Collagen Structure in Solution. III. Effect of Cross-Links on Thermal Stability and Refolding Kinetics*

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ABSTRACT: Comparisons of thermal stability and refolding properties are made between two cross-linked collagens (native *Ascaris* and formaldehyde cross-linked ichthyocol) and their noncross-linked counterparts (RCM-*Ascaris* and native ichthyocol). The pH dependence of the melting temperature (T_m) parallels the ionization of side-chain carboxyl groups.

Over the pH range 2.5 to 6, T_m increases $\sim 5°$ for the ichthyocol species and $\sim 12°$ for *Ascaris* collagen, the larger effect of pH on the latter material being due to the more even distribution of its ionizable residues throughout the triple-helical structure. Initial rates of renaturation of unfolded collagens showed negative temperature dependence and were studied as a function of pH at constant undercooling. All species exhibited maximum rates of refolding near their isoelectric points, suggesting that even the very earliest stages of refolding involve interaction of *several* polypeptide chain segments. The variation of initial rate with pH was small (< 2-fold), except in the case of native ichthyocol where a 19-fold change in rate was observed between pH 2.2 and 4.4. Cross-linking abolished the large pH dependence of rate in ichthyocol by covalently joining the polar (repulsive) regions of the gelatin chain. Homogeneous polar residue distribution in *Ascaris* collagen obviated such an effect of cross-linking on the pH dependence of the refolding rate.

The slow and generally incomplete refolding exhibited by denatured single-chain (α) gelatins is believed to be a consequence of the extremely small probability of three separate chains becoming aligned in proper register for formation of the triple-helical native collagen structure. At low protein concentrations (<0.1 mg/ml), where interchain interaction is virtually eliminated, the refolding process is intramolecular (Rao and Harrington, 1966; Harrington and Rao, 1970). Structural studies of such "backfolded" gelatins show broad melting profiles with T_m[1] about 10–15° below that of the parent native collagen (Harrington and von Hippel, 1961; Piez and Carrillo, 1964; Harrington and Rao, 1967). Judging from the final value of the specific optical rotation, approximately 70% of the total residues in the chain may be involved in the formation of this loosely cooperative structure. As the protein concentration is increased above about 0.1 mg/ml, interchain interactions become significant so that multistranded products are possible, with the limiting case being the formation of rigid gels where each chain interacts with *many* neighboring chains (Harrington and Rao, 1970). Melting profiles of these multistranded materials are similar to those of the backfolded gelatins, indicating that the structural imperfections are essentially independent of concentration when isothermal folding is employed. Kühn *et al.* (1964) have demonstrated that small amounts of perfect native collagen can form in cooled gelatin solutions, but temperature fluctuation between 4 and 20° was required to anneal the improperly folded regions. The same native product forms very slowly at low levels of isothermal undercooling (Beier and Engel, 1966).

Collagens which have been covalently cross-linked in the native state generally are not subject to the refolding difficulties mentioned above because the proper chain register is maintained in the denatured state. Altgelt *et al.* (1961) showed that triple-stranded γ-gelatin from calfskin collagen would refold at a far greater rate than either double-stranded β-gelatin or single-chain α-gelatin. That the rate increase was a consequence of the natural cross-linkages in the γ-gelatin was proved by Veis and Drake (1963) who prepared synthetically cross-linked ichthyocol collagen and showed it to refold 30 times faster than the noncross-linked material. The resulting product was virtually identical with the native collagen in both thermal stability and specific optical rotation. Similar findings were reported by Josse and Harrington (1964) and McBride and Harrington (1967b) for the collagen from *Ascaris* cuticle. Native *Ascaris* collagen, which is naturally cross-linked by disulfide bonds, can be completely refolded after denaturation; single-chain *Ascaris* gelatin, prepared by reduction and carboxymethylation of the disulfides (RCM-*Ascaris*), exhibits the slow and relatively imperfect refolding characteristic of all α chains.

In the following three papers, the mechanism of refolding of cross-linked collagen has been examined in detail. First, comparisons are made between two cross-linked species (native *Ascaris* and formaldehyde cross-linked ichthyocol) and their noncross-linked counterparts (RCM-*Ascaris* and native ichthyocol). The role of the cross-links in the pH and temperature dependence of the refolding reaction is examined. Second, the thermal stabilities of the refolded

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[1] Abbreviations used are: T_m, midpoint of thermal transition; Gd·HCl, guanidine hydrochloride.

gelatins are analyzed as a function of the reaction conditions. Annealing of imperfectly structured regions is demonstrated. Finally, a kinetic mechanism is proposed for the refolding of cross-linked collagens. Discussion of the mechanism concerns the pH and temperature dependence of those component reactions, "nucleation," "growth," and "annealing," which are found to comprise the total process. Remarkable similarities exist between the refolding mechanism for single-chain α-gelatins (Harrington and Karr, 1970) and cross-linked gelatins, thus supporting the view that collagen-fold formation, and probably all polypeptide folding, proceeds by intramolecular and intermolecular nucleated crystallization.

Materials and Methods

Purified ichthyocol was prepared from swim bladders of the carp (*Cyprinus carpio*) according to the method of Gallop and Seifter (1963). This material is henceforth referred to as native ichthyocol or, in the event of denaturation, as native ichthyocol gelatin. Cross-linked ichthyocol was prepared at 4° from native ichthyocol (c = 1.5 mg/ml) by reaction with 0.1% formaldehyde at pH 8.2 as described by Drake and Veis (1964).

Ascaris lumbricoides (var. *suis*) from hog intestines were obtained at a slaughterhouse (Schluderberg-Kurdle Co., Baltimore, Md.). All preparative procedures were at 4° unless otherwise indicated. The worms were washed and then frozen rapidly by placement in a stainless steel beaker surrounded by Dry Ice–2-propanol. After thawing in tap water at 4° for 12–24 hr, the cuticles were removed surgically and washed exhaustively in water. Cuticles prepared in this fashion were exceptionally clean and showed no contamination by muscle fibers. Native *Ascaris* collagen was prepared from the cuticles by the method of Josse and Harrington (1964).

