the kinetic mechanism of refolding in single-chain and cross-linked collagen systems.

Materials and Methods

The α_1 and α_2 chains of ratskin, ichthyocol, and codskin collagen were prepared by chromatographic fractionation according to the method of Piez *et al.* (1960). They were kindly supplied by Dr. Karl Piez. Earthworm cuticle collagen was obtained by the procedure of Josse and Harrington (1964). Reduced alkylated *Ascaris* cuticle collagen (RCM-*Ascaris*)

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was prepared according to McBride and Harrington (1967a).

Preparation of Gelatin Solutions. Gelatin chains were dissolved by overnight stirring in the cold (4°) in 0.15 M NaCl–0.05 M NaAc (pH 4.8) aqueous solution and then dialyzed against 50 volumes of the buffer for 3 days with frequent changes of the dialysate. The resulting protein solution was centrifuged at 30,000 rpm for 1 hr at 4°[1] and the clear supernatant collected. The amounts of the gelatin and buffer were so adjusted in the early steps that the final stock solution (supernatant after centrifugation) had a protein concentration of 1–2 mg/ml. All lower concentrations were achieved by appropriate dilution of the stock solution with dialysate. The pH of some of the solutions examined at low wavelength in the Cary spectropolarimeter ($\lambda < 300$ mμ) was adjusted with HCl rather than with acetate ion in order to eliminate light absorption problems.

Determination of Gelatin Concentrations. Concentrations were determined routinely by the microbiuret method of Zamenhof (1957) which had been standardized for two gelatins of widely differing composition (ichthyocol and RCM-*Ascaris*) by micro-Kjeldahl analyses. Since both of these materials gave identical color yields per milligram of nitrogen, the same relationship was assumed for the other species of collagen.

Optical Rotation Measurements. A Rudolph Model 200 spectropolarimeter utilizing a Hg lamp as light source was employed for some of the work at a wavelength of 313 mμ and for gelatin concentrations greater than 0.20 mg/ml. The Cary 60 spectropolarimeter was used for low wavelength ($\lambda < 300$ mμ) and low protein concentration studies. Both instruments were calibrated before initiating the present study with solutions of sucrose (NBS) and gave the expected rotations within $\pm$ 1% over the useable range of each instrument.

Jacketed cells were used throughout this investigation and the temperature of the gelatin solution inside the cell was maintained by circulating water from a large water bath held at constant temperature. Temperature control, measured as the average of influx and efflux circulant, was better than $\pm 0.1°$. In order to prevent condensation of moisture on the windows of the polarimeter cell, dry air was circulated inside the cell compartment of the Rudolph instrument. Nitrogen flushing was always present whenever the Cary 60 was used. Solvent blanks were determined before and after each experiment and appropriate corrections made.

In the kinetic experiments, the gelatin solution was heated at 50° for 10 min (except for RCM-*Ascaris* gelatin which was heated at 60° for 10 min) to ensure complete conversion into the random coil form, precooled to the desired temperature for about 20 sec, then quickly transferred into the polarimeter tube or cell with a precooled pipet or syringe. This operation generally took 2–4 min. Zero time for the experiment was assumed to be the time at which cooling of the melted gelatin solution was initiated. Cells of 1 and 2-dm path length were used with the Rudolph polarimeter, whereas 0.01-, 0.1-, and 1.0-dm cells with quartz windows were routinely used in the Cary measurements. The path length of the cell used in experiments with the Cary 60 was chosen to give an optical density of less than 1 (*e.g.*, a dynode voltage difference between solution and solvent of less than 150 V). Measurements of mutarotation in the deep ultraviolet (215 mμ) have provided information at concentrations approximately two orders of magnitude lower than reported heretofore.

[1] Unless otherwise indicated temperatures are given in degrees Celsius.

Results

The effect of protein concentration on the isothermal renaturation kinetics of single-chain polypeptides derived from several collagens is shown in Figure 1. Solutions were rapidly cooled to 10° following a preliminary heating step (50–60°). Plots of specific rotation, $[\alpha]^{10}_{215}$, *vs.* time for α_1-ratskin and α_1- and α_2-ichthyocol chains show a definite concentration dependence above about 0.05–0.1 mg/ml. Below this level the $[\alpha]^{10}_{215}$ *vs.* time profiles are independent of protein concentration, indicating that renaturation in these gelatin systems becomes first-order with respect to protein at low concentration. This behavior stands in contrast to that observed for the single polypeptide chains derived from *Ascaris* cuticle collagen, RCM-*Ascaris*, where renaturation is independent of concentration over the range 0.004–2.2 mg/ml (McBride and Harrington, 1967b). The effect of concentration on the initial rate of reaction for these collagen systems is shown in Figure 2 where $\log_{10} -[\mathrm{d}[\alpha]_{215}/\mathrm{d}t]_0$ is plotted against log protein concentration. Since the measured value of optical rotation, α, is proportional to concentration, we may write a general expression for the initial rate dependence of specific rotation as

$$\frac{\mathrm{d}[\alpha]_\lambda}{\mathrm{d}t} = kc^{n-1} \qquad (1)$$

where the rate constant, k, includes the constant of proportionality (= $[\alpha]l/100$, where concentration is expressed in units of grams/100 ml and l, the path length, in decimeters) and n is the order of reaction.

Initial rates of renaturation are first order ($n = 1$) with respect to protein concentration, indicative of an intramolecular process below ~0.1 mg/ml in the case of the three vertebrate chains. Above this concentration the order increases with increasing concentration, but the restricted solubility of these gelatin systems prevents assessment of the limiting value of n at very high protein concentrations. The initial rate of α_1-ratskin gelatin has also been determined by Piez and Carrillo (1964) at a temperature of 15° (λ 313 mμ) over the concentration range 0.25–1.61 mg/ml. Their results (normalized for temperature and wavelength differences) are also plotted in Figure 2 where it will be seen that the concentration dependence of the initial rate is in good agreement with that obtained in the present study. The lowest protein concentration examined by Piez and Carrillo (0.25 mg/ml) is above the concentration-dependence threshold, and these authors were, therefore, unable to detect the first-order dependence of renaturation displayed in Figure 2.

Renaturation studies of α_1-ratskin in the high temperature range (20°) show a similar first-order dependence of the initial rate at low protein concentration (Figure 2, lower). Although the very slow mutarotation reaction observed at this temperature results in a wider scatter in the data, it appears that the initial rate of renaturation at 20° follows first-order behavior over a wider concentration range than is observed for this gelatin system at 10°.

