Helix–Coil Transition in Collagen. Evidence for a Single-Stranded Triple Helix*

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ABSTRACT: The effect of the disulfide cross-linkages on the stability and regeneration of the collagen-fold conformation in polypeptide chains of *Ascaris* collagen has been investigated. Cleavage of the disulfide cross-links lowers the helix–coil thermal transition temperature, T_m, about 19° and reduces the helix regeneration rate by a factor of 10–20 below that of the native collagen. Reoxidation following reductive cleavage of the SS bridges increases the regeneration rate to a level comparable to the native collagen and elevates T_m to an intermediate value between the native and fully reduced systems. The mechanism of formation and stabilization of the collagen fold in the reduced carboxymethylated polypeptide chains of *Ascaris* collagen (RCM *Ascaris*) has been examined. The rate of renaturation is shown to be independent of concentration over about a 500-fold range. Kinetic analysis of tritium–hydrogen-exchange curves reveals that approximately 230 slowly exchanging hydrogens/subunit chain (mol wt 62,000) are formed during the regeneration of the polyproline II type helix, in good agreement with the number determined for the native collagen per helical triplet.

Tryptic digestion experiments indicate that a large portion (75–85%) of the renaturated RCM *Ascaris* chain remains intact at 5° following proteolysis. These results, when taken in conjunction with the invariance in molecular weight observed during transformation of the helix to the coil forms, suggest that the subunit of *Ascaris* is a single polypeptide chain which folds back upon itself to form a stable, collagen-type triple helix at low temperature.

Evidence presented in the previous paper (McBride and Harrington, 1967) has demonstrated that the neutral salt-soluble collagen derived from the cuticle of *Ascaris lumbricoides* is a multichain structure held together by disulfide cross-linkages. Moreover, the subunit polypeptide chains isolated following reductive cleavage of the SS cross bridges have a molecular weight of 62,000, that is, roughly two-thirds of the chain weight commonly found for the individual subunits (α chains) of the vertebrate collagen species. Optical rotatory dispersion studies, in conjunction with other physical measurements, indicate that the isolated subunits can exist in a stable, collagen-type structural pattern which undergoes a helix–coil transition without any alteration in the molecular weight. In view of these unusual features we have investigated the mechanism of stabilization of the chain conformation and the process of regeneration of the collagen-type structure at low temperatures in both the cross-linked and noncross-linked subunits. Since the cross-links in *Ascaris* collagen are chemically well defined and undergo specific cleavage in the presence of reducing agents, their effect on the stabilization and regeneration of the collagen fold can be investigated under well-controlled conditions.

The mechanism of formation of the collagen fold in single-chain gelatin systems has been under study in several laboratories in recent years (von Hippel and Harrington, 1959, 1960; Harrington and von Hippel, 1961a,b; Flory and Weaver, 1960; Engel, 1962; Drake and Veis, 1964; Piez and Carillo, 1964; Bensusan and Nielsen, 1964). As is well known, when solutions of randomly coiled gelatin molecules are cooled to low temperature, the helical conformational pattern of the polypeptide chains characteristic of collagen is slowly regenerated as evidenced by a number of physical and physicochemical techniques. The fundamental processes involved in the renaturation reaction are as yet incompletely understood, but two general theories have been proposed (von Hippel and Harrington, 1959; Flory and Weaver, 1960) to account for the main features of the regeneration reaction. Both theories are based on the observation that the kinetics of regeneration appear to be closely first order in protein concentration and both attempt to resolve the apparent contradiction of a unimolecular reaction forming a *stable*, collagen-type triple helix.

Although the studies to be reported below have been carried out entirely on the *Ascaris* collagen system,

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we believe that the basic findings have general application to the mechanism of refolding in all gelatin species. Thus, it appears that in all single-chain gelatin systems the refolding process is strictly first order at low protein concentration (<0.05 mg/ml; Rao and Harrington, 1966a) and it seems likely that the processes involved in forming the collagen-fold conformation are analogous to those operating in the *Ascaris* subunits. Our studies provide evidence supporting the view that the stable, collagen-type conformational pattern in the *Ascaris* subunits is formed within a single strand through reverse folding of the polypeptide chain.

Experimental Methods

Chemicals. Bovine pancreatic trypsin (two times recrystallized) was a product of the Worthington Biochemical Corp. Tritiated water (500 mc/ml) was obtained from the Volk Radiochemical Corp. All chemicals were reagent grade. Water was deionized and glass distilled.

Optical Rotation. A Model 80 Rudolph spectropolarimeter equipped with an oscillating polarizing prism was routinely utilized for the optical rotation studies at high protein concentrations (milligrams per milliliter range). The 366-mμ emission band of the mercury arc was used for most measurements. Thermal denaturation studies were carried out by the equilibrium technique of von Hippel and Wong (1963) and the temperature was maintained constant until no further change in optical rotation could be detected (as judged by measurement at 30-min intervals).

In the mutarotation experiments, solutions were heated at 70° for 20 min. At time $t = 0$, the samples were quickly quenched to the temperature of the experiment and transferred to a jacketed polarimeter tube which had been equilibrated at the same temperature. The optical rotation measurements were always followed during the entire primary phase of the mutarotation (over approximately 1440 min and occasionally for longer periods of time).

Measurements of mutarotation rates at very low concentrations (micrograms per milliliter range) were made in a Model 60 Cary recording spectropolarimeter at a wavelength of 215 mμ, *i.e.*, near the cotton trough of collagen. We are indebted to Dr. N. V. Rao for the use of this data.

Hydrogen–Tritium-Exchange Methods. The rate of exchange of protons between tritiated protein and nontritiated solvent was determined by utilizing the G-25 Sephadex one-column technique of Englander (1963) for the rapid separation of protein from bulk solvent. The solvent employed was 0.2 M acetic acid adjusted to pH 4.00 with concentrated NaOH. Since the one-column exchange-out technique was used, the duration of each experiment at any particular temperature was limited by the time required for diffusion of the trailing solvent THO[1] down the column into the zone of exchange between tritiated protein and nontritiated solvent.

Radioactivity and protein concentration in aliquots of column effluent containing the eluted protein peak were determined by liquid scintillation counting and biuret assay, respectively, and the aliquots showing constant specific activity were averaged. Tritium activity in the original solution was similarly determined. The number of tritium atoms per protein molecule remaining at each time interval of exchange was calculated from the equation H_t/molecule $= 111 \cdot (C/C_0)(k/D)$, where C_0 and C are the counts per minute per milliliter of tritiated protein solution and of column effluent, respectively, k is the optical density per millimole of protein, and D is the optical density of column effluent. The values of H_t/molecule were plotted against time and a smooth curve was drawn through the points and extrapolated to zero time to determine the total number of exchangeable hydrogens.

The size and rates of exchange of various classes of hydrogens were obtained from analysis of this curve by two different procedures. (1) Log H_t *vs.* time was plotted and the slow linear phase was extrapolated to zero time to obtain $H_{s,0}$, the total number of exchangeable hydrogens in the slow class. Using the value of $H_{s,0}$ and the rate constant of exchange, k_s, obtained from the slope of the log H_t *vs.* time plot, the number of unexchanged hydrogen atoms in this class at time, t, was calculated from the equation $H_s = H_{s,0}e^{-k_s t}$. Plots of log $(H_t - H_s)$ *vs.* time were sensibly linear with intercept, $H_{f,0}$ (the total number of rapidly exchanging hydrogens), and rate constant, k_f. (2) Log (dH_t/dt) *vs.* time was plotted to obtain $H_{s,0}$ and k_s from the intercept at $t = 0$ and the slope, respectively. Again, the plots exhibited nonlinearity in the initial phase of the reaction followed by an extended linear phase. Subtraction of the slow exchange reaction from the H_t *vs.* t plot gave a rapid first-order exchange reaction which was analyzed as in 1 above. The specific optical rotation of the protein solutions used for tritium-exchange studies was determined to enable comparison of estimated helix content and the number of slowly exchanging hydrogens.

Proteolytic Fragmentation Studies. The kinetics of digestion of RCM *Ascaris*[2] collagen by trypsin (1:250, w/w) was followed in a capillary viscometer and in the pH-Stat. At the completion of digestion, a twofold excess (w/w) of soybean trypsin inhibitor was added and the solutions were further examined by velocity sedimentation, equilibrium sedimentation, optical rotation, and gel filtration studies.

The pK of the α-amino groups liberated during tryptic digestion was determined by the following procedure. A solution of RCM *Ascaris* in 0.2 M NaCl

[1] Abbreviations used: THO, tritiated water; FDNB, fluorodinitrobenzene.

[2] No significant physical or chemical differences have been detected between the RCM *Ascaris* collagen subunits prepared from the neutral salt-soluble fraction and the insoluble matrix fraction of *Ascaris* cuticle collagen (see McBride and Harrington, 1967). The RCM *Ascaris* collagen described in this paper has been prepared from both sources and used interchangeably.