RCM-*Ascaris* collagen was prepared in large quantity by a newly devised procedure based on the knowledge that the amino acid composition of whole *Ascaris* cuticle is essentially the same as that of the soluble collagen derived from it (Watson and Silvester, 1959; McBride and Harrington, 1965). Homogenized cuticles (100 g wet weight), which had previously been extracted for native *Ascaris* collagen, were suspended in 2 l. of 1% β-mercaptoethanol–0.5 M NaCl (pH 8.0), and stirred for 12 hr. Following centrifugation to remove residual cuticles (15,000g, 20 min), the extract was brought to 30% saturation with ammonium sulfate (saturated, pH 8.0). The heavy white precipitate (170 g wet weight) was collected after 2 hr by centrifugation and redissolved in 1.5 l. of 1% β-mercaptoethanol–0.5 M NaCl (pH 8.0). This solution was then brought to 30% saturation with ammonium sulfate, and the precipitate was collected as before. Exhaustive dialysis of the second ammonium sulfate precipitate against water resulted in a gray-white gel (8.6 mg of protein/g) which hardened over a period of days as the sulfhydryl groups oxidized to disulfide cross-linkages. The fully reduced gel contained 31.5 SH groups/1000 residues (mean residue weight = 93.6 g/mole). The gel was reduced and carboxymethylated at a protein concentration of 2.5 g/l. following the procedure of Crestfield *et al.* (1963) as described by McBride and Harrington (1967a). After dialysis against 40 volumes of 0.05 M ammonium acetate, the solution was dialyzed for 1 week against many changes of water. The water clear solution was centrifuged (70,000g, 1 hr) and lyophilized, yielding about 4 g of fluffy, white RCM-*Ascaris* collagen. This material was identical with that of McBride and Harrington (1967a) by amino acid analysis, sedimentation coefficient, and chromatographic behavior on CM-cellulose, where two gelatin peaks of approximately equal size were observed. The unretarded gelatin material was dialyzed against 0.01 M Tris buffer, pH 7.5, and rechromatographed on DEAE-cellulose (0.01 M Tris to 0.1 M Tris–0.5 M NaCl gradient elution pH 7.5) from which it was eluted as a single peak. RCM-*Ascaris* collagen used for melting and renaturation studies consisted of the normal mixture of the two types of gelatin chains described above.

Lyophilized collagens and gelatins were readily dissolved in 0.001 N HCl and then dialyzed extensively against the appropriate buffer. Stock protein solutions were prepared at concentrations of 0.5–2.0 mg/ml and stored at 4°; all protein solutions were centrifuged for 1 hr at 70,000g before use. Dilutions of stock solutions were prepared by weighing out the desired amount of protein stock and mixing with the required weight of fresh buffer.

Two solvent systems were used in this study. All experiments with native and RCM-*Ascaris* collagen were done in 0.2 M NaCl–0.009 M citrate (pH 2.6 to 7.2). While it would have been desirable to use the same solvent for ichthyocol, this was impossible because of the heat precipitation characteristics of this protein. It was found that 0.1 M citrate buffer (pH 2.2 to 6.0) abolished heat precipitation, hence this solvent was used for native and cross-linked ichthyocol. The pH of buffers and protein solutions was measured at 25° with a Radiometer Model 4 pH meter standardized with Beckman buffers. Temperature dependence of pH of the citrate buffer system was found to vary in sign and magnitude; it was small ($\partial pH/\partial T \leq 0.002$/deg) and was therefore ignored in the present study.

All chemicals were reagent grade. Water was glass distilled. Gd·HCl was prepared from guanidine carbonate by the method of Anson (1941). Protein concentration was determined by the microbiuret method of Zamenhof (1957), where [protein] = $(OD_{310} - OD_{390})$/(color factor). Optical density was measured against appropriate solvent blanks using a Zeiss PMQII spectrophotometer. A color factor of 1.11 ml/mg was determined for ichthyocol by micro-Kjeldahl analysis (Miller and Houghton, 1945), assuming a nitrogen content of 19.52%. For native *Ascaris* collagen and RCM-*Ascaris* collagen, the color factor of 1.12 ml/mg determined by McBride and Harrington (1967a) was assumed. Protein concentrations in 5 M Gd·HCl were calculated from the density data of Kielley and Harrington (1960) after addition of a weighed amount of the solid salt to a collagen solution of known concentration.

Agarose gel electrophoresis of ichthyocol and *Ascaris* gelatins was done at various pH values according to the method of Wieme (1964). The agarose (Nutritional Biochemical Co., electrophoretic grade) concentration was 0.8% in 0.2 M NaCl–0.009 M citrate (pH 3.28 to 7.52). Gel slabs were prepared at a thickness of 1.6 mm on 8 × 10 cm glass plates. Microliter volumes containing 5–15 μg of protein or blue dextran (Pharmacia) in the same solvent as that of the slab were applied to 8-mm slits in the gel. Electrophoresis conditions were the following: 25 V (4 V/cm), 80 mA, T =

20° ± 2°, t = 40–60 min. Gels were fixed in 12.5% trichloroacetic acid for several hours and then stained with coomassie brilliant blue R-250 (0.02% in 12.5% trichloroacetic acid). Migration was estimated (±0.1 mm) with a calibrated occular. Blue dextran moved toward the cathode at a pH-independent velocity of 0.9 mm/100 min; this velocity was assumed to be a measure of the endosmotic flow and was subtracted from the gelatin mobilities. Electrophoresis was performed for at least three different time intervals at each pH; observed mobilities were sensibly independent of time.