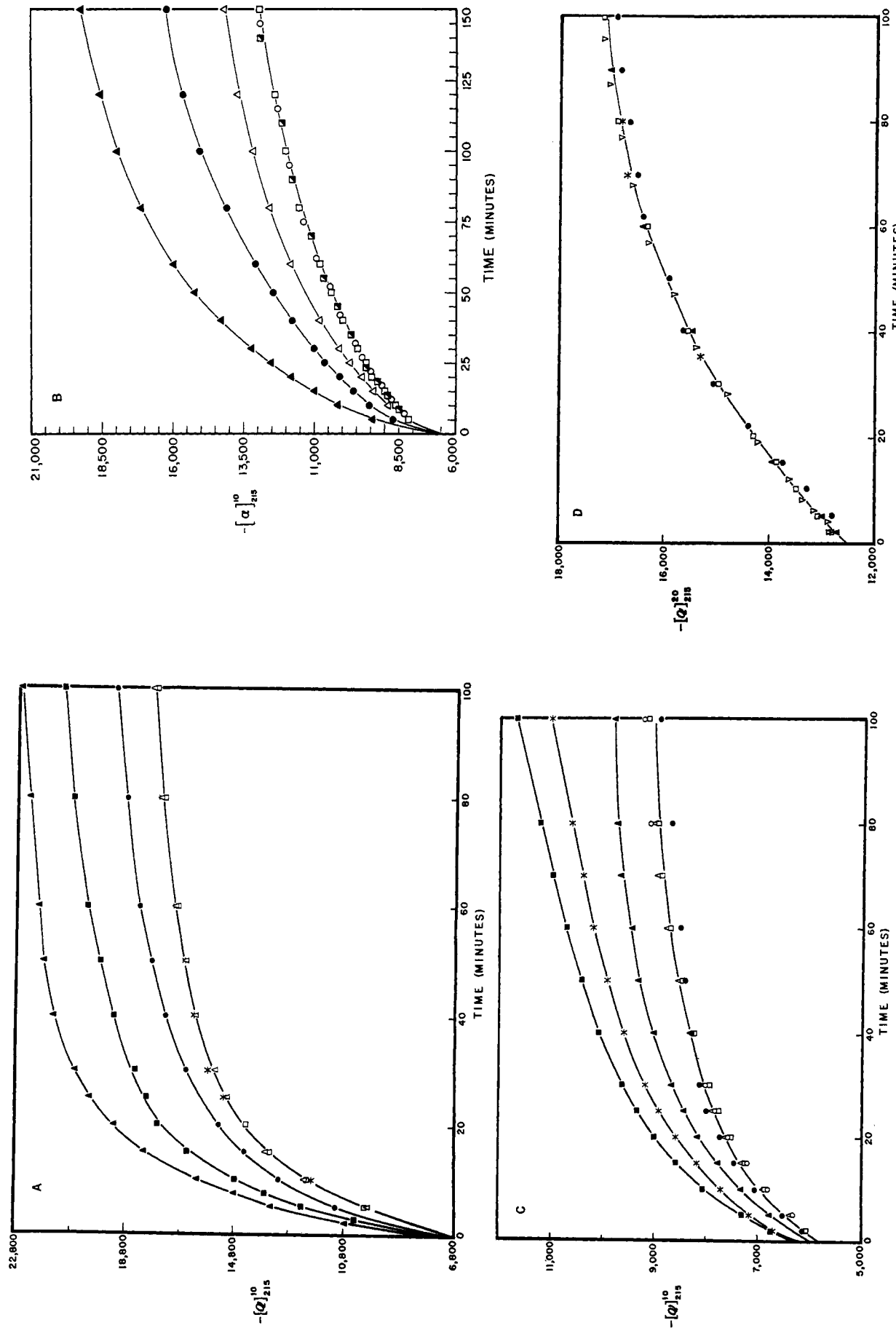

FIGURE 1: Effect of protein concentration on the mutarotation of various single-chain gelatins: temperature 10°, solvent is 0.15 M NaCl–0.05 M NaAc, pH 4.8. (A) α_1-Ratskin concentrations (mg/ml) are: (□) 0.004; (△) 0.02; (*) 0.04; (●) 0.06; (■) 0.20; (▲) 0.40. (B) α_1-Ichthyocol concentrations (mg/ml) are: (□) 0.004; (◪) 0.02; (○) 0.04; (△) 0.08; (●) 0.20; (▲) 0.40. (C) α_2-Ichthyocol concentrations (mg/ml) are: (□) 0.004; (△) 0.04; (○) 0.08; (●) 0.12; (▲) 0.26; (*) 0.52; (■) 1.05. (D) RCM-*Ascaris* (From McBride and Harrington, 1967b) concentrations (mg/ml) are: (□) 0.004; (*) 0.03; (▲) 0.06; (●) 0.09; (▽) 0.60.

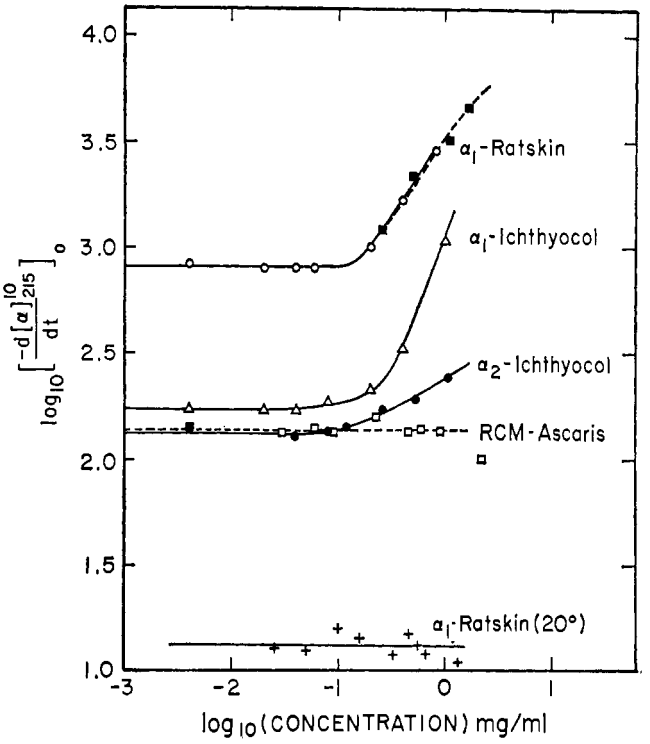

FIGURE 2: Effect of protein concentration on the kinetic order of mutarotation. Log initial rate is plotted as a function of log protein concentration for various single-chain gelatins: solvent is 0.15 M NaCl–0.05 M NaAc, pH 4.8; (■) normalized rate data of Piez and Carrillo (1964) on α_1-ratskin.