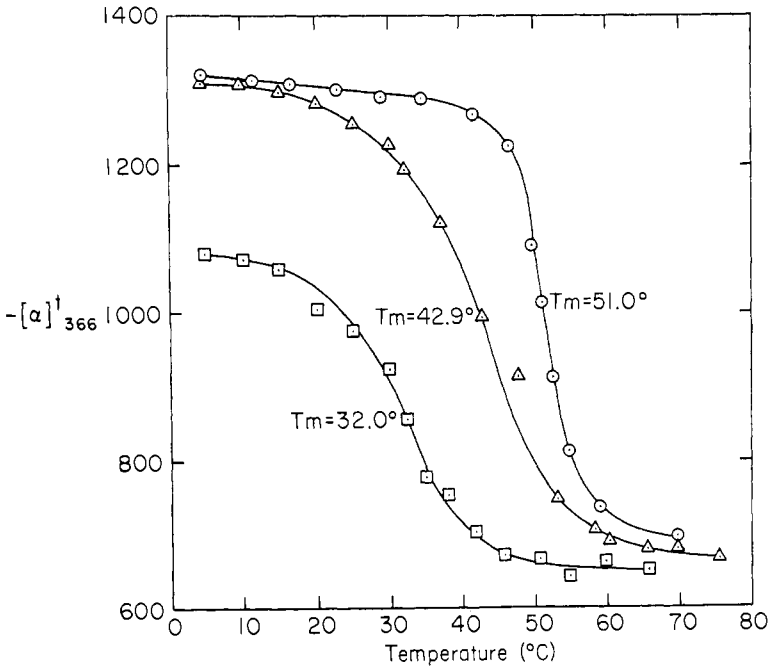


FIGURE 1: The collagen-fold–gelatin thermal transitions of native (○), reoxidized (△), and reduced carboxymethylated (□) *Ascaris* cuticle collagen in 0.2 M NaCl at pH 6 as measured by specific optical rotation ($[\alpha]_{366}$). Protein concentrations were 0.12–0.23 g/100 ml. Each point on all curves represents the value reached after the reading had become invariant with time (see Methods).

was titrated through the pH range 6.2–10.0 at 5°, trypsin (1:250 w/w) was added, and the digestion was allowed to proceed to completion (pH 8) at 40°. The solution was titrated through the same pH range at 5° after the addition of trypsin inhibitor. A plot of the difference between these two titration curves showed a characteristic smooth titration curve with pK of 8.44 (5°). Although there is a total of 52 lysines and arginines/molecule, the base uptake indicated a total of only 28 α-amino groups released during proteolysis (54% of theoretical maximum).[3] The number of bonds cleaved during proteolysis at 5° was determined from the total alkali uptake and the pK of the liberated α-amino groups.

The molecular weight of the material resulting from tryptic digestion was determined by low-speed, short-column sedimentation equilibrium at 5° and it was also examined at 35–40° after a preliminary heating step at 50° for 10 min. In some of these studies, 3-mm liquid columns were employed and the mass distribution within the column was determined by measuring the fringe displacement of a single fringe at intervals of 100 μ along the radial direction. The products of proteolysis were also fractionated by gel filtration employing columns of G-75 Sephadex equilibrated with 1 M ammonium bicarbonate. The eluted fraction containing small peptides was evaporated to a small volume and applied to a column of P-2 acrylamide gel to remove salt.

N-Terminal Analyses. Solutions of RCM *Ascaris* were dinitrophenylated in aqueous solution in a pH-Stat at 40° according to the method described by Fraenkel-Conrat *et al.* (1955). The dinitrophenylation reaction was allowed to proceed to completion (*i.e.*, to the stage where the rate of base addition owing to hydrolysis of FDNB became constant). All of the procedures were carried out in the dark or in subdued light as far as possible. Dinitrophenylated protein was separated from salt, dinitrophenol (DNP), and dinitroaniline by application to columns of G-25 Sephadex and hydrolyzed anaerobically in 6 N HCl at 110° for 16 hr. The ether-soluble DNP-amino acids were separated by two-dimensional paper chromatography employing the conventional solvent systems consisting of toluene–pyridine–2-chloroethanol–NH_4OH and 1.5 M Na-phosphate (pH 6). Spots were identified by comparison with the migration of standard DNP-amino acids along the edge of the chromatogram

[3] The lysylprolyl bonds in model peptides and several denatured proteins (see Hill, 1965) have been found to be completely resistant to tryptic hydrolysis. Considering the fact that position 1 of most triplet sequences is occupied by glycine and assuming a random distribution of arginine, lysine, and proline (including hydroxyproline) residues between positions 2 and 3 of these triplets, we would anticipate that 25% of the total arginine and lysine residues would be involved in lysylprolyl or arginylprolyl bonds and hence not susceptible to attack by trypsin. The proximity of polar groups to the trypsin-susceptible bonds also generally decreases the rate or extent of tryptic cleavage (see Hill (1965) and Rigbi as quoted by Katchalski *et al.*, 1964).

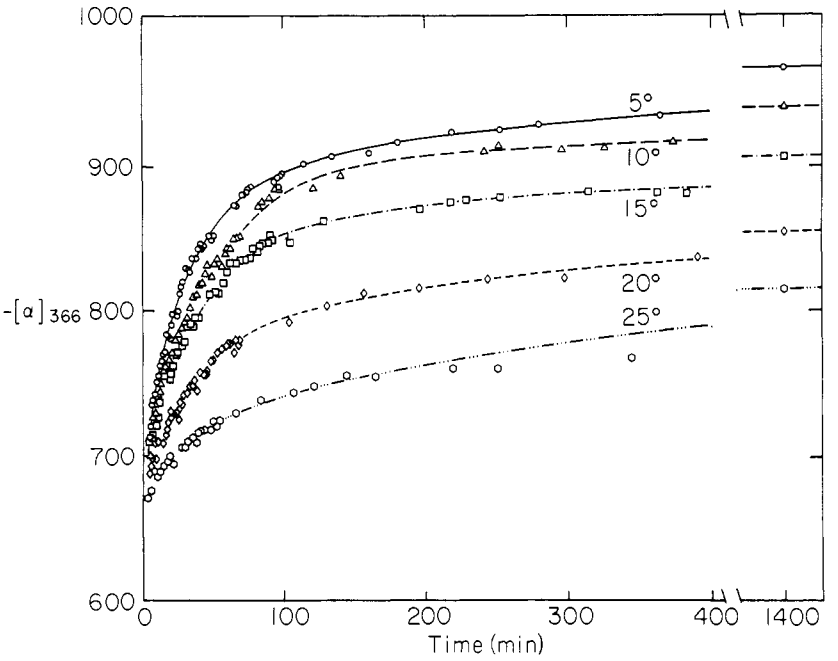


FIGURE 2: Changes in the specific rotation ($[\alpha]_{366}$) of RCM *Ascaris* gelatin (0.15 g/100 ml) in 0.2 M NaCl at various temperatures following thermal denaturation at 60° (20 min). Optical rotatory measurements were made at 366 mμ.

in both dimensions. In some instances further identification of the separated DNP-amino acids was obtained by amino acid analysis after alkaline hydrolysis in concentrated ammonium hydroxide and in other cases by rechromatography in a buffered phthalate system. The DNP-amino acids were quantitated spectrophotometrically after elution from the paper.

Water-soluble DNP-amino acids were separated either by high-voltage electrophoresis on paper in 1 M ammonium hydroxide or by paper chromatography employing a buffered phthalate system. Amino acid analysis of an aliquot of the water-soluble phase showed that hydrolysis was complete and that recovery of mass was quantitative.

Aliquots of standard DNP-amino acids were carried through the identical hydrolysis in the presence of RCM *Ascaris* followed by extraction and chromatography to determine the per cent losses. These correction values were employed to estimate the quantity of each N-terminal amino acid in RCM *Ascaris* collagen.

Results

Thermal Denaturation of Native, Reduced, and Reoxidized Ascaris Collagen. The thermal denaturation of RCM *Ascaris*, reoxidized *Ascaris*, and the native protein in 0.2 M NaCl is presented for comparison in Figure 1. The RCM and reoxidized *Ascaris* solutions were held at 5° for at least 30 days prior to measurement to allow the collagen-fold conformation to be established. After 30 days at 5° the specific levorotation of RCM *Ascaris* solutions shows that about 70% of the collagen-fold conformation of the native protein has been regenerated, while recoveries as high as 75% have been found in solutions standing for longer periods of time at low temperatures. Preparation of the reoxidized *Ascaris* collagen is described in the previous paper. Briefly, the native *Ascaris* collagen was reduced in the presence of 5 M guanidine·HCl, dialyzed at pH 2.5 to remove the reducing and denaturing agents, and reoxidation was initiated by dialysis against a pH 8.5 buffer. After standing 30 days at pH 8.5, the specific rotation of this material was virtually identical with that of native *Ascaris*. The width of the thermal transition profile of RCM *Ascaris* ($\Delta T_{1/2}$ = 11°) is, like those of other gelatins which have been studied, significantly greater than that of the native protein ($\Delta T_{1/2}$ = 5°) and the midpoint of the transition, T_m = 32°, is about 19° below that observed for native *Ascaris*. Reoxidiation of reduced *Ascaris* chains leads to an increase in T_m (T_m = 42.9°), but there is little apparent change in the width of the transition profile.