Optical rotation was measured with the Cary Model 60 spectropolarimeter. Wavelength was calibrated at the 252.9-mμ emission line of the 500-W xenon arc. Verification with standard sucrose solutions (National Bureau of Standards) showed that absolute values of optical activity could be read directly from the recording chart. The sample solution (9.5 ml) was contained in a coaxially jacketed cell of 10.0-cm path length (Opticell, Brentwood, Md.). Temperature was controlled to within ± 0.01° in the range of 0–40° and to within ±0.05° above 40° by circulation of water from a thermostatted bath through the cell jacket. Rapid temperature changes during the course of an experiment (as, for example, in the quenching of a hot gelatin solution to initiate mutarotation) were achieved by a valving system between two constant-temperature baths; the cell jacket temperature could be changed from 80 to 0° in about 5 sec. For the typical procedure of 60–80° melting followed by 0–20° quenching, the sample solution approached the new temperature asymptotically to within 5° at 50 sec and 1° at 100 sec.

Specific optical rotation was calculated by the familiar expression

$$[\alpha]_{313}^{T} = \frac{100\alpha_{313}^{T}(\text{obsd})}{lc}$$

where l is the optical path length in decimeters, and c is the protein concentration in g/100 ml. Optical rotation data obtained in 5 M Gd·HCl were corrected for refractive index (relative to 0.2 M NaCl) by interpolation of the wavelength dependence of refractive index tables of Fasman (1963) and the relationship

$$[\alpha]_{\lambda} = \frac{3}{n_{\lambda}^{2} + 2}[\alpha]_{\lambda}(\text{obsd})$$

No refractive index corrections were made for the dilute salt solvent systems which were normally employed.

Most measurements were carried out at protein concentrations of 0.08–0.15 mg/ml. Observed rotations of such solutions were in the range −0.05 to −0.30°. Overall stability of the instrument was checked by measuring solvent base lines before and after each sample measurement. Even for the longest measuring intervals of several days, base-line drift was less than ±0.0003°; for shorter periods it was usually unobservable (<±0.0001°). Fundamental to the accuracy of the measurements was the stability of the birefringence of the jacketed cell. The 10.0-cm cell was chosen because it was devoid of birefringence hysteresis. Although the solvent base line shifted with temperature (total change = 0.0010° from 0 to 80 °C), it was always constant (±0.0001°) at any given temperature.

Thermal stability of collagen and renatured gelatin was measured in two separate ways, yielding either equilibrium or nonequilibrium melting curves. Equilibrium measurements were obtained on single samples. The temperature of the sample was raised incrementally and maintained at a constant value until no further change in optical rotation was observed. Nonequilibrium melting curves required the use of many separate samples of each collagen or refolded gelatin solution. Each sample was placed in the jacketed cell at low temperature (usually 0–5°), well below T_m. After measurement of the optical rotation at this temperature for 10 min, the temperature was rapidly shifted to a new value as previously described. Decrease in levorotation was recorded for a period of 20–60 min, after which the sample was discarded. Sets of curves indicating the kinetics of melting were obtained at temperatures spanning the region of T_m. By plotting the optical rotation values at a constant time of melting (*e.g.*, 30 min) against the temperature, a melting curve could be generated from such data. Because the melting was incomplete in such short times, the melting temperature so derived was greater than the equilibrium T_m. The deviation for melting times of 3, 10, 30, and 100 min was 2.5, 1.8, 1.4, and 0.9°, respectively, for ichthyocol at pH 5.95. Melting temperature is defined by the following expression, where, at $T = T_m$

$$[\alpha]^{T} = \frac{1}{2}([\alpha]^{T}(\text{collagen}) + [\alpha]^{T}(\text{gelatin}))$$

The width of the thermal transition, ΔT_t, is defined as the temperature increment between 25 and 75% of the total transition. Assuming a linear relationship between optical rotation and helical content, "per cent helix" was calculated by the relationship

$$\%\ \text{helix} = \frac{100\,[[\alpha]^{T}(\text{obsd}) - [\alpha]^{T}(\text{gelatin})]}{[[\alpha]^{T}(\text{collagen}) - [\alpha]^{T}(\text{gelatin})]}$$

Refolding studies were carried out with solutions of collagen which had been melted directly in the polarimeter cell. Letting T_d be the denaturation temperature (well above T_m) and T_r the temperature of refolding, optical rotation of both the collagen solution and the solvent was measured according to the following schedule: 600 sec at T_r, 600 sec at T_d, then rapidly to T_r, with recording of the refolding kinetics commencing 20 sec after switching to T_r. Generally, renaturation reactions were followed for anywhere from 10,000 sec (native *Ascaris* collagen) to 100,000 sec (cross-linked ichthyocol). Superimposed on the observed renaturation curve was the perturbation due to density changes in the solvent during quenching, but this artefact could be subtracted, and the true renaturation curve was thereby obtained. Reproducibility of such renaturation curves was better than ±0.0003° (±0.1 to ±0.6%) over the time period 10,000–100,000 sec.

Results

pH Dependence of Melting Temperature. von Hippel (1967) has pointed out some of the dangers of interpreting melting curves obtained with rapid heating rates. To assess the presently used method of nonequilibrium melt curves,

a study of T_m *vs.* time of heating was carried out for ichthyocol at pH 5.95. The observed T_m was less than 2° higher than the equilibrium T_m of 32.2°, as long as the heating time was at least 10 min at each temperature. The chosen time of 20–30 min was judged to be quite satisfactory for determination of meaningful T_m values.