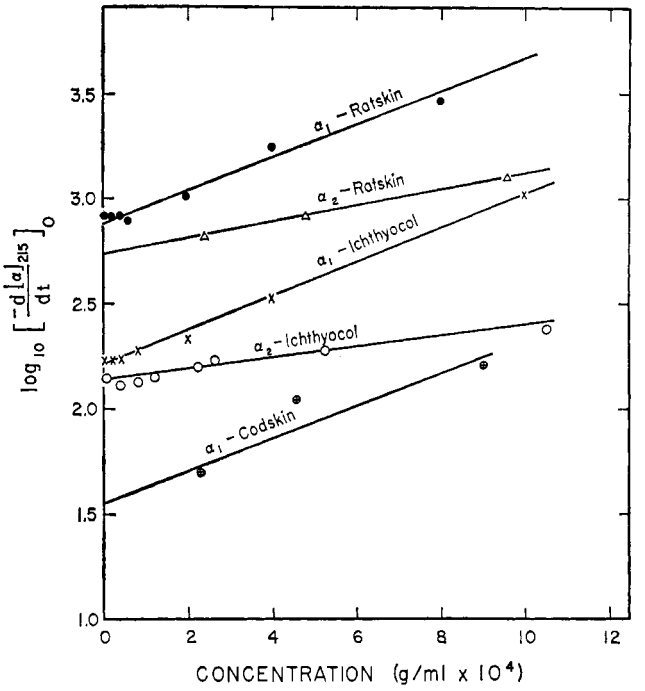

FIGURE 3: Concentration dependence of initial rate of renaturation of α_1 and α_2 chains at high protein concentration. Log initial rate is plotted as a function of concentration. Solvent is 0.15 M NaCl–0.05 M NaAc, pH 4.8. α_2-Ratskin plot has been displaced along the ordinate to avoid confusion.

In addition to the gelatin species presented in Figure 2 we have also examined the concentration dependence of mutarotation of α_2-ratskin, α_1-codskin, and earthworm cuticle gelatin chains over a more limited concentration range (0.2–1.0 mg/ml) and find that their behavior is closely similar to that of the more extensively investigated gelatin systems. The initial rates of renaturation of these polypeptide chains were determined at a wavelength of 313 mμ.

Although Piez and Carrillo observed a twofold increase in molecular weight of cooled (15°) α_1-ratskin gelatin over a 24-hr period, this study was carried out at a protein concentration of 0.24 mg/ml, which is above the concentration-dependent threshold (Figure 2). The molecular weight change and the apparent second-order dependence of mutarotation with respect to protein concentration led these authors to propose association of chains to form double-chain helices in the initial stages of collagen-fold regeneration. We have investigated the molecular weight changes in α_1-ratskin gelatin following the regeneration of collagen structure at 5°. The average molecular weights, obtained from duplicate high-speed sedimentation equilibrium runs after 5 days renaturation of a 0.01% solution were found to be 85,000 ($\bar{M}_n$), 111,000 ($\bar{M}_w$), and 135,000 ($\bar{M}_z$). The molecular weight of random coil α_1-ratskin gelatin approximates 100,000 g/mole (Lewis and Piez, 1964). Thus it is clear that collagen-fold regeneration can proceed *via* a completely intramolecular mechanism in this species below the concentration-dependent threshold. Also, in the case of RCM-*Ascaris*, molecular weight changes are not observed during the renaturation process (McBride and Harrington, 1967b). Moreover, von Hippel (1967) has reported that mutarotation in ichthyocol gelatin can occur under conditions in which molecular weight changes are negligible clearly indicating that stable intramolecular helix formation within a single-chain gelatin molecule is possible. These observations, when taken in conjunction with the results summarized in Figures 1 and 2, lead to the conclusion that renaturation in all single-chain gelatin molecules is probably intramolecular at low protein concentration.

Another noteworthy aspect of Figure 2 is the marked difference in concentration dependence between the α_1 and α_2 chains derived from the same collagen species. Plots of the logarithm of the initial rate *vs.* concentration (Figure 3) show apparent linearity in the concentration-dependent region and all of the chains may therefore be fitted by the general expression

$$\log [R_i]_c = \log [R_i]_0 + k_c c \qquad (2)$$

where $[R_i]_0$ is the initial rate of helix formation at infinite dilution, $[R_i]_c$ the rate at the concentration c and k_c is a characteristic constant for a given species of α chain.

Values of $[R_i]_0$ and k_c estimated for each of the gelatin species are presented in Table I. Since kinetic studies on earthworm cuticle and α_1-codskin utilized a spectral wavelength of 313 mμ, initial rates of mutarotation of these materials have been estimated at λ 215 mμ (from optical rotatory dispersion curves) to facilitate comparison with the other chains. Values of $[R_i]_0$ for earthworm cuticle and α_1-codskin gelatin were determined at 1° and have been

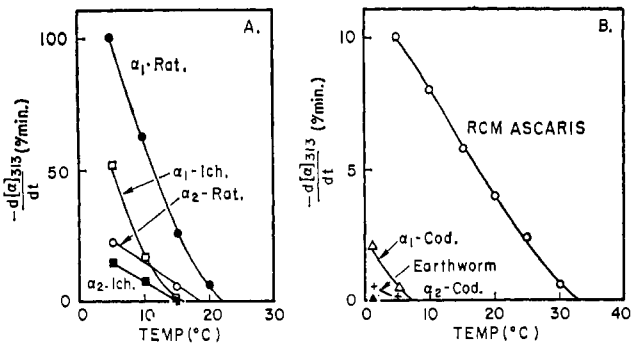

FIGURE 4: Temperature dependence of initial rate of renaturation of single-chain gelatins at high protein concentration (1 mg/ml). Solvent is 0.15 M NaCl–0.05 M NaAc, pH 4.8.

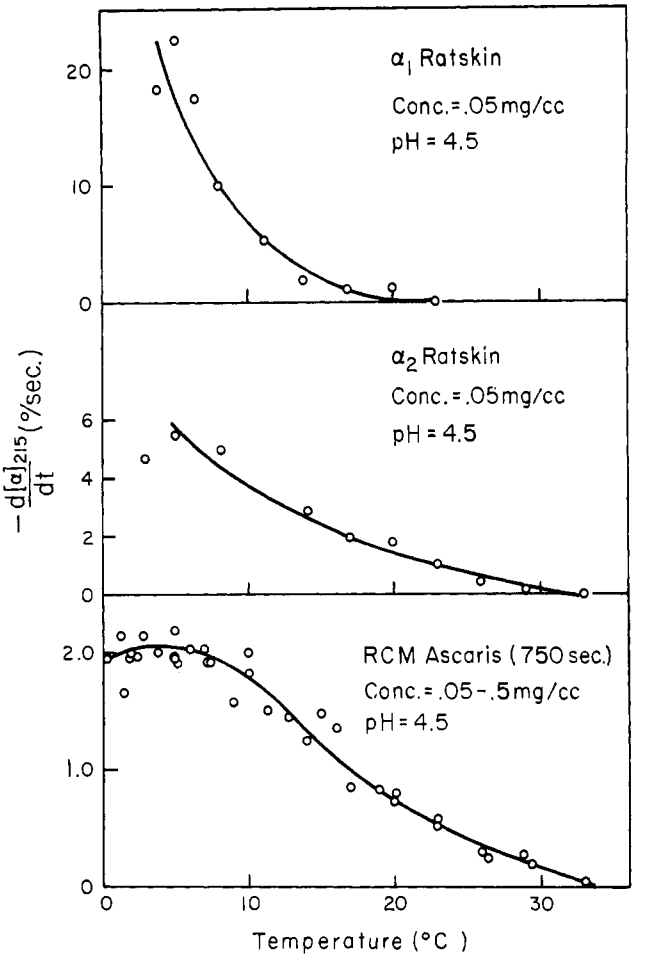

FIGURE 5: Temperature dependence of initial rate of renaturation of single-chain gelatins at low concentration. Solvent is 0.15 M NaCl–0.01 M NaAc, pH 4.5. RCM-*Ascaris* rates are given at 750 sec after initiation of mutarotation.

corrected to 10° by means of the temperature-dependence data of Figure 4. The three α_1 chains show a much larger concentration dependence of helix formation than the α_2 chains. This behavior has been noted earlier for the α chains of ratskin (Piez and Carrillo, 1964) and may be pertinent to the process of tropocollagen formation *in vivo*.