It was also of interest to examine the effect of the alkylating groups on the thermal transition. Reduction was carried out in the presence of 5 M guanidine·HCl and the system was dialyzed against a low pH buffer (0.18 M NaCl–0.05 M sodium acetate, pH 4.8) to minimize reoxidation. The thermal transition profile of this material (after standing for 4 days at 5°, pH 4.8) was closely similar to that of the RCM *Ascaris*.

Kinetics of Mutarotation of RCM Ascaris. EFFECT OF TEMPERATURE ON MUTAROTATION. Figure 2 presents a series of plots showing the time dependence of specific levorotation ($[\alpha]_{366}$) of RCM *Ascaris* solutions at various temperatures following thermal denaturation

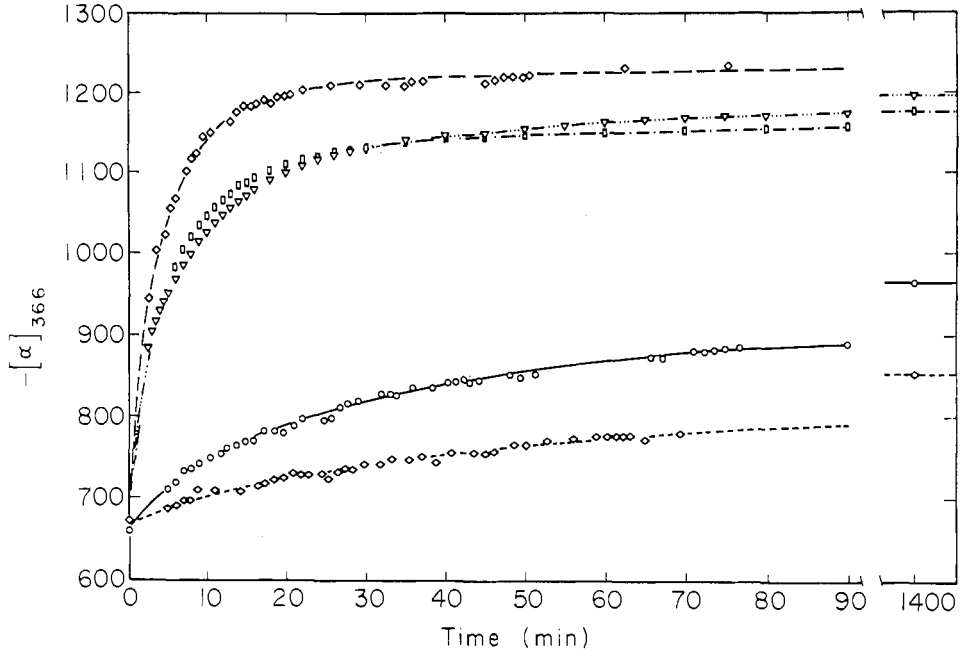


FIGURE 3: Changes in the specific rotation ($[\alpha]_{366}$) of RCM *Ascaris* cuticle gelatin at 5 (○) and 20° (◇), reoxidized *Ascaris* cuticle gelatin at 12 (▽) and 22° (□), and native *Ascaris* cuticle gelatin at 20° (◇) following thermal denaturation at 60° (20 min). Solvent was 0.2 M NaCl (pH 6). Protein concentrations were 0.9–1.5 mg/ml. Optical rotatory data relevant to reoxidized *Ascaris* was obtained at 313 mμ and corrected to 366 mμ by means of the optical rotatory dispersion constant.

at 60° (20 min). As demonstrated in Figure 2, the specific levorotation of RCM *Ascaris*[4] increases rapidly in the early phase of the reaction, but following this stage, continues to change at a much reduced rate over a prolonged period of time. This type of behavior in optical rotation has been consistently observed in all of the predominantly single-chain vertebrate and invertebrate gelatin systems which have been studied. Two other important features of the regeneration reaction are: (1) a strong negative temperature dependence of the primary phase of the reaction; and (2) the extent of renaturation after prolonged standing (>60 days) is dependent on the temperature and approximates 70–75% of the conformational rotation of the native collagen at the lowest temperature examined. All of these features stand in contrast to cross-linked native *Ascaris*. At all temperatures, even in the collagen–gelatin transition range, 85–90% of the terminal specific rotation is recovered in less than 5 min and the rate of regeneration is virtually independent of temperature. Moreover, it appears that the helix–coil transition in native *Ascaris* is completely reversible, the final specific rotation at a given temperature being the same for the melting and renaturation processes. These effects are also seen in the mutarotation of reoxidized *Ascaris*. The time dependence of regeneration of the collagen fold is shown in Figure 3 for native, reoxidized, and RCM *Ascaris* at comparable temperatures of undercooling below their transition temperatures. It will be seen that cross-linking gives a striking increase in the regeneration rate and, moreover, the negative temperature dependence observed in the noncross-linked RCM *Ascaris* is

[4] The course of mutarotation of reduced nonalkylated *Ascaris* (pH 4.85) was not significantly different from RCM *Ascaris* indicating that the alkylating groups have little effect on the reestablishment of the helical-chain conformation.

TABLE I: Kinetic Parameters Derived from Mutarotation Curves of RCM, Reoxidized, and Native *Ascaris* Collagen.

Material	Temp (°C)	Initial Rate, $d[\alpha]_{366}/dt$ (deg/min)	Half-Time[a] (min)	Kinetic Order (n)[b]
RCM-A	5	10	28	2.3
	10	8.1	32	2.0
	15	5.8	35	2.0
	20	4.0	47	1.9
	25	2.4	90	2.5
Reoxidized A	12	97	3.9	2.0
	22	88	3.7	1.8
Native	20	125	2.5	–

[a] Half-time of primary phase of reaction. [b] Derived from Van't Hoff plot (see text).

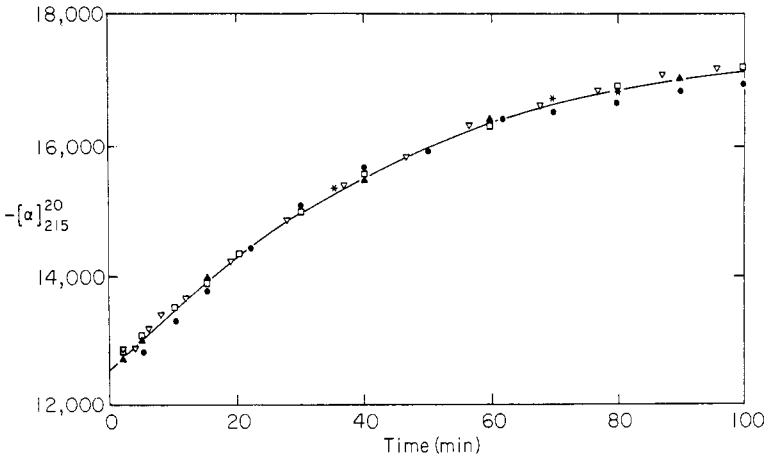


FIGURE 4: The effect of protein concentration on the rate of mutarotation of solutions of RCM *Ascaris* gelatin in 0.2 M NaCl. All optical rotatory measurements were made at 215 mμ and 20°. Protein concentrations (milligrams per milliliter) were 0.004 (□), 0.03 (*), 0.06 (▲), 0.09 (●), and 0.60 (▽).

virtually eliminated in the cross-linked (reoxidized) material. Since the melting profile and physical properties (molecular weight, reduced viscosity, and sedimentation coefficient) are grossly different between the native and reoxidized *Ascaris* (McBride and Harrington, 1967), it is apparent that the organization of cross-links in the reoxidized structure cannot be identical with that of the native structure. The kinetics of mutarotation of RCM, reduced, and reoxidized *Ascaris* at various temperatures are summarized in Table I.

A number of authors have demonstrated that the over-all mutarotation reaction of various gelatin systems appears to be a reflection of two temporally distinct processes proceeding at markedly differing rates. The initial primary phase of the reaction, which accounts for a major fraction of the change in optical rotation, is generally completed within a few hours at low temperature. During the secondary phase of the reaction the specific levorotation increases gradually over a period of many weeks. By virtue of the large difference in rates, the primary and secondary phases are clearly differentiated at low temperature. However, at temperatures approaching T_m, the two phases are less clearly defined and the total change in specific rotation of the primary reaction cannot be estimated accurately. The kinetics of the primary phase of RCM *Ascaris* mutarotation have been analyzed according to the general equation

$$\frac{d[\alpha]}{dt} = k([\alpha]_\infty - [\alpha]_t)^n$$

where n is the apparent order of the reaction with respect to the concentration of chain elements in the unfolded form, $[\alpha]_\infty$ is the specific rotation at the termination of the primary phase, and $[\alpha]_t$ the specific rotation at time t. In view of the difficulty in evaluating $[\alpha]_\infty$ at the higher temperatures, we have assumed the value reached after 1440 min for calculation of the order of the reaction, n. At this time the rate of change of mutarotation was virtually zero within the limits of experimental measurement (see Figure 2). Plots of log $d[\alpha]/dt$ *vs.* log $([\alpha]_\infty - [\alpha]_t)$ were linear over about 90% of the primary phase of the reaction giving values of $n = 2.2 \pm 0.2$ for all temperatures examined in agreement with previous studies on various gelatin species (Harrington and von Hippel, 1961a).