Decrease in optical rotation of dilute native ichthyocol solutions was followed for 30 min or more under various conditions of pH and temperature. It was found that the thermal stability of native ichthyocol is extremely sensitive to pH in the region of T_m. For example, after 30 min at 30.0°, ichthyocol at pH 2.16 had lost more than 75% of its helical content, while at pH 5.95 less than 6% of the structure had been destroyed. This procedure of using melting kinetics in the measurement of T_m is far more sensitive to small changes in T_m than the equilibrium melt curve method. Melting temperatures for native ichthyocol as a function of pH are presented in Figure 1 along with similar data for cross-linked ichthyocol. The behavior of the cross-linked ichthyocol is identical with that of the native material, except that its thermal stability has been increased 1.8 ± 0.2° (30-min T_m values) or 1.4° (equilibrium T_m values) over the entire pH range studied. Veis and Drake (1963) observed a 1.4° increase in T_m at pH 2.8 after introduction of methylene cross-links into native ichthyocol. There are about ten such cross-links introduced per molecule (300,000 daltons) by reaction with formaldehyde. This value has been measured independently by Veis and Drake (1963) who observed the decrease in reactive ε-amino groups of lysine, and by von Hippel (1967) who measured the stable incorporation of [^{14}C]formaldehyde into ichthyocol. The location of the methylene cross-links would appear to be between amino groups and amide or guanidyl groups (Veis and Drake, 1963). *Increases* in T_m are expected when cross-links are introduced into those regions of an axially ordered polymer structure which participate in the melting and crystallization processes (Mandelkern, 1964). The observed T_m increase is in rough agreement with calculations based on the theories of Flory (1956) and Kühn and Majer (1956) (see Hauschka, 1969).

From the investigations of McBride and Harrington (1967a,b) it is known that the carboxymethylated *Ascaris* collagen subunit RCM-*Ascaris* can undergo mutarotation to form a cooperative, collagen-fold structure by an entirely intramolecular process. The structure which is formed is similar to the collagen-fold developed by α chains of vertebrate collagens at low concentrations. This similarity extends to the melting profiles of the regenerated structures. In all cases the transition is rather broad ($\Delta T_t \sim$ 7–12°) and is centered about 10–20° below the T_m of the parent native collagen. Before examining the effect of pH on the thermal stability of RCM-*Ascaris* collagen it was necessary to obtain material with a large helical content. This was done by storing a fresh solution of the protein (c = 1.4 mg/ml in 0.2 M NaCl, pH 5.5) at 4° for 2 weeks, by which time approximately 87% of the native *Ascaris* collagen laevorotation had been regenerated. In this case the melting curves were plotted using the 20-min values of $-[\alpha]^T_{313}$ (Figure 2). While the shapes of the melt curves are identical, with a uniform transition width, ΔT_t = 11°, the midpoint T_m values vary by more than 10° with changes in pH. By comparison with the equilibrium T_m of 32.0° for RCM-*Ascaris* collagen in 0.2 M NaCl, pH 6, measured by McBride and Harrington (1967b), it is estimated that the 20-min T_m values are higher than the corresponding equilibrium T_m by 2.8°.

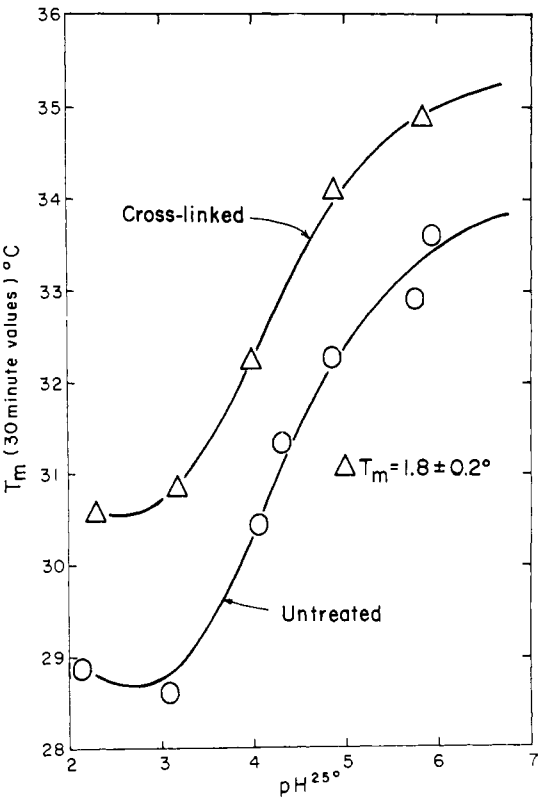

FIGURE 1: pH dependence of the melting temperatures of native and cross-linked ichthyocol: protein concentration = 0.080 mg/ml in 0.1 M citrate.

Equilibrium melting curves of native *Ascaris* collagen are presented in Figure 3. Because of the uncertainty in estimating $-[\alpha]^T_{313}$ for the fully denatured native *Ascaris* gelatin (required for determination of T_m), it was necessary to measure the rotation as a function of temperature in 5 M Gd·HCl. This was assumed to be the gelatin base line for calculation of T_m by the midpoint method. Variation of the thermal stability of *Ascaris* collagen with pH (Figure 4) is in line with previous findings for other collagen systems; an inflection point ($\partial^2 T_m/\partial pH^2 = 0$) occurs at pH 4.0. The magnitude of the change in T_m with pH for native *Ascaris* is the largest [T_m(pH 6) − T_m (pH 2.5) = 12.3°] of all the collagens studied. Native *Ascaris* collagen is considerably more stable than its noncross-linked counterpart RCM-*Ascaris*. Part of the 20° difference in *equilibrium* T_m values may be attributed to imperfections in the RCM-*Ascaris* collagen structure, because melting studies can only be done on refolded material. Also, the stabilization caused by the presence of disulfide cross-links (16 S–S/1000 residues) is probably about 10° (see McBride and Harrington, 1967b). That the ΔT_m for cross-linked ichthyocol is only 1.4° (Figure 1) agrees with the fivefold lower degree of cross-linking in this collagen compared to native *Ascaris*.