Kinetic Order with Respect to Random-Chain Content. Previous work in several laboratories (Harrington and von Hippel, 1961; Drake and Veis, 1964; McBride and Harrington, 1967b; Bensusan and Nielsen, 1964; von Hippel and Wong, 1963; Smith, 1919) has demonstrated that the kinetics of mutarotation in various unfractionated gelatin systems approximate a second-order process with respect to the amount of the chain in random-coil form. The results of the present study on purified noncross-linked single-chain gelatins are in agreement with the earlier findings. Plots of log $d[\alpha]/dt$ *vs.* log $\{[\alpha]_\infty - [\alpha]_t\}$ were linear over a period of 2–3 half-lives yielding an order of reaction, $n = 2.0 \pm 0.15$ for all of the gelatin systems. No variation in the apparent kinetic order was observed over a 25-fold change in protein concentration. Thus the apparent order $n \cong 2$ is characteristic of single-chain gelatins in the concentration dependent range as well as under conditions where chain–chain interactions have been minimized ($c < 0.1$ mg/ml). Moreover, van't Hoff plots of mutarotation reactions followed at various temperatures gave no indication of any trend in the apparent order of reaction over the temperature range 5–20°. Although a number of workers have attempted to develop a satisfactory theoretical explanation for this general kinetic behavior, to date results have been largely unsuccessful. As we will demonstrate in subsequent papers of this series, it seems likely that the apparent second-order dependence on the amount of chain elements in disordered form is a consequence of differing temperature dependencies of nucleation and growth reactions within the chain. We defer detailed consideration of this problem to the following paper (Harrington and Karr, 1970).

Temperature Dependence of Renaturation Rate. The dependence of the initial rate on the renaturation temperature of various single-chain gelatin molecules is presented in Figures 4 and 5. All of the chains show negative temperature dependence of mutarotation, *i.e.*, a fall in rate with increasing temperature. Rather striking differences are seen in the temperature dependence of the α_1 and α_2 chains in that the

TABLE I: Initial Rates and Concentration Dependence Parameters for Renaturation of Single-Chain Gelatins at 10°.

Gelatin Chain	Initial Rate $\left(\frac{d[\alpha]_{215}^{10}}{dt}\right)$ (deg/min)	k_c[b] (g/ml $\times 10^2$)	ΔT (°C)
Earthworm cuticle[a]	19	1.8	12
α_1-Codskin[a]	34	7.1	11
α_2-Ichthyocol	138	2.5	22
α_1-Ichthyocol	158	7.8	24
α_2-Ratskin	364	3.8	25
α_1-Ratskin	777	7.6	26
RCM-*Ascaris*	151	0	35

[a] Initial rates for these chains were measured at 1 and 5° and have been estimated at 10°. [b] k_c is defined by the equation $\log [R_i]_c = \log [R_i]_0 + k_c c$ where $[R_i]_0$ is the initial rate at infinite dilution and c is concentration in g/ml.

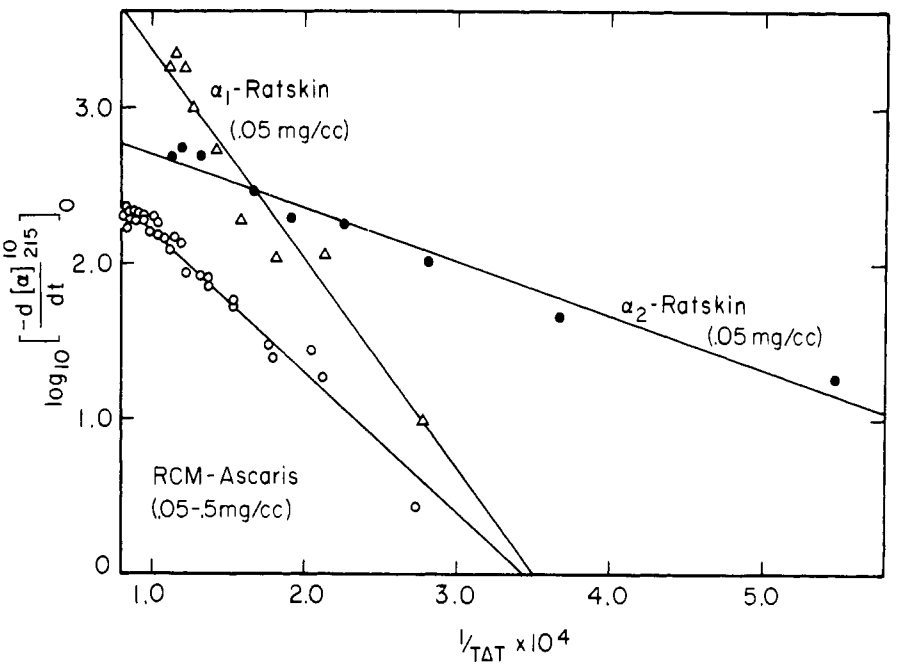

FIGURE 6: Flory–Weaver plots of initial rates of three single-chain gelatins. Solvent is as in Figure 5.

renaturation rates of α_1 chains of ratskin and ichthyocol show a much greater temperature dependence than do the corresponding α_2 chains. A similar comparison of the codskin system is prevented by virtue of the limited temperature interval (1–5°) available for mutarotation studies. However at very low temperature (1°) the initial rate of α_1-codskin is about tenfold greater than α_2. The data of Figure 4 were obtained at a constant protein concentration of 0.1%, but qualitatively similar temperature behavior is observed below the concentration-dependent threshold (see Figure 5).

An unusual feature in the rate–temperature profile of RCM-*Ascaris*, presented in Figure 5, can be seen in the low-temperature range in that the rate appears to pass through a maximum near 3°. This finding has special significance in understanding the kinetic mechanism of refolding within collagen-type polypeptide chains and it will be considered in detail in the following paper (Harrington and Karr, 1970).

Examination of Figures 4 and 5 reveals that the fractional change in initial rate with temperature, *i.e.*, the slope of $\ln\{-d[\alpha]/dt\}$ *vs.* $1/T$ increases as the midpoint of the helix → coil transition temperature is approached. Thus the apparent (negative) Arrhenius activation energy will increase progressively as the regeneration temperature is elevated. As Flory and Weaver (1960) have pointed out, this behavior is characteristic of nucleated crystallization reactions. Assuming the nucleation step to be rate limiting, these authors derive an expression for the rate constant of renaturation in which the rate is an exponential function of $T\Delta T$. They find

$$k = B\exp\left(\frac{-A}{RT\Delta T}\right) \qquad (3)$$

where B and A are constants, T (in °K) the renaturation temperature and ΔT, the degree of undercooling ($T_m - T$). In accordance with this equation Flory and Weaver observed that a plot of the logarithm of the half-time *vs.* $1/T\Delta T$ was sensibly linear for the mutarotation kinetics of unfractionated rat-tail gelatin.