EFFECT OF PROTEIN CONCENTRATION ON MUTAROTATION. The order of the mutarotation reaction has been shown to be $n = 2.2$. This value is based on the concentration of elements of the polypeptide chain (as measured by rotation) in the random chain form which can be transformed into a helical structure. To determine whether this process is dependent on association of individual chains, studies have been carried out on the mutarotation at various protein concentrations at a single temperature. Figure 4 demonstrates that over the range 0.6–0.004 mg/ml, *i.e.*, over a 150-fold range in concentration, the rate of mutarotation is independent of protein concentration indicating that the order of the reaction, $n = 2.2$, refers to an intramolecular process. Rao and Harrington (1966a) have shown that the concentration independence of mutarotation extends over a 500-fold range, *i.e.*, 0.004–2.0 mg/ml. This conclusion is supported by the results presented in the previous paper (McBride and Harrington (1967), Table III) demonstrating that the helix–coil transition in RCM *Ascaris* occurs with no alteration in molecular weight.

Tritium-Exchange Studies of RCM Ascaris. The finding that the formation of the collagen-fold conformation takes place in RCM *Ascaris* without chain association raises the question of the mechanism of stabilization. If the structure formed at low temperatures in RCM *Ascaris* is the triple-helical, coiled-coil arrangement of polypeptide chains proposed for collagen by Rich and Crick (1961) and Ramachandran

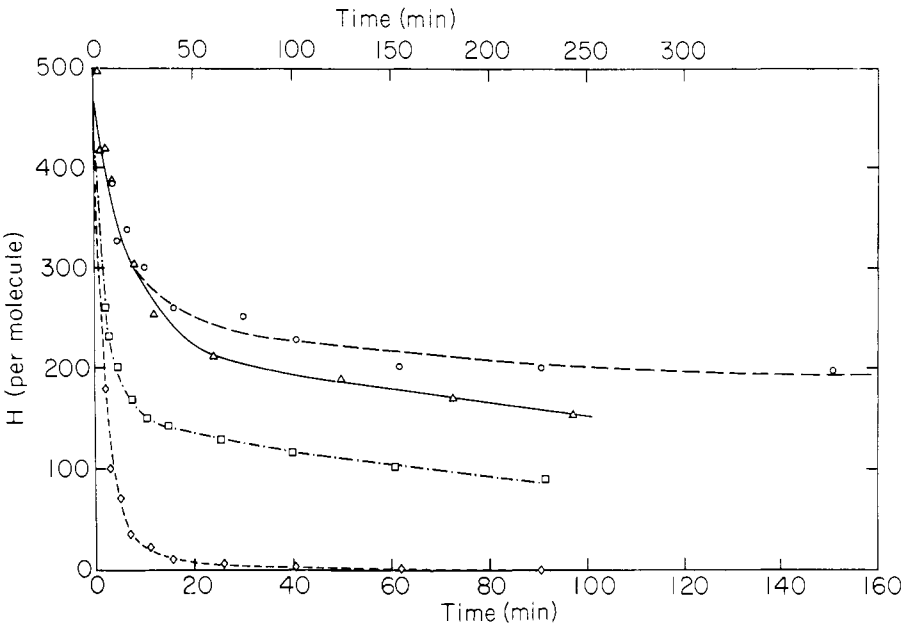


FIGURE 5: Exchange-out curves (by loss of tritium) of RCM *Ascaris* cuticle collagen at 5 (Δ) and 22.4° (□), native *Ascaris* collagen at 22.4° (○), and thermally denatured RCM *Ascaris* gelatin at 22.4° (◇) against water at pH 4.0 (0.2 M acetic acid–Na-acetate). The upper abscissa (time) refers to the experiment at 5° and the lower abscissa refers to studies at 22.4°.

et al. (1962), we would expect that part of the stabilization energy would be derived from a systematic set of lateral hydrogen bonds. Such a hydrogen-bonded, coiled-coil structure could be generated by a reverse folding of a single polypeptide chain upon itself, as suggested by Drake and Veis (1964), or could result if the 62,000 mol wt subunit were a multichain structure maintained in parallel register by a set of nondisulfide cross-linkages. In either case the renaturation of RCM *Ascaris* would be accompanied by the formation of a large number of slowly exchanging peptide hydrogen atoms.

The results of tritium-exchange studies on RCM *Ascaris* at various temperatures according to the method of Englander (1963) are summarized in Figure 5. Following a preliminary heating step (60°, 1 hr), solutions of RCM *Ascaris* in the presence of tritiated water (1 mc/ml of THO) were cooled to 5° and allowed to renature over 24–48 hr at this temperature. The solutions were then equilibrated at the temperature of the experiment for 4 hr, aliquots were applied sequentially to jacketed Sephadex columns, and the rate of exchange-out of tritium from the protein was measured as described in Methods.

It is clear from the time dependence of exchange-out of tritium from RCM *Ascaris* plotted in Figure 5 that the coil–helix transition of this material results in the formation of a large number of slowly exchanging protons. The kinetics of exchange have been analyzed according to the two analytical procedures described in Materials and Methods. Resulting plots invariably showed a nonlinear phase in the early part of the reaction suggesting the presence of more than one class of exchangeable hydrogens.

Following regeneration of the collagen fold in RCM *Ascaris*, about 150 hydrogens are found in the slowly exchanging class with half-time ($t_{1/2}$) = 115 min at 22° and about 230 with $t_{1/2}$ = 460 min at 5°. On the other hand, when RCM *Ascaris* is thermally denatured and the resulting gelatin system is applied to the Sephadex columns immediately after cooling to 22°, virtually all protons exchange in a single class with velocity constant ($t_{1/2}$ = 2 min) similar to that estimated for the fast-reaction class of both RCM renatured and native collagen at the same temperature (see Table II). It will be noted that exchange studies on gelatin systems can be carried out on both the helix and random coil forms under identical conditions since the rate of renaturation just below T_m is slow compared to the time of exchange measurements. At 5° the rates of exchange of the two reaction classes of renatured RCM *Ascaris* are sufficiently slow to permit a determination of the total number of peptide hydrogens with considerable confidence. This number (478) is in excellent agreement with the number of peptide hydrogens expected for the subunit (mol wt 62,000).[5]

Although the over-all exchange curves of RCM *Ascaris* could be analyzed as the sum of two first-order reactions, we do not wish to imply that the slowly

[5] Based on the total number of peptide bonds minus the number of pyrrolidine residues.

TABLE II: Kinetic Analysis of Hydrogen–Tritium Exchange.

Material	Temp (°C)	Class Size[a]	$t_{1/2}$ (min)	Class Size[a]	$t_{1/2}$ (min)	% Helix[c]
RCM	5	233	455	245	11.3	52
RCM	22.4	153	113	188	2.4	44
RCM[b]	22.4	6	20	335	2.0	0
Native	22.4	291	>3 hr[d]	197	2.8	100

[a] Number of hydrogens per subunit (mol wt 62,000). [b] Tritiated solution was heated to 60° (1 hr), then cooled to 22.4°, and applied immediately to a Sephadex G-25 column. [c] Estimated from specific optical rotation. [d] Sums all classes with half-lives greater than 25 min.

exchanging hydrogens belong necessarily to only a single class. Our exchange studies have utilized a one-column technique and were, therefore, carried out over a restricted length of time. Examination of the reaction over longer time intervals and at varying conditions of pH may well reveal that the hydrogen atoms exchanging in the slowest virtual class are composed of more than one group.

It was shown in earlier studies (Josse and Harrington, 1964) that all of the physical properties of the native *Ascaris* collagen are regained at low temperatures following thermal denaturation. This behavior, which has been attributed to the presence of the disulfide linkages, permits an examination of the tritium-exchange kinetics of the "native" protein following equilibration with THO under denaturing conditions (*i.e.*, at 60° for 1 hr). This situation does not obtain in most collagens and many other proteins where complete equilibration with THO cannot be obtained in the absence of irreversible denaturation. Analysis of the exchange curve of "native" *Ascaris* collagen at 22° indicates that about 290 hydrogens (/62,000 mol wt subunit) exchange with half-time of several hours. Since the per cent helical conformation (as measured by specific optical rotation) of tritiated *Ascaris* collagen at 22° is greater than 95% of that exhibited at 5°, we believe that this number is a close approximation of the slowly exchanging protons in the native structure.

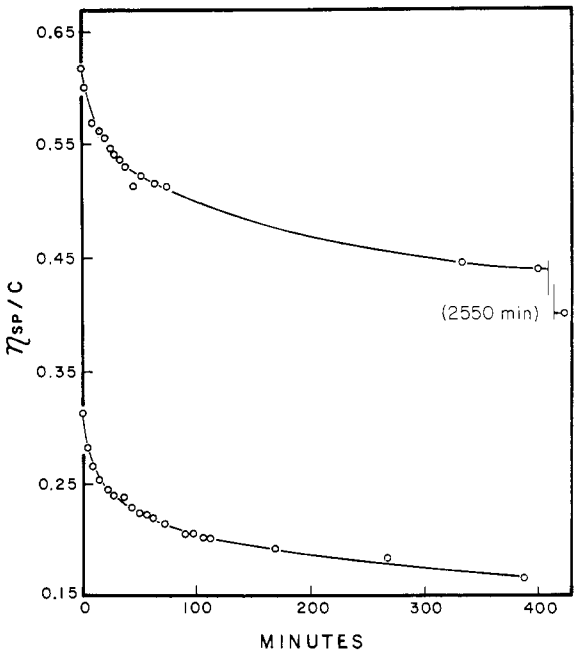


FIGURE 6: Reduced viscosity of a solution of RCM *Ascaris* cuticle collagen (0.21 g/100 ml) in 0.18 M NaCl–0.10 M Tris-HCl (pH 8.4) at 5° as function of time in the presence of trypsin (1:250, w/w, trypsin:protein). Folded, primarily helical collagen (upper curve) and heat-denatured, quick-cooled gelatin (lower curve) each subjected to proteolysis under identical conditions (see text for details).