In Figure 5, differentiated native *Ascaris* collagen melt curves for pH 2.58 and 7.15 are plotted. The *Ascaris* curves differ from normal collagen (Kühn *et al.*, 1964) and cross-

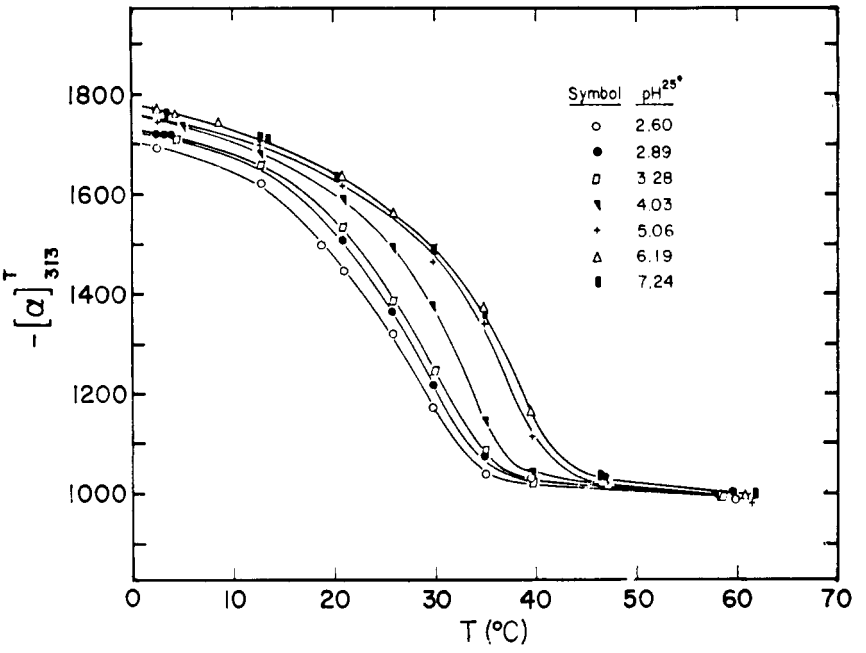

FIGURE 2: Melting curves of refolded RCM-*Ascaris* gelatin. The protein was allowed to fold at 4° (c = 1.5 mg/ml in 0.2 M NaCl, pH 5.5) for 2 weeks prior to this experiment. Twenty-minute values of specific rotation are plotted on the ordinate; protein concentration = 0.140 mg/ml in 0.2 M NaCl–0.009 M citrate.

linked ichthyocol (Figure 5c) only at high temperatures (greater than T_m + 5°), where they should have followed the course shown by the dashed lines in returning to the "random chain" base line. Integration of these curves gives the total change, in degrees of specific rotation, for the thermal transition. Amounts of noncooperative structure were calculated from the shaded areas. The cooperative collagen-fold structure of native *Ascaris* contributes about 77–88% of the observed structural levorotation (above the random chain rotation), while the remaining 12–23% results from noncooperative structure. As the pH was lowered from 7.15 to 2.58, about half of the noncooperative structure

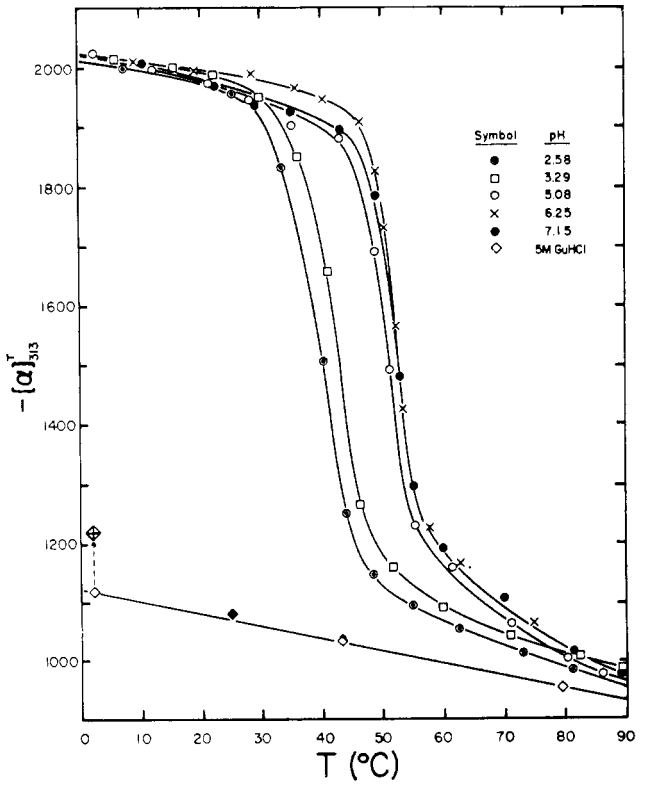

FIGURE 3: Equilibrium melting curves of native *Ascaris* collagen; protein concentration = 0.107 mg/ml in 0.2 M NaCl–0.009 M citrate. Specific rotation in 5 M Gd·HCl (◇) agrees with the value (◆) measured by Josse and Harrington (1964); mutarotation occurred at 2° in 5 M Gd·HCl to the extent of about 10% helix over a period of several hours (⊕).

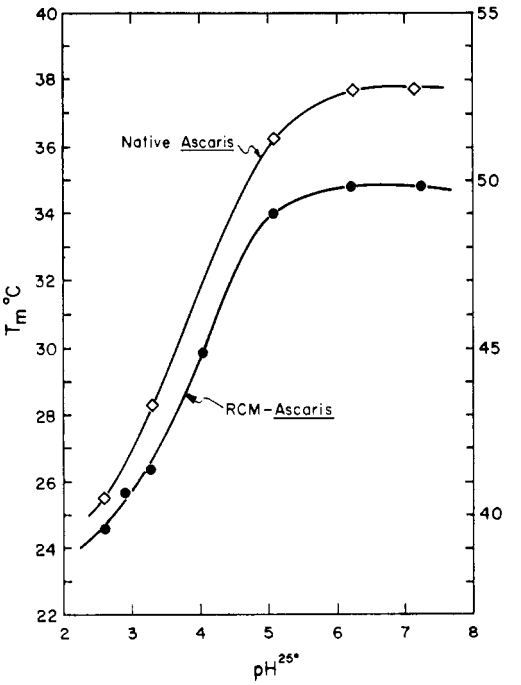

FIGURE 4: pH dependence of the melting temperature of RCM-*Ascaris* and native *Ascaris* collagen: left ordinate, 20-min T_m values for RCM-*Ascaris* from Figure 2; right ordinate, equilibrium T_m values for native *Ascaris* collagen from Figure 3.