Plots of eq 3 for the three collagen chains of Figure 5 are presented in Figure 6 demonstrating that initial rates of renaturation in the concentration-independent range also obey the Flory–Weaver relationship. Table II lists the parameters A and T_m which give the best fit with the rate data. The values of T_m are in close correspondence with the expected thermal transition temperatures of the respective collagens estimated from the melting curves of purified α-type gelatin chains (Harrington and Rao, 1967). As Flory and Weaver have emphasized, the temperature at which *all* structure has been melted in these systems should correspond to the T_m of the native collagen.

The activation free energy of nucleation, $A/\Delta T$, of the α_1 chains at a given level of undercooling is always greater than that of the α_2 chains, in keeping with the greater temperature dependence of this species seen in Figures 4 and 5.

Role of Imino Acids in Controlling Renaturation Rate. Earlier studies (Harrington and von Hippel, 1961; Josse and Harrington, 1964) have called attention to the apparent dependence of renaturation rate of gelatin chains, compared

TABLE II: Flory–Weaver Parameters for Single-Chain Gelatins at Low Concentration.

Gelatin Chain	T_m (°C)	A (cal-deg mole^{-1})
α_1-Ichthyocol	34	88,000
α_2-Ichthyocol	32	43,000
α_1-Ratskin	36	61,000
α_2-Ratskin	35	15,000
RCM-*Ascaris*	44	44,000

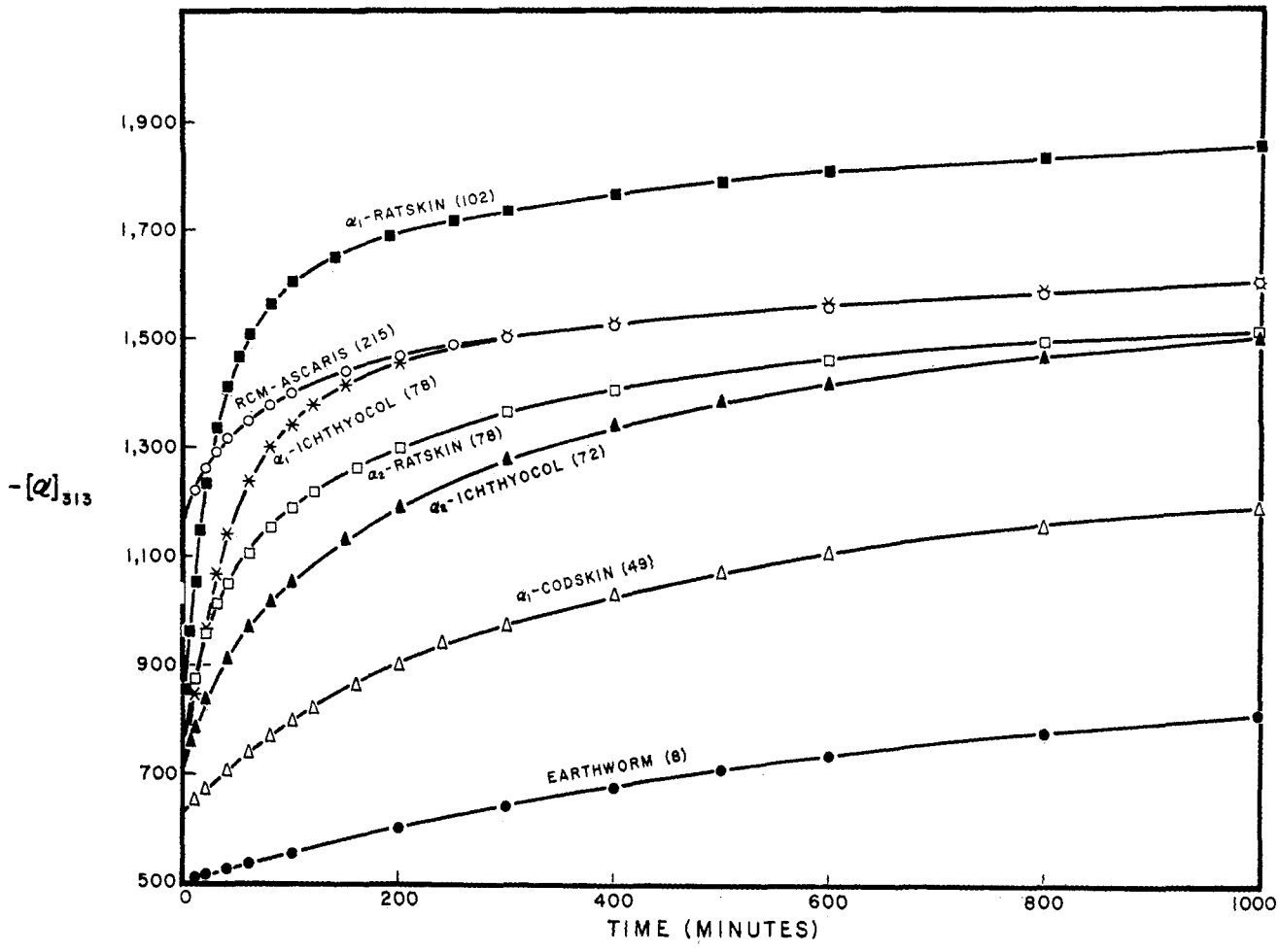

FIGURE 7: Isothermal mutarotation curves of various single-chain gelatins at a constant level of undercooling (ΔT) below the transition temperatures of the refolded structures. Solvent is 0.15 M NaCl–0.05 M NaAc, pH 4.8; protein concentration = 1 mg/ml.

at a fixed temperature, T, on the number and distribution of imino acid residues within the chain. A relationship between the rate and the sum of pyrrolidine residues would be expected if the initial rates were simply a function of the degree of undercooling ($T_m - T$), since it is now well established that T_m of the native collagens increases monotonically with the fractional increase in pyrrolidine content. In these studies mutarotation was followed at relatively high concentrations of protein (>0.5 mg/ml) and no account was taken of the possible concentration dependence of renaturation. Moreover, the presence of cross-links between chains as well as the use of unfractionated chains have introduced additional complications and precluded a definitive correlation. The effect of cross-linking on the renaturation kinetics is especially difficult to assess. Altgelt *et al.* (1961) and Drake and Veis (1964) have shown that covalent cross-linking of individual α chains increases the mutarotation rate. In the case of *Ascaris* cuticle collagen, the initial regeneration rate of the highly cross-linked native structure following thermal denaturation is about 20-fold greater than that of the noncross-linked RCM-*Ascaris* chains at 4°.