Proteolytic Fragmentation of the Renatured and Random Chain Conformations of RCM Ascaris. The optical rotatory and tritium-exchange studies presented above have provided evidence favoring the view that RCM *Ascaris* forms a helical, hydrogen-bonded, collagen-type structure on renaturation in the absence of interchain association. Assuming RCM *Ascaris* to be a single polypeptide chain, various folding arrangements can be imagined which would account for these results. For example, reverse folding could occur over small local segments of the chain, these regions being extensively hydrogen bonded; or, one might imagine the chain to be back-folded only twice to form a single, triple-helical structure in which two chain segments are parallel and one is antiparallel. In principle it should be possible to differentiate between these extremes through a study of the fragmentation induced by the action of a proteolytic enzyme such as trypsin. It would be expected that the segmented multifolded structure would be rapidly cleaved to low molecular weight fragments in the presence of the enzyme, whereas the single, coiled-coil structure should, like the vertebrate tropocollagen molecules, be largely resistant to proteolytic attack (Gallop *et al.*, 1957; Rubin *et al.*, 1965; Drake *et al.*, 1966).

Figure 6 summarizes the time dependence of reduced viscosity (η_{sp}/c) of renatured RCM *Ascaris*

(concentration = 0.21%) at 5° following addition of trypsin. The reduced viscosity of this system decreased continuously from 0.62 to 0.41 dl/g, over a period of about 1200 min when it became invariant with time. The half-life of the over-all reaction was about 65 min. This situation stands in contrast to the changes observed during tryptic digestion of the random coil form of RCM *Ascaris*. An aliquot of the protein solution used in the preceding experiment was heated to 60° (10 min) to unfold the structure and the enzyme was added immediately after cooling to 5°. Under these conditions η_{sp}/c decreased from 0.32 to 0.15 dl/g with a half-life of 35 min. Thus the high terminal-reduced viscosity following tryptic hydrolysis of renatured RCM *Ascaris* suggests that the product of digestion is still highly asymmetric and provides evidence against the segmented multifolded model. The specific rotation, $[\alpha]^5_{366}$, of renatured RCM *Ascaris* (−1250°) before addition of the enzyme and (−1210°) after completion of the reaction indicates that the helical conformation of the polypeptide chains is essentially unaffected by proteolysis.

To assess directly the extent of proteolysis of the renatured RCM *Ascaris*, the kinetics of bond cleavage were measured in the pH-Stat. A solution of RCM *Ascaris* (0.25%) was digested with trypsin (5°, 1:250, w/w, trypsin:protein) at pH 8.44. Analysis of the kinetics of peptide-bond cleavage showed that the process was first order over greater than 90% of the reaction with half-life of about 45 min. Only 12 peptide bonds were cleaved/subunit chain. Thus, it is clear that the renatured RCM *Ascaris* structure is much less susceptible to cleavage than is the unfolded polypeptide chain in which 28 bonds were cleaved (Materials and Methods).

TABLE III: Molecular Weight of Products of Tryptic Digestion.[a]

Expt	Temp (°C)	Method	Mol Wt
I	5	Low-speed sedimentation equilibrium ($\bar{M}_W$)	52,000
	40[b]	Low-speed sedimentation equilibrium ($\bar{M}_W$)	8,000
	5	pH-Stat (M_N)	4,700
II	5	Low-speed sedimentation equilibrium ($\bar{M}_W$)	50,000
	40[b]	Low-speed sedimentation equilibrium ($\bar{M}_W$)	14,000
	5	$s^0_{20,w}$ and $[\eta]$	42,000
	5	pH-Stat (M_N)	4,800
III	5	$s^0_{20,w}$ and $[\eta]$	36,000
IV	5	Low-speed sedimentation equilibrium ($\bar{M}_W$)	13,500
	5	$s^0_{20,w}$ and $[\eta]$	13,200

[a] In expt I–III the helical form of renatured RCM *Ascaris* was digested with trypsin at 5°. In expt IV RCM *Ascaris* was heat denatured at 50° (10 min) and trypsin was added immediately after lowering the temperature to 5°; reaction was terminated after 400 min. [b] Solutions were heated at 50° (10 min) immediately before centrifugation at 40°.

Molecular weights of samples of renatured RCM *Ascaris* which had been digested to completion at 5° are presented in Table III. In these studies a twofold excess (w/w) of trypsin inhibitor was added at the termination of the cleavage reaction in the pH-Stat and an aliquot was removed and examined by low-speed equilibrium sedimentation (5°, 8225 rpm). The resulting log c *vs.* r^2 plots showed upward curvature and the weight-average molecular weight, $\bar{M}_w$, over the whole column was calculated to be about 50,000. On heating these solutions (50°, 10 min) followed by low-speed equilibrium sedimentation, the molecular weight dropped precipitously to $\bar{M}_w$ = 8,000–13,000 (Table III). Velocity sedimentation studies also demonstrated that the product from low-temperature proteolysis of renatured RCM *Ascaris* retained the sedimentation properties of a high molecular weight species. After 2500 min at 5° in the presence of trypsin (1:250, w/w, trypsin:protein) a single, asymmetric boundary with $s_{20,w}$ = 1.73 S was observed (concentration = 2 mg/ml). Undigested RCM *Ascaris* at this concentration exhibits a sedimentation coefficient ($s_{20,w}$ = 2.0 S).

Amino Acid Compositions of Proteolytic Fragments. Samples (approximately 30 mg in 10 ml of 0.2 M NaCl) of the renatured RCM *Ascaris* which had been digested to completion in the pH-stat at 5° were applied to G-75 Sephadex columns which had been equilibrated with 1 M NH_4HCO_3 at 5°. Approximately 75–85% of the mass applied to the column was eluted as a single sharp peak at the void volume (see Figure 7). The remainder of the material showed appreciable hold-up and was eluted with a broad profile in slightly less than a column volume. Tubes containing the high molecular weight component and those containing the low molecular weight peptides were pooled separately, the low molecular weight fraction was desalted by filtration through a P-2 acrylamide gel column, and the two fractions were concentrated and subjected to amino acid analysis. Results presented in Table IV show that the low molecular weight peptide material has a much lower glycine and proline content than undigested RCM *Ascaris* and has a markedly higher half-cystine content. On the other hand, the high molecular weight component shows a significant increase in glycine and proline contents and a marked reduction in half-cystine content. The high glycine content of this fragment, although still below the required 33% for a perfect collagen-type structure, is consistent with our interpretation that this segment of the renatured RCM *Ascaris* molecule is folded into the collagen conformation.

End-Group Analysis of RCM Ascaris. Evidence presented in the previous paper (McBride and Harrington, 1967) indicates that RCM *Ascaris* consists of at least two chemically distinct collagen subunits of

TABLE IV: Amino Acid Composition of Tryptic Digest of RCM *Ascaris* (residues/1000 total residues).

		Tryptic Digest	
	RCM	High Mol Wt Component[a]	Small Peptides[b]
Lys	37	35	52
His	9.7	5.2	15.5
Arg	40	34	34
Hyp	(16)	—	—
SCMC	36	22	55
Asp	65	60	92
Thr	15	13	21
Ser	16	12	43
Glu	68	67	80
Pro	308	332	214
Gly	285	305	234
Ala	63	62	72
Val	15	9.8	32
Met	6.2	7.1	5.4
Ile	12	11	13.3
Leu	16	16	27
Tyr	0.8	0.3	1.7
Phe	7.2	5.3	10.5

[a] Average of three analyses. [b] Average of two analyses.

TABLE V: End-Group Analysis of RCM *Ascaris* by the DNP Method.[a]

	I	II
Quantity of protein used in analysis (mg)	34.7	22.7
Glutamic acid (μmole)	0.081	0.063
Glycine	0.191	0.145
Proline or valine	0.121	0.123
Others (sum of 2–4 minor spots)	0.055	0.087
Sum of amino acids recovered (μmole)	0.448	0.418
Molecular weight chain (weight of protein/Σamino acids)	77,500	54,300

[a] Recovery values of individual amino acids determined by acid hydrolysis of known amounts of DNP-amino acids in the presence of RCM *Ascaris*.

identical mass (62,000). It is possible that these subunits are not single polypeptide chains but rather multichain structures held together by nondisulfide covalent linkages. Information relevant to this question has been obtained from end-group analysis.