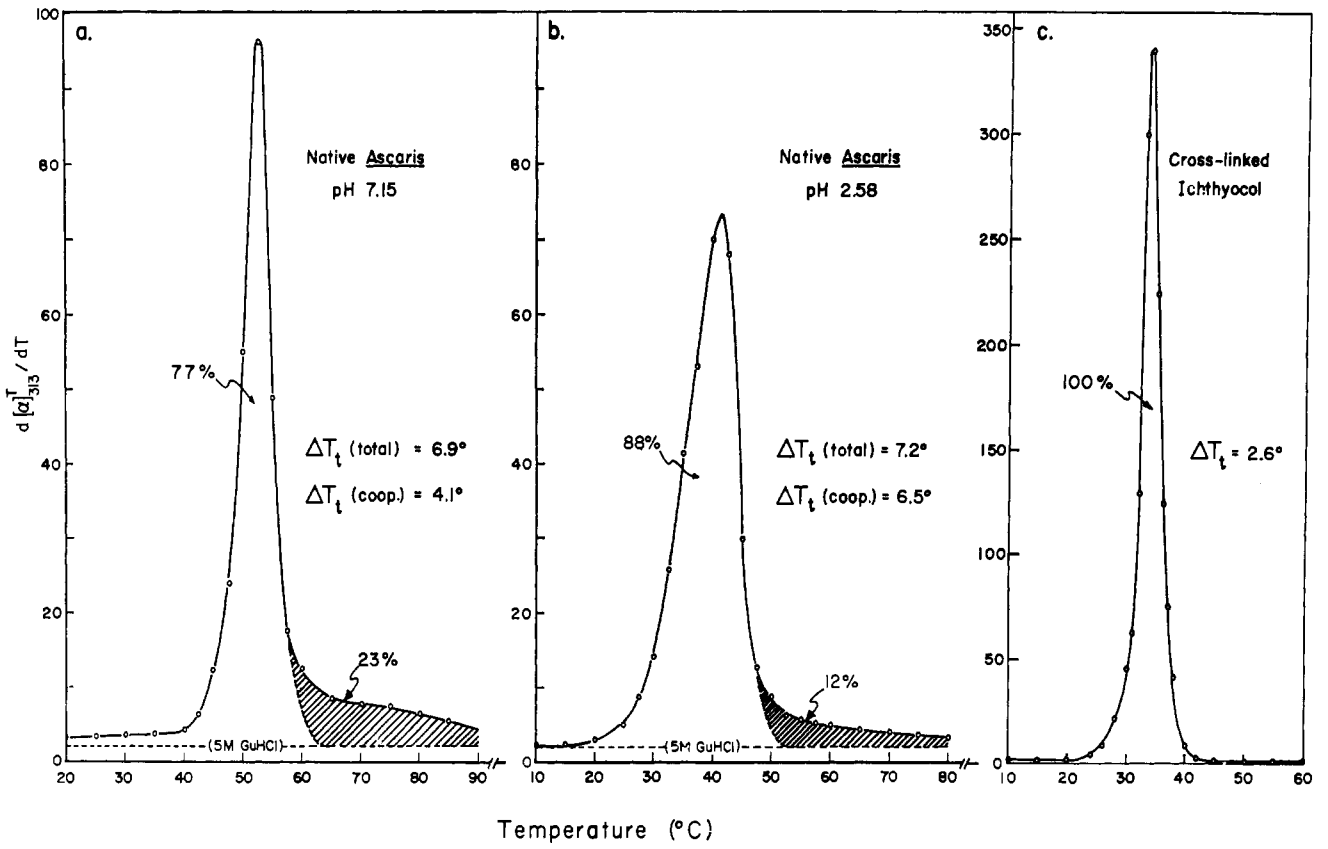

FIGURE 5: Differentiated equilibrium melt curves: (a) native *Ascaris* collagen at pH 7.15; (b) native *Ascaris* collagen at pH 2.58 (same conditions as in Figure 3); (c) cross-linked ichthyocol at pH 5.95, 0.1 M citrate, c = 0.080 mg/ml. Curves in a and b were extended to the gelatin baseline (5 M Gd·HCl) in accordance with the shape of the symmetrical, "100% cooperative" curve in c. Shaded regions represent melting of noncooperative structure.

was destroyed. Furthermore, a broadening of the differentiated melt curve at low pH may be clearly seen in Figure 5. While the width of the overall thermal transition increases only slightly (ΔT_t = 6.9° at pH 7.15, and ΔT_t = 7.2° at pH 2.58), the cooperative portion is markedly broadened at low pH (ΔT_t = 4.1° at pH 7.15, and ΔT_t = 6.5° at pH 2.58). Following the treatment of Flory (1961) and Harrington and Rao (1967), eq 1 may be used to compute the average size of the "cooperative unit," $\sigma^{-1/2}$, which is destroyed by thermal denaturation, provided that $\sigma \ll 1$. In eq 1, R is the gas constant, T_m is in degrees Kelvin, ΔH_{res} is the enthalpy change per residue upon denaturation, and ΔT_t is the thermal transition width. Lowering the pH from 7.15 to 2.58 results

$$\sigma^{-1/2} = \frac{2RT_m^2}{\Delta H_{res}\Delta T_t} \quad (1)$$

in a decrease in the average size of the cooperative unit from 97 to 57 residues. Polar residues are therefore implicated in both types of structures. Protonation of these residues destroys the noncooperative structure, and apparently causes an increased interruption frequency of the helical segments comprising the cooperative structure.