A comparison of the initial rates of mutarotation of the various noncross-linked single chains below the concentration-dependent threshold is presented in Table I. Rates are given at the same renaturation temperature, 10°, and show a rather dramatic elevation as the imino acid content (ΔT) of the chains is increased. The apparent correlation is not absolute, however, since RCM-*Ascaris* and earthworm show anomalous behavior. Earthworm has a higher total pyrrolidine content than α_1-codskin but a significantly lower rate of mutarotation. The mole fraction of imino acid residues in *Ascaris* is greater than in any of the chains examined, yet RCM-*Ascaris* renatures at a relatively low rate. It seems clear that the rate of refolding is not a simple function of pyrrolidine content. This lack of dependence is also brought out in Figure 7 where optical rotatory changes accompanying renaturation of the various chains have been determined at a constant level of undercooling ($\Delta T = 10°$ below T_m of the respective gelatins). Although this comparison would be expected to eliminate the pyrrolidine-rate dependence, since measurements were made at a constant ΔT, an increase in rate is observed paralleling that found at constant temperature. Again, earthworm and *Ascaris* gelatins show anomalously low renaturation rates. All of the experiments summarized in Figure 7 were carried out at a constant protein concentration of 1 mg/ml, but the same relative behavior is observed in the low concentration range.

An appreciable fraction of the gelatin chain retains the

poly-L-proline II type conformational pattern above the helix → random coil transition temperature. The residual specific levorotation, in excess of that expected on the basis of amino acid composition for a completely random structure, varies with the collagen species being smallest for earthworm and largest for *Ascaris* cuticle collagen (Josse and Harrington, 1964). This behavior has been attributed to the presense of contiguous pyrrolidine residues, which, by virtue of their stereochemistry, impose rotational restraints on the backbone chain (Harrington, 1958; von Hippel and Harrington, 1959; Schimmel and Flory, 1968) and fix the relative orientation of nine successive backbone bonds in the left-handed poly-L-proline II conformation. This interpretation is consistent with the observed initial values of specific rotation seen in Figure 7. All of the chains approach a terminal specific rotation, $[\alpha]_{313}^{10} - 1930°$, after several weeks of isothermal incubation at low temperature. Thus a comparison of the effect of primary structure on initial rates of renaturation must take into account the variation in $([\alpha]_\infty - [\alpha]_{initial})$ among the various species, which in turn is proportional to the "random chain content" of the unfolded chains.

Although the mutarotation rates of the various single-chain gelatins do not correlate directly with pyrrolidine content, we would expect the probability of nucleation to be maximal in regions of the chain which have a high density of imino acid residues. The reduced conformational entropy change accompanying the coil → helix transformation favors initiation of the collagen fold in these regions. Since the lowest average entropic change will occur in Gly-X-Y triplets containing contiguous pyrrolidine residues, it has been suggested (Josse and Harrington, 1964) that these "locked" contiguous residues may act as the primary initiating sites for the refolding process. In Table III the "infinite dilution" initial renaturation rates of the various single-chain gelatins are given at the same level of undercooling ($\Delta T = 20°$) below T_m of the respective collagens. Rates have been normalized for the random-coil content, (C_r), of each chain assuming that the specific rotatory change of earthworm $([\alpha]_\infty - [\alpha]_{initial})$ represents 100% random coil. Since renaturation rate is approximately proportional to the second power of the random chain content, initial rates have been normalized by dividing by $(C_r)^2$. The number of pyrrolidine doublets in the various chains has been estimated according to the statistical analysis presented earlier (Josse and Harrington, 1964). It will be seen that the marked variation in normalized initial rates correlates fairly well with the predicted contiguous pairs of pyrrolidine residues except for the case of RCM-*Ascaris*. The reasons for this discrepancy are not clear at the present time.

TABLE III: Effect of Imino Acid Residues on Initial Renaturation Rate of Single-Chain Gelatins ($\Delta T = 20°$).[a]

Gelatin	Σ Pyrrolidines (Per 1000 Residues)	Predicted Contiguous Pyrrolidines[b] (Per 1000 Triplets)	Initial Rate[c] $-\frac{d[\alpha]_{215}}{dt}$ (°/min)
Earthworm cuticle	169	10	34
α_1-Codskin	155	49	92
Dogfish sharkskin	166	57	94
α_2-Ichthyocol	187	72	88
α_1-Ichthyocol	199	78	124
α_2-Ratskin	197	78	144
α_1-Ratskin	226	102	200
α_1-Calfskin	232	110	234
RCM-*Ascaris*	310	211	84

[a] Solvent is 0.15 M NaCl–0.01 M acetate, pH 4.8. [b] Predicted frequency of neighboring pyrrolidine residues estimated according to Josse and Harrington (1964). [c] Normalized for random chain content.

Discussion

In the original three-step proposal for the mechanism of the isothermal gelatin → collagen-fold transformation (von Hippel and Harrington, 1959; Harrington and von Hippel, 1961) the primary event was considered to be nucleation of the poly-L-proline II type helix in the pyrrolidine-rich regions of the polypeptide chain. Growth of the helix from the initiating sites was thought to occur along single polypeptide chains which, in turn, made possible a specific association between single-chain helices. This single-chain refolding mechanism was based primarily on the observation that helix formation appeared to be independent of the gelatin concentration. An alternate mechanism proposed by Flory and Weaver (1960) included the formation of a transient, unstable intermediate formed by "unimolecular rearrangement" of a single random-coil molecule as the first, rate-determining step. This would be followed by rapid association of three such intermediates to form the compound triple helix of collagen. The Flory–Weaver scheme accounts for the apparent first-order kinetics by considering the initial conformational change in a single chain to be rate determining.

Although both mechanisms proposed a single-chain intermediate to account for the first-order kinetics (with respect to protein), they differed in the view taken of the stability of this intermediate. In the three-step scheme the collagen fold is developed along single chains and is a structurally stable entity, whereas in the Flory–Weaver mechanism the single-chain helix has only a transitory existence and stabilization is accomplished through association of three single-chain intermediates to form the compound triple helix.

The major difficulty in the three-step scheme was the stabilization of the single-chain helix. Although it was suggested that stabilization might be accomplished through a systematic solvation of the chain, this mechanism was later shown to be unlikely by the hydrogen exchange studies of Bensusan and Nielsen (1964) who observed rates of deuterium–hydrogen exchange incompatible with such a structure. Moreover, it has recently been shown that the formation of the poly-L-proline II type helix in the synthetic polymer poly-L-proline is not influenced by the presence or absence of water, clearly indicating that this solvent does not play a unique role in the stabilization (Kurtz and Harrington, 1966). On the other hand the Flory–Weaver mechanism assumes association between individual polypeptide chains

to occur so rapidly as to have a negligible effect on the overall kinetics, but it is clear from the variation in mutarotation rate as a function of concentration presented in Figures 1 and 2 that chain association is a relatively slow process and is reflected in a small increase in the mutarotation rate which can be eliminated at sufficiently low protein concentration. The association of individual chains, although clearly essential in the regeneration of the triple helix of tropocollagen, appears to be of secondary importance in the mechanism of collagen-fold formation at low concentration. This interpretation is in keeping with the order of reaction determined for the coil → helix kinetics. All of the single-chain gelatin species exhibit first-order dependence in protein concentrations below 0.1 mg/ml, while showing at the same time an apparent second-order reaction with respect to the amount of the chain in random-coil form. This latter kinetic parameter is invariant with protein concentration. Thus it seems likely that the same type of intramolecular mechanism is operating below the concentration-dependent threshold in all gelatin species gelatin species, both vertebrate and invertebrate evidence supporting this view will be given in the following papers of this series.