Table V summarizes the results of N-terminal analyses of unfractionated RCM *Ascaris* according to the method of Fraenkel-Conrat *et al.* (1955). Two-dimensional paper chromatography of the DNP-amino acids from acid hydrolysates reveals the presence of three major spots (accounting for more than 80% of the total DNP-amino acids) corresponding to DNP-glutamic acid, glycine, and valine (or the degradation product of proline).[6]

Since the molecular weight of each of the subunits is identical, the sum of all N-terminal amino acids should be present in the ratio of 1 mole/62,000 g if each subunit represents a single, unbranched polypeptide chain. Although there is variation in the amounts of each end group recovered in the two experiments (Table V), the total recovery of N-terminal residues indicates a subunit weight of 55,000–75,000 in reasonable agreement with the molecular weight established by the physical methods.

It will be clear that the possibility of masked α-amino groups in *Ascaris* collagens cannot be excluded by the present studies. In this regard it should be noted that several workers have reported the absence of significant quantities of free α-amino groups in the vertebrate collagens (Bowes and Moss, 1953; Grassman and Hörmann, 1953; Joseph and Bose, 1958; Steven and Tristram, 1962). Acetylation of the N-terminal residues of these collagen chains may be responsible for these results, since Hörmann and Joseph (1965) have found approximately 6 *N*-acetyl residues/1000 residues in calf skin collagen.

Discussion

Effect of Disulfide Cross-Links on the Helix–Coil Transition. Ascaris cuticle collagen offers a particularly useful system for examining the effect of cross-linking on the thermal stability of collagen and on the regeneration process since a large number of chemically well defined cross-links are present which are susceptible to cleavage without any alteration in the primary structure of the polypeptide chains. Numerous studies of cross-linking of polymeric systems (see Flory, 1956; Mandelkern, 1964) have demonstrated that the effect of cross-links on the thermal melting temperature depends on the state of the structure at the time of introducing cross-linkages. If cross-linked networks are formed at random between randomly coiled chains, the cross-linking process does not influence the configurational entropy characteristic of the random noncross-linked chains. Such systems generally exhibit a decrease in melting temperature compared to the

[6] End-group analysis by the phenylthiohydantoin method also revealed glycine and glutamic acid as N-terminal residues, but quantitative recovery of amino acids was thwarted by incomplete cyclization of the phenylthiocarbamyl peptide to give the phenylthiohydantoin.

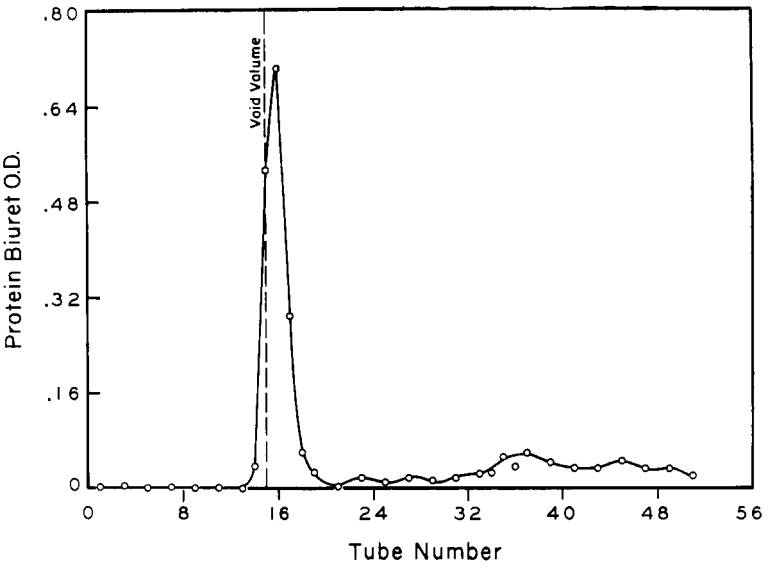


FIGURE 7: Gel filtration elution profile of a tryptic digest of RCM *Ascaris* cuticle collagen. The collagen (30 mg of RCM in 15 ml of 0.2 M NaCl) was applied to a column of 3.5-cm diameter × 93-cm height) of G-75 Sephadex (preequilibrated with 1 M $(NH_4)HCO_3$ and eluted with the same buffer under gravity at a flow rate of 1.5 ml/min and 15-ml fractions were collected. The protein concentration of aliquots was determined by the biuret reaction. The vertical dashed line indicates the void volume (elution volume of Blue Dextran 2000) of this column.

noncross-linked structure. On the other hand, if a parallel array of chains, each in an ordered conformation, is cross-linked without alteration in the crystalline structure, the entropy loss owing to restrictions in the conformational freedom of the random chains leads to an increase in the melting temperature. It has been amply demonstrated that these considerations apply to single molecules in solution as well as fibers (Scheraga, 1963). In the case of cross-linking in the crystalline state, the entropy loss, ΔS_x°, can be expressed in general as $\Delta S_x^\circ = -(3R\nu/4)(\ln n' + 3)$, where ν is twice the number of cross-links and n' is the number of statistical elements between cross-links (Flory, 1956). Thus the contribution of cross-linking to the free energy of stabilization of the ordered structure will be $\Delta F_x^\circ = -T\Delta S_x^\circ$ and the over-all free-energy change for the helix–coil transition of a structure containing n peptide units $\Delta F_{unf} = n\Delta H_{residue} - nT_m\Delta S_{residue} - T_m\Delta S_x^\circ = 0$, where $\Delta H_{residue}$ and $\Delta S_{residue}$ are the enthalpy and entropy changes per residue. Values of $\Delta H_{residue} = 1200$ cal and $\Delta S_{residue} = 4.1$ eu have been reported by Flory and Spurr (1961) based on the relationship between the tensile force and the equilibrium melting temperature of rattail tendon collagen. More recent studies on the helix–coil transitions of single gelatin chains derived from a variety of collagens give similar values. In particular, the estimated parameters for RCM *Ascaris* are $\Delta H_{residue} = 1050$ cal and $\Delta S_{residue} = 3.2$ eu (N. V. Rao and W. F. Harrington, in preparation).

Assuming a statistical element of five to nine residues[7] for RCM *Ascaris* in the random chain form and ten cystine cross-links per chain, $\Delta S_x^\circ \cong 150$ eu and the expected difference in T_m between the cross-linked and noncross-linked *Ascaris* chains would be about 25°. (A similar temperature differential has been calculated from Kühn's equation; see Schellman, 1955.) Since this estimate is based on the assumption that the cross-links are introduced into a perfectly ordered structure it must be considered an upper limit and in this connection it will be noted that cross-linking of the renatured *Ascaris* subunits (60% helix) at 5° (reoxidized *Ascaris*) increases T_m about 10°. Moreover, the calculation of temperature differential between native and RCM *Ascaris* does not take into account the difference in the degree of cooperative order in the native and renatured structures. The decrease in the size of the cooperative unit of RCM compared to native *Ascaris* is reflected in the increased width of the transition profile. Judging from the reported differences in T_m between essentially noncross-linked vertebrate collagens and their renatured single-chain gelatins (Harrington and von Hippel, 1961b; von Hippel and Wong, 1963), a reduction in T_m of 7–10° is to be expected from this source. We conclude from this discussion and the observed differential in T_m (20°) between RCM and native *Ascaris* (Figure 1), that the *in vivo* formation of the

[7] Brant and Flory (1965) have determined the characteristic dimensionless ratio, $\langle r\rangle_0^2/\eta_p l_p^2 = 9$, for a number of random polypeptide chains. However for gelatin chains with their high glycine content this ratio is decreased significantly (Flory, 1960). A value of $\langle r\rangle_0^2/\eta_p l_p^2 = 5$ has been estimated for RCM *Ascaris* from its molecular parameters (molecular weight, $[\eta]$, and the second virial coefficient).

disulfide cross-links occurred under conditions in which the polypeptide chains were already existing predominantly in helical conformation.

It has been clear for several years from the work of Altgelt *et al.* (1961) and Veis and his collaborators (see Drake and Veis, 1962, 1964) that cross-linking induces a marked elevation in the rate of regeneration of the collagen fold. A particularly striking example of this behavior is seen in Figure 2 demonstrating that the presence of the disulfide linkages results in an approximately 10–20-fold increase in the rate of renaturation of the *Ascaris* chains. Although the rate of helix regeneration in reoxidized *Ascaris* is qualitatively similar to that observed for the native protein, a smaller conformational change occurs in the primary phase of mutarotation of this material, indicating significant differences in the assembly pattern of the polypeptide chains in the native and reoxidized systems. Native *Ascaris* and the reoxidized material show little temperature dependence of mutarotation in contrast to the single-chain gelatin systems. In terms of a nucleated crystallization process (see below) the marked increase in regeneration rate and the lack of a negative temperature dependence suggests that the cross-links act to augment the stabilization of viable nuclei. That is, only a local region in the vicinity of the initiating sites need be involved, in contrast to gross chain alignment. A highly specific chain-segment alignment seems unlikely in reoxidized *Ascaris* in view of the broad thermal transition profile observed for this material.