The dependence of T_m on pH (Figures 1 and 4) and the location of the inflection point at about pH 4 is an observation which is not unique to ichthyocol and *Ascaris* collagens. Burge and Hynes (1959), Dick and Nordwig (1966); Bianchi *et al.* (1967); and Woodlock and Harrap (1968) have published similar findings for several soluble vertebrate collagens. The shape of the curves in Figures 1 and 4 is not unlike the titration curves of collagen over this pH range (see Bowes and Kenten, 1948). In the titration data, the inflection point represents the pK_a of glutamic and aspartic acid side-chain carboxyl groups. It is probable that the value of T_m is controlled, in part, by the state of ionization of the free carboxyl groups. In ichthyocol, for example, there are about 80 carboxyls per 1000 amino acid residues. Below the isoelectric pH (about 5.2), ichthyocol bears a net positive charge as the carboxyl groups are neutralized and the protonated basic groups (53 Arg, 26 Lys, and 4 His per 1000 residues) dominate. As the net charge builds up, electrostatic repulsion between charged residues favors unfolding of the collagen structure to the randomly coiled gelatin. Similar arguments apply to the *Ascaris* system. Because there is apparently little or no involvement of polar side chains in intramolecular hydrogen bonding (Ramachandran, 1967; von Hippel, 1967), the major interactions between these charged groups are of the coulombic and dipole–dipole type. McLarin and Lewis (1950) have shown that electrostatic repulsion can generate large disruptive potentials in polypeptide chains. The helix → coil transition of polyglutamic acid is caused by such disruptive forces. Electrostatic arguments have been invoked by previous authors in considering pH effects on collagen stability (Burge and Hynes, 1959; Dick and Nordwig, 1966).

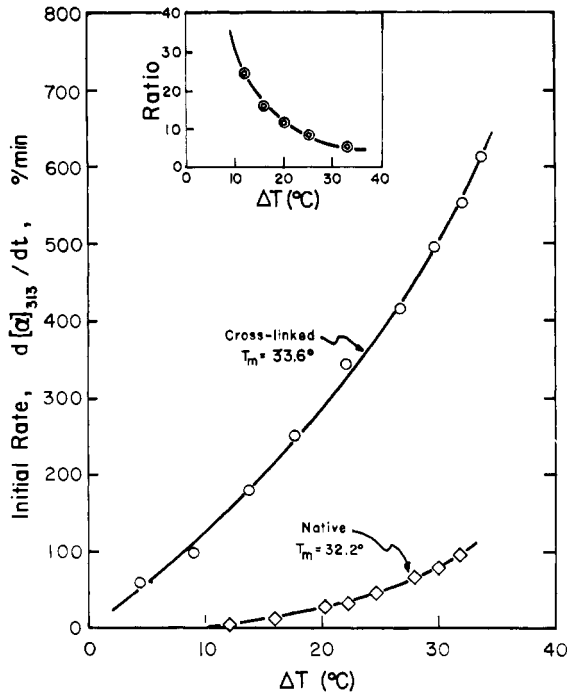

FIGURE 6: Effect of cross-linking on the initial refolding rate of ichthyocol (c = 0.080 mg/ml in 0.1 M citrate, pH 5.93). ΔT is the difference between T_m and the refolding temperature. Inset: ratio of cross-linked to native initial rates as a function of ΔT. Collagens were melted at 40° for 10 min before refolding.

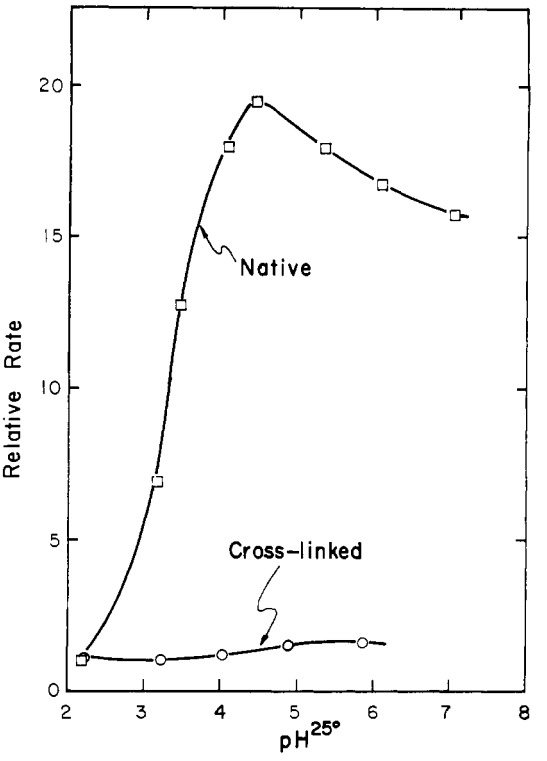

FIGURE 7: pH dependence of ichthyocol gelatin initial refolding rates at constant undercooling. Native ichthyocol was refolded at $T\Delta T$ = 7000 deg² ($\Delta T \sim 25°$); cross-linked ichthyocol was refolded at $T\Delta T$ = 2950 deg² ($\Delta T \sim 10°$). Protein concentration = 0.080 mg/ml in 0.1 M citrate, pH 5.93. Collagens were melted at 40° for 10 min before refolding. A relative rate of 1.0 corresponds to an absolute initial rate of 2.1 deg/min for native ichthyocol and 67 deg/min for cross-linked ichthyocol.