Drake and Veis (1964) have made an important contribution to the problem in proposing that the first-order dependence (in protein) of the refolding process could result from an "intramolecular reaction in which different segments of the same molecular chain must interact to form stable collagen-fold units." They pointed out that the weight concentration within the domain of an average random-coil gelatin molecule would be expected to be about 2 mg/ml and that interchain and intrachain segment contacts are about equally probable at this concentration. Dilution favors intrachain contacts. The suggestion that reverse-folded triple helices may form in cooled gelatin systems at low concentrations is consistent with the behavior of RCM-*Ascaris*. As we have noted earlier, these chains reform the collagen-type helix in a completely intramolecular fashion at all concentrations. Moreover, the reverse-folded triple helix appears to be the stable conformational state of the subunit chain in native *Ascaris* cuticle collagen (McBride and Harrington, 1967a,b).

The results of the present paper provide additional insight into the problem. It is clear that at low protein concentration the renaturation process is truly first-order with respect to protein for all of the single-chain gelatins, whereas at concentrations above about 0.1 mg/ml the reaction order of the vertebrate chains increases exponentially. It seems likely that, as in the case of RCM-*Ascaris*, the first-order reaction reflects a *completely intramolecular* folding process to form a stable, reverse folded structure in all of the single-chain species at low protein concentration. Since the process of chain folding is intramolecular, there is no need to invoke a transient intermediate as Flory and Weaver have done to account for the first-order dependence of the rate. We visualize the rate-limiting step at low concentration and at moderate levels of undercooling (ΔT) as the formation of a prototypic, hydrogen-bonded, triple-helical nucleus formed through reverse folding of the polypeptide chain. Once the basic hydrogen-bonded pattern is established, propagation of the structure proceeds stepwise as the chain segments pendant to the nucleus wrap about themselves to form the triple helix. At high protein concentration the rate-limiting step is considered to be formation of a hydrogen-bonded nucleus through interaction of peptide segments belonging to three separate chains. Formation of the prototypic nucleus in this case is assumed to be a third-order reaction. Again, it is unnecessary to invoke a transient intermediate since the higher reaction order seen above the concentration-dependent threshold in Figure 2 indicates an intermolecular process. Chain folding may proceed along either of these two pathways and the extent of reverse-folded (intramolecular) and parallel-folded (intermolecular) patterns of the triple helix will depend both on protein concentration and temperature (see Figure 8). Since both reactions are possible it is essential to go to very low concentration (<0.1 mg/ml) or to a very high concentration (≫2 mg/ml) in order to eliminate one or the other of the triple-helical structures as end products of the reaction. Measurement of the kinetic order of renaturation in the intermediate concentration region will give an "average" value for this parameter which is completely misleading in assessing the kinetic mechanism of refolding.

The degree of cooperativity in refolded gelatin systems depends markedly on the temperature of renaturation, as might be expected for a nucleated crystallization process. A reasonable yield of native tropocollagen molecules can be obtained if renaturation of unfractionated gelatin is carried out at low levels of undercooling, (Beier and Engel, 1966) or if the system is annealed through temperature cycling or through gradual cooling (Engel, 1962; Kuhn *et al.*, 1964). Kuhn and Zimmerman (1965) have demonstrated that even pure α_1 chains will completely renature to native-type collagen molecules by alternate warming and cooling or by gradual cooling. In general, relatively high protein concentrations (>1 mg/ml) were used in the temperature fluctuation studies thus favoring multichain, parallel-type folding patterns. Beier and Engel (1966) also employed relatively high protein concentrations (>2.0 mg/ml) in their investigations on the optimum conditions for isothermal regeneration of native collagen structure from unfractionated calfskin gelatin. From an analysis of time-dependent changes in optical rotation, viscosity, and sedimentation profiles they concluded that an optimum high temperature exists for the formation of native molecules to the exclusion of other stabilization forms, notably high molecular weight aggregates. Beier and Engel (1966) observed a significant concentration dependence of renaturation in the high temperature range, (22°) and, like Piez and Carrillo (1964), concluded that an association between chains is the rate-limiting step in the regeneration process.

However, the essential paradox of the kinetics was not treated by either of these groups. If the renaturation involves as its rate-limiting step the association of three chains, then the process should be third order in protein concentration. The studies of Beier and Engel on mutarotation in the high temperature range (22°) where, they suggest, native collagen molecules are reformed, show that the initial rate increases by a factor of 2–4 when the concentration is increased by about eightfold (from 0.6 to 4.9 mg per ml). For a process in which association between three chains is rate limiting, the rate should have been elevated by a factor of about 500. Piez and Carrillo propose from their results at low temperature (15°) that association between two α chains is the rate-limiting step under these conditions, but it can be seen from