Regeneration of the Collagen-Fold in Single-Chain Gelatin Species. Since the mechanism of regeneration of the polyproline II type helical conformation in the subunit polypeptide chains of collagen has been the subject of intensive study in recent years, it is well at this point to briefly review some of the salient experimental evidence pertaining to this process. Most of the studies heretofore reported have been carried out on gelatin solutions derived from vertebrate collagens (for a detailed discussion of early literature, see Harrington and von Hippel, 1961b; Veis, 1964) and have led to two general mechanisms for the formation of the collagen fold when these systems are cooled below the helix–coil thermal transition temperature (T_m).

As a result of an investigation of the changes in various physicochemical properties of dilute gelatin solutions following cooling below T_m, it was proposed (von Hippel and Harrington, 1959) that the regeneration of the collagen fold could be considered in terms of three structurally and temporally distinct steps: (1) nucleation of the poly-L-proline II type helix in the imino acid rich portions of the polypeptide chains; (2) growth of the polyproline II helix from the initiation sites along single polypeptide chains; and (3) specific association between the single chain helices.

Experimental support for the nucleation concept was derived from the kinetics of peptide-bond cleavage by collagenase. Above the thermal transition temperature, T_m, the action of collagenase could be described by a single first-order rate constant for all bonds split. Below T_m the kinetics of cleavage became complex, the change in the form of the kinetics developing virtually immediately on lowering the temperature below T_m and long before any significant development of the collagen fold as measured by changes in optical rotation. Since collagenase requires the specific local sequence X-Pro-Y-Gly-Pro-Z (where cleavage occurs between Y and Gly) these results suggested a rapid local conformational transition in the pyrrolidine-rich segments of the chain. The idea of a nucleated growth process is also compatible with the negative temperature coefficient of helix formation observed in all gelatin systems as was first pointed out by Flory (see Becker and Doring, 1935; Volmer, 1939; Mandelkern, 1956; Mandelkern *et al.*, 1954; Flory and Weaver, 1960).

Step 2 of the mechanism, the growth of the helix from the initiation sites, was considered to be an intramolecular process resulting in the formation of a relatively stable polyproline II type conformation since the rate of mutarotation was found to be independent of protein concentration in dilute solutions of ichthyocol gelatin and rattail gelatin (Flory and Weaver, 1960) over a wide range of protein concentrations. In all gelatin systems which have been examined, the major changes in specific rotation occur over a period of a few hours, reaching a maximum in a time interval which is sensibly independent of protein concentration for a given gelatin species. Following this primary phase in mutarotation, the specific levorotation increases at a much reduced rate for many days. On the other hand, viscosity and light-scattering studies (Engel, 1962) reveal that the molecular parameters measured by these techniques are highly concentration dependent. In dilute gelatin systems only a fractional increase is observed in the reduced viscosity and weight-average molecular weight in the time period required for completion of the primary phase of mutarotation (Engel, 1962; von Hippel, 1967).

In their analysis of the problem, Flory and Weaver (1960) have assumed the transformation process in dilute solution to be three-strand helix (tropocollagen) $\rightleftharpoons$ three random coil molecules (gelatin). They have attempted to account for the marked negative temperature coefficient of collagen-fold formation and to develop an adequate explanation for the apparent contradiction that the kinetics of reversion to form the three-stranded superhelix is first order in protein concentration. In order to circumvent this difficulty it was necessary to postulate an unstable intermediate which could be a single-strand helix or a segment of a single chain in helical conformation of the polyproline II type. The scheme may be represented as

$$C \underset{k_1}{\overset{k'_1}{\rightleftharpoons}} I \underset{k_2}{\overset{k'_2}{\rightleftharpoons}} (1/3)H$$

where C, I, and H represent the random coil, the intermediate, and the three-chain helix, respectively. The formation of intermediate was conceived as a

unimolecular rearrangement of segments of the chain into the polyproline II helix and is rate controlling. The concentration of the intermediate is considered to be at all times small compared to C and conversion to the compound helix sufficiently rapid to have no effect on the over-all rate. Thus the rate of reversion may be given as $R' = k_1'[C]$.

The essential feature of the three-step scheme (von Hippel and Harrington, 1959, 1960; Harrington and von Hippel, 1961a,b) is that the collagen fold is developed along single chains and is a structurally stable entity, whereas in the mechanism of Flory and Weaver the single-chain helix can have only a transitory existence and stabilization of the helix is accomplished through rapid association of three single-chain intermediates to form the compound triple helix.

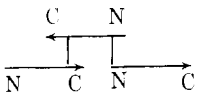
It has been difficult to establish the categorical presence or absence of a stable, single-chain helix from a comparison of the kinetics of mutarotation and those of viscosity and light scattering since denatured vertebrate collagen generally contains some covalently linked double chains (β-gelatin) and triple chains (γ-gelatin), the amount varying from 18 to 66 (β) and 0 to 10% (γ) depending on the source and method of isolation (Piez *et al.*, 1961, 1963). Moreover, in all of the gelatin systems examined heretofore, some time-dependent chain–chain association does occur over the concentration range which has been available for study.

In order to assess the relative contribution of cross-linking to the regeneration process, Piez and Carillo (1964) examined the kinetics of optical rotatory changes in single-chain (α) and double-chain (β) rat skin gelatins isolated in pure form by chromatography. Contrary to the results of Harrington and von Hippel (1961a) on ichthyocol gelatin (β = 30%) and Flory and Weaver (1960) on rattail tendon (β = 60%) they observed a significant concentration dependence of mutarotation and molecular weight of the α chains over the range 0.25–1.6 mg/ml. Helix formation (at 15°) involving pure β component proceeded at a concentration-independent rate with invariant molecular weight below 0.2 mg/ml. Their results were interpreted as evidence for stabilization of the collagen fold by interchain interactions to form double-chain helices.

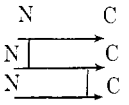
The reasons for the discrepancy between the data of Piez and Carillo and that of Harrington and von Hippel on primarily single-chain gelatin (ichthyocol) are not clear, but may well be related to differences in the temperature coefficients of the helix growth (negative temperature coefficient) and the interchain association (positive temperature coefficient) processes. Piez and Carillo have followed collagen-fold formation at 15° while the results on ichthyocol were obtained at 8°. In any event it is clear from the results presented in Figure 4 that the mutarotation of the gelatin chains of *Ascaris* is *independent* of concentration over an approximately 500-fold range. Moreover, recent studies on a number of single α-type gelatin chains isolated from various sources by the Piez *et al.* (1963) procedure demonstrates that collagen-fold formation in all of these chains, both vertebrate and invertebrate, becomes independent of concentration at low concentrations (below about 0.05 mg/ml). At higher concentrations, in agreement with the findings of Piez and Carillo, mutarotation shows significant concentration dependence. Nevertheless, it should be noted that over a 100–200-fold increase in concentration within the range 0–2 mg/ml the rates were found to be elevated by only two- to threefold (Rao and Harrington, 1966a). For a second-order process with respect to protein concentration, the reaction rate over this concentration range would be expected to change by about 10^4. In this respect it should be noted that Piez and Carillo also observed only an approximately threefold increase in the rate of mutarotation over the range 0.25–1.61 mg/ml. Thus it seems clear that the dominating reaction over this concentration range is close to first order as proposed earlier.

The question now arises as to the detailed arrangement of the chain conformation in the stable single-chain helix and the mechanism of stabilization. In the experiments referred to above on ichthyocol gelatin (Harrington and von Hippel, 1961a) it was postulated that mutarotation must be accompanied by some type of hydrogen bonding since no mutarotation was observed in the presence of high concentrations of urea and guanidine·HCl. Moreover, T_m of the melting profiles of the collagen fold generated in chilled gelatin solutions is increased significantly (3.7°) in D_2O over that in H_2O. Yet it is well known that a linear, systematic, hydrogen-bonded system is not possible along the length of the polyproline II type helix because of the steep pitch of this helix. Referring to pertinent evidence (Rougvie and Bear, 1953; Gustavson, 1956; Burge *et al.*, 1958; Bradbury *et al.*, 1958; Esipova *et al.*, 1958; Fraser and MacRae, 1959) on the structural role of water in the native collagen protofibril, Harrington and von Hippel proposed that water molecules might be involved in the stabilization mechanism forming a systematic set of hydrogen-bonded water bridges along the helix. If such a structure exists for gelatin chains at low temperature, it might also be expected that the synthetic polymer poly-L-proline II would have a similar type of stabilized solvation pattern, since the chain geometry is identical and the carbonyl oxygen atoms of the polymer are all available for solvation. Nevertheless, a recent study (Kurtz and Harrington, 1966), dealing with the form I–form II interconversion in anhydrous solvents and in D_2O and the related question of the effect of the neutral salts, has demonstrated that water does not play a unique structural role in the formation and stabilization of the linear polyproline II helices. Bensusan and Nielsen (1964) have provided additional information on this point from hydrogen-exchange studies. It would be expected that hydrogen-bonded water bridges of the type envisioned by Harrington and von Hippel should exchange at rates comparable to those found for random polypeptide chains, since the hydrogen-bonded water molecules are presumably readily accessible to the bulk solvent. However, these workers observed a more than 1000-fold reduction (below that of parent

CHART I



A, high temperature (unfolded)



B, low temperature (folded)

gelatin at zero time) in the rate of exchange of peptide hydrogen atoms during regeneration of the collagen-fold conformation on cooling calfskin gelatin from a temperature above T_m to 14°.