pH Dependence of Refolding Rates. Determination of the effect of pH on T_m, while interesting in its own right, was merely a prerequisite for carrying out experiments on the effect of pH on collagen renaturation. Because of the strong negative temperature dependence of the initial rate of the refolding reaction (Flory and Weaver, 1960; von Hippel and Harrington, 1960), comparison of refolding rates in different solvent systems cannot be done at a single temperature. Variation of the initial rate with temperature is described by the Flory–Weaver equation

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

where A and B are constants and $\Delta T = T_m - T$ is the degree of "undercooling." Clearly, any change in T_m brought about by a different solvent will alter the observed refolding rate through the ΔT term. pH has been shown to exert a strong effect on T_m, consequently all investigations of the effect of pH on the initial refolding rate were carried out at constant $T\Delta T$ (±2%). Initial refolding rates were determined from the slopes of tangents to the mutarotation curves drawn at the earliest time where accurate comparison was possible; this time was 60 sec for all gelatins except the extremely rapid native *Ascaris*, where 150-sec tangents were used.

It is well known that under identical solvent conditions, the gelatins of cross-linked collagens will refold much faster than their noncross-linked counterparts (Altgelt *et al.*, 1961; Drake and Veis, 1964; McBride and Harrington, 1967b). This acceleration results from a positioning effect, whereby the polypeptide chains are held together in register by the covalent cross-links. Proper positioning increases the probability of viable intersegment interactions which can lead to collagen-fold formation. Cross-linking also decreases the entropy change for the entry of a residue into a helical segment. Nucleation theory shows that this entropy decrease will accelerate nucleus formation in an exponential fashion (Flory and Weaver, 1960; Mandelkern, 1964). The effect of cross-linking on the initial rates (Figure 6) grows from a minimum fivefold increase at large ΔT to more than a 30-fold increase for $\Delta T < 10°$.

The effect of pH on the initial rate of mutarotation (refolding) of native ichthyocol was studied at a protein concentration of 0.08 mg/ml in 0.1 M citrate (pH 2.21–7.04). As in all of the presently reported renaturation studies, the low gelatin concentration ensures that intramolecular folding is the dominant process (Rao and Harrington, 1966; Harrington and Rao, 1970). Solutions were melted at 40° for 10 min and then rapidly quenched; renaturation temperatures were chosen so that at each pH the ichthyocol would be renatured at about 25° below its melting temperature. Hence T_r increased from 3.5° at pH 2.2 to 8.7° at pH 7.04, and $T\Delta T$ was constant (7000 deg²). Relative initial mutarotation rates are plotted as a function of pH in Figure 7. The magnitude of the effect of pH on rate is striking and suggests immediately the importance of electrostatic interactions in the renaturation process. Over the pH range 2.2–4.4

the rate increases by a factor of 19. A preliminary experiment had been carried out at constant temperature of renaturation (T_r = 7°), rather than at constant $T\Delta T$. In that case a 30-fold change in rate was observed over the same pH range. The larger effect for the isothermal experiment is expected because of increasing values of ΔT with increasing pH.

Several additional observations were made in conjunction with these experiments. First of all, the specific rotation, $[\alpha]^T_{313}$, of the native ichthyocol at low temperatures (3–8°) was independent of pH. Hence the potentially recoverable levorotation after denaturation was identical for all samples. Attainment of these potential values was not observed, nor was it expected, as the isothermal renaturation of non-cross-linked collagens is notoriously imperfect. Yet the differences in $[\alpha]^{6°}_{313}$ after 4 days of refolding at 4° were not nearly so dependent on pH as the pH dependence of *initial rates* might lead one to suspect. At pH 2.21, 30% of the native ichthyocol levorotation was recovered, while 57% was recovered at pH 4.42. Had the experiment been done at constant $T\Delta T$ for the long-term refolding, the range of recovery of native structure would have been increased slightly to 30–70%.

For native ichthyocol, the shape of the dependence of initial rate on pH, with an inflection point at pH 3.3, suggests that the state of ionization of the side-chain carboxyl groups has important consequences on the rate-determining step of the reaction. In fact, the net charge on the ichthyocol gelatin chain would seem to be controlling the rate in this particular case, because the rate passes through a maximum value at pH 4.5 which is close to the isoelectric point of the molecule. If backfolding of the gelatin chain segments is actually occurring in the early stages of the reaction, it is reasonable for net charge to affect this process. At the isoelectric point, unfavorable electrostatic interactions between like charges would be at a minimum, statistically, hence the isoelectric pH should be optimum for intersegment association within the gelatin chain. As net negative or positive charge is acquired at pH values above or below the isoelectric point, repulsive interactions would be expected to increase, with a concomitant drop in renaturation rate. Anticipating results of similar pH–rate experiments with cross-linked ichthyocol, native *Ascaris* and RCM-*Ascaris* collagen, the renaturation rate is also maximum in these systems near the isoelectric point.

Dilute solutions of cross-linked ichthyocol (c = 0.060 mg/ml) were prepared in 0.1 M citrate (pH 2.28–5.86). After 10 min of melting at 40° in the polarimeter cell, the samples were quenched to the desired renaturation temperature, 19.9–24.5°, and monitored for increasing levorotation. A relatively low value of $\Delta T \sim 10°$ (hence $T\Delta T$ = 2950 deg²) was chosen so that the initial rates of this much accelerated mutarotation could be accurately determined. Results are shown in Figure 7 where *relative* initial rates are plotted against pH. Several important differences may be noted between the two types of ichthyocol. First, the absolute values of the renaturation rates are higher for cross-linked ichthyocol than for native ichthyocol. While the ratio of rates is about 3 at pH 5.8, it would have been about 9 if both materials had been renatured at $T\Delta T$ = 7000 deg². Second, a striking difference in the magnitude of the pH dependence of refolding rate is observed. The 19-fold change in rate for untreated ichthyocol is almost completely abolished