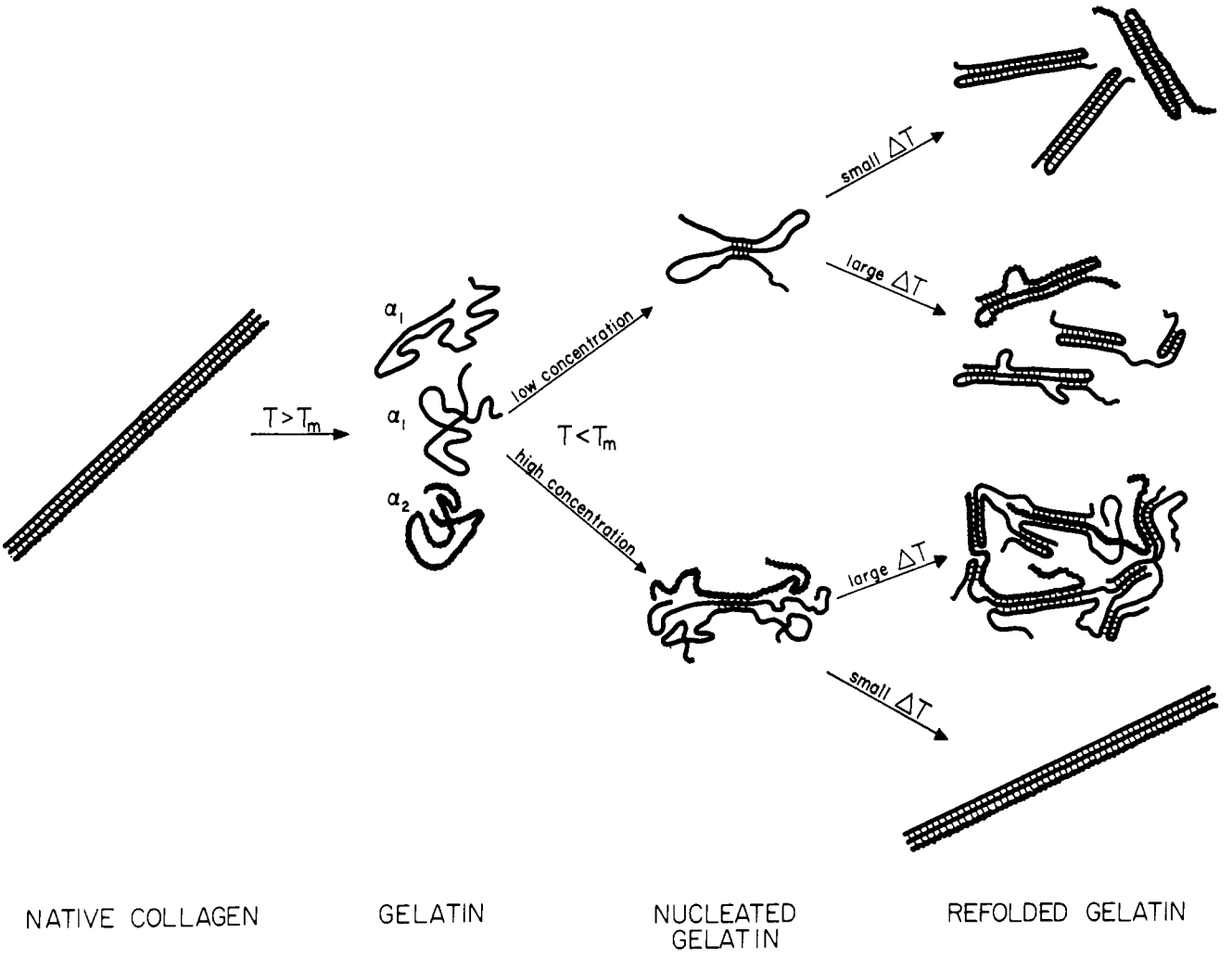

FIGURE 8: Suggested scheme for the concentration-dependent dual pathway for chain folding below T_m in single-chain polypeptides derived from collagen.

Figures 1 and 2 that with a 100- to 200-fold increase in concentration, the rates are elevated only two- to threefold. For a second-order process with respect to concentration the reaction rate over this concentration range would be expected to change by about 10^4. We believe that the apparent discrepancy results from the presence of both intramolecular and intermolecular reactions as discussed above. A very significant fraction of the overall mutarotation reaction involves reverse folding even at concentrations well above 2 mg/ml. This is also true in the high temperature range as indicated by the lack of concentration dependence of the initial rate of α_1-ratskin at 20° (see Figure 2, lower).

Considerable insight into the mechanism of chain folding in single-chain collagen-type molecules can be obtained from the temperature dependence of the kinetics. In the Flory–Weaver mechanism the formation of the transient intermediate is conceived as a unimolecular rearrangement of a small segment of the chain into the poly-L-proline II type helical conformation. Although we view the formation of a hydrogen-bonded triple-helical nucleus as the rate-determining step at low values of undercooling, the rate–temperature dependence of this process should also follow the Flory–Weaver formulation (eq 3) as the energetics of the two mechanisms differ only in the extent of contribution of the enthalpy term to the free energy change involved in the formation of the nucleus. The postulated hydrogen-bonded nucleus must attain a minimum size before it can be assured of a stable existence and this critical size will be dependent on the positive surface free energy, ΔF_s, arising from the helix-coil junctions as well as the degree of undercooling, ΔT, below the melting temperature of the native collagen (T_m). As Flory and Miller (1966) have pointed out, ΔF_s is related to the helix interruption constant, σ, in the Zimm–Bragg theory (1959) through the expression $\Delta F_s = -RT \ln \sigma$ (see also Flory, 1961; Flory and Weaver, 1960). A stable nucleus will be formed, either through reverse folding of the chain (low concentration) or association of three separate chains (high concentration), when the number of residues, n, is large enough to give an overall *negative* free energy equal to or greater than ΔF_s. The free energy of formation of the nucleus, ΔF_n, may then be written as $\Delta F_n = n\Delta F' + \Delta F_s$ where $\Delta F'$ is the negative free-energy change accompanying transfer of a single residue from the random coil state to the interior, H-bonded state of the

nucleus.[1] Since $n\Delta F' + \Delta F_s = 0$ for the achievement of a stable nucleus, the critical size (n^*) required for growth at the temperature T will be given by

$$n^* = \frac{\Delta F_s}{\Delta S \Delta T} \quad (4)$$

Thus, following the treatment given by Flory and Weaver (1960), the rate constant k (eq 3) can be shown to be exponentially related to $1/T\Delta T$. The results of Figure 5 demonstrate that the initial rate-temperature profile associated with the *intramolecular* folding reaction is compatible with eq 3. It has also been shown in earlier work (Flory and Weaver, 1961; von Hippel and Wong, 1963) that mutarotation data obtained on unfractionated noncross-linked and cross-linked chains at high protein concentration conform to the Flory–Weaver formulation.

Referring back to eq 4 it may be noted that the critical size of the nucleus required for subsequent growth will increase as T_m is approached. For instance, in the case of α_1-ratskin collagen which has a T_m of 36° (Table II), renaturation of the corresponding gelatin at 30° requires a nucleus which is 5.2 times larger than that needed at 5°. Since the size of a stable helical segment even at low temperatures is at least 20–30 residues (Harrington and Rao, 1967), and perhaps more, the mounting difficulties encountered in the attainment of molecular cooperation in a nucleus of much longer sequence of peptide units will drastically slow down the renaturation rate in the high-temperature range (20–30°). This mechanism of nucleation not only explains the observed negative temperature coefficient but also gives an insight into the reasons for formation of randomly oriented small helices with a low degree of asymmetry in gelatins renatured at low temperatures (4–5°) in contrast to the much more perfect, large, and stable helices formed in the temperature range of 20–30° (Hauschka and Harrington, 1970).

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Added in Proof

Recent studies by Piez and Sherman (1970) with the 36-residue peptide α1-CB2 from α_1-ratskin have provided important information on the mechanism of collagen-fold formation in the concentration-dependent range (>0.1 mg/ml). While this peptide is probably of insufficient length to permit a reverse-folded triple-helical structure, it has been shown to undergo *reversible* aggregation to form a trimeric collagen-fold structure at low temperatures. The kinetics of helix regeneration are third order in protein concentration, suggesting that the α1-CB2 system represents an idealized case of *intermolecular* nucleated crystallization of the collagen fold.

[1] At the temperature, T, $\Delta F' = (\Delta H - T_m\Delta S) - (\Delta H - T\Delta S) = -\Delta S \cdot \Delta T$ where ΔS is the overall change in entropy per peptide unit. We assume ΔH and ΔS to be independent of temperature.

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