Bensusan and Nielsen (1964) have reported that helix formation at low temperature in rattail gelatin is accompanied by the formation of a large number of slowly exchanging peptide hydrogen atoms and the present hydrogen-exchange studies on RCM *Ascaris* yield similar results. At 5° approximately 233 slowly exchanging hydrogens were detected per subunit, *i.e.*, per 640 amino acid residues, at the end of the rapid primary phase of the renaturation process. About 52% of the helical content of native *Ascaris* is present at this stage of the reaction, judging from the specific optical rotation, and we, therefore, estimate 2.1 slowly exchanging hydrogens/three-residue helical element. When the temperature of the *Ascaris* solution at this stage of renaturation is raised to 22°, the fraction of helix is reduced to 44% and 1.6 slowly exchanging hydrogens are found/helical triplet. These results are comparable to the findings of Bensusan and Nielsen and, assuming that the slowly exchanging hydrogens are a measure of peptide hydrogen bonds, provide evidence favoring a two-bonded, Ramachandran-type collagen structure. In fact the number of slowly exchanging hydrogens is significantly larger than expected for a two-bonded model if the high proline content of *Ascaris* (29%) is taken into account. We estimate that 50% of the triplet sequences of *Ascaris* collagen should contain proline residues in position 2 (residue X in the common triplet sequence Gly-X-Y (Josse and Harrington, 1964; Rao and Harrington, 1966b)). In the Ramachandran-type structure only one hydrogen bond is possible for each triplet containing a pyrrolidine residue in position 2 since the systematic hydrogen-bonding pattern requires the peptide nitrogen atom of this position to be a proton donor. Thus the maximum number of slowly exchanging hydrogens expected in *Ascaris* would be about 1.5/triplet. These considerations suggest that the hydrogen-bonded helical conformation formed in the rapid, primary phase of the renaturation reaction is formed predominantly from segments of the polypeptide chain which can form the two-bonded pattern and which are therefore devoid of imino acid residues in position 2 of the triplets. This conclusion is consistent with the proposal (Rao and Harrington, 1966b) that the most stable helical unit would consist of doubly hydrogen-bonded triplets containing pyrrolidine residues only (and exclusively) in position 3.

Approximately 291 slowly exchanging hydrogen atoms/subunit are observed in native *Ascaris* collagen at 22°, which is equivalent to 1.4/helical triplet.[8] Englander and von Hippel (1962) have reported a similar close correspondence of the number of slowly exchanging hydrogens with that expected for the two-bonded model based on tritium-exchange studies of ichthyocol collagen. Jordan and Speakman (1965) have also found approximately two slowly exchanging hydrogens per triplet by following the increase in weight when D_2O vapor is allowed to equilibrate with calfskin collagen in the solid state. In summary then, it appears that all of the exchange studies favor a two-bonded model but this conclusion should be treated with some reservation, in view of the potential sources of error in the various methods (see Hvidt and Nielsen, 1966).

In the discussion above we have tacitly assumed that a systematic hydrogen-bonding scheme of either the Rich–Crick or Ramachandran type is possible if one of the polypeptide chains (or chain segments) of the triple helix is antiparallel. Rich and Crick (1961) have reported that a satisfactory one-bonded scheme can be obtained, apart from the possible side-chain interference, by reversing the chain sense of one chain of the complex. At the present time, it is not known if a systematic two-bonded pattern of the Ramachandran type can be formed within a triple helix in which one of the chains is antiparallel. This question is currently under study.

An alternative possibility to a single, reverse-folded polypeptide chain would be a structure in which three polypeptide segments of mol wt 20,000 are arranged as shown in Chart I (A) below with reversal of chain sense in the central segment. The three segments are assumed to be held together by linkages other than α-peptide bonds. Such a structure would have solution behavior characteristic of a single, linear polypeptide chain at high temperature, but the three segments could fold to form the standard triple-helical pattern with all polypeptide segments arranged in parallel array at low temperatures (B). It will be clear that this primary structural organization would be compatible with Gallop's (1964) proposal that the polypeptide chains of collagen are made up of structural units of mol wt 20,000–30,000 linked together by ester or imide bonds (see also Bailey and Hodge, 1965). The low-temperature structure (B) would accommodate the two-bonded hydrogen-bonding scheme of Ramachandran since all chain segments are parallel. Because of the possible presence of masked α-amino groups, structure A

[8] If we assume that only $^{28}/_{33}$ of the native *Ascaris* can be arranged in the triple-helical-chain conformation as a result of the abnormally low glycine content of this collagen (*Ascaris* has about 28% glycine), the value would be raised to 1.6. Similarly, 2.4 and 2.0 slowly exchanging hydrogens/helical triplet would be calculated for RCM *Ascaris* at 5 and 22°, respectively.

cannot be excluded at the present time (see Results).

A γ-type structure in which the individual chains of 20,000 are cross-linked together by several non-disulfide cross bridges seems unlikely in view of the regeneration rate of RCM *Ascaris* at low temperatures. Cross-linked γ-type structures would be expected to refold rapidly at low temperature, whereas RCM *Ascaris* exhibits regeneration rates characteristic of the single α-type collagen chains (Figure 2). The broad helix–coil transition profile (Figure 1) is also characteristic of renatured α chains, whereas the common γ-type cross-linked structures exhibit relatively sharp transitions.

Regardless of the specific type of hydrogen-bonding pattern assumed, our studies indicate that an organized arrangement of intrachain peptide hydrogen bonds develop in the renaturation reaction of RCM *Ascaris* and our present evidence suggests the formation of a hydrogen-bonded, triple-helical collagen-type structure by reverse folding of a single polypeptide chain. This type of structural pattern was first proposed by Drake and Veis (1964) to resolve the apparent contradiction of the formation of a triple-stranded helix in a unimolecular reaction. They pointed out that intrachain interactions are to be expected since within the domain of an average single-chain gelatin molecule the concentration of chain segments is of the order of 2 mg/ml. Thus they reasoned that segment–segment interactions will be preferred to associations between chains at low concentrations.

Engel *et al.* (1966) have also suggested that the synthetic collagen model $(\text{Pro-Gly-Pro})_n$, which they believe to be hydrogen bonded in solution, may be stabilized as a single-stranded double or triple helix through reverse folding of the chain. Their interpretation would account for the observations that (a) the end-group molecular weights are very close to the particle molecular weights, (b) particle weights are the same in water and in the denaturing agent, guanidine thiocyanate, and (c) the annealing of a labeled polymer with an unlabeled one shows no tendency to form hybrid chains.

It is clear that the high molecular weight particle remaining after tryptic digestion of renatured RCM *Ascaris* (Figure 6 and Table III) can be readily interpreted if the chain is folded back upon itself to form a double- or triple-chain unit. In contrast, the molecular weight of a single linear helix would be expected to decrease rapidly on peptide-bond cleavage since each scission would lead to two particles. Evidently the lateral secondary forces between chain segments are sufficient to maintain the structural integrity of a major fraction of the chain at low temperatures since a dramatic fall in molecular weight (from mol wt 50,000 to 8000–12,000) occurs on raising the temperature above T_m. The invariance in specific optical rotation during proteolysis at low temperature is consistent with this interpretation.

Although the tryptic digestion studies would be compatible with either a double- or triple-folded chain, hydrodynamic parameters of RCM *Ascaris* presented in the previous paper would seem to favor a conventional, triple-chain collagen-type helix. The length of a rigid rod of mol wt 62,000 consisting of a coiled coil of two polyproline II type helices should be about 1000 A; that of a triple-chain collagen-type structure about 600 A. The length of the folded RCM *Ascaris* particle at 25° assuming an equivalent prolate ellipsoid (from measurements of $[\eta]$ and M)[9] is 540 A. We also note that a two-chain helix would contain only one hydrogen bond per triple-helical three-residue element assuming a Ramachandran-type hydrogen-bonding pattern or one-half hydrogen bond per three-residue element for a systematic Rich–Crick-type pattern. The tritium-exchange results are not compatible with either of these possibilities. Moreover, calculations assuming reasonable values for $\Delta H_{\text{residue}}$ and $\Delta S_{\text{residue}}$, the enthalpy and entropy changes per mole of peptide residue, and including the restrictions to rotational freedom about the backbone linkages of the pyrrolidine residues, indicate that such an RCM *Ascaris* structure would be completely unstable at all available temperatures (Harrington, 1964; Rao and Harrington, 1966b).

Amino acid analysis of the high molecular weight fragment isolated chromatographically from the tryptic digest of folded RCM *Ascaris* demonstrates that this segment of the structure has an appropriate composition for forming nearly a perfect collagen triple helix. As was mentioned earlier, the glycine content of this region approaches that of the vertebrate collagens, while the very high proline content (330 residues/1000) should provide great thermal stability. We think it likely that the low molecular weight fragments released from helical RCM *Ascaris* arise from the loop regions and possibly also near the chain ends. The low glycine content of these segments would prevent them from participating in a systematic triple-helical structure.

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[9] Using the equation $L = 6.80 \times 10^{-8} [(\eta]M)^{1/3}(p^2/\nu)^{1/3}$ where p is the axial ratio and ν, the viscosity increment (see Yang, 1961).

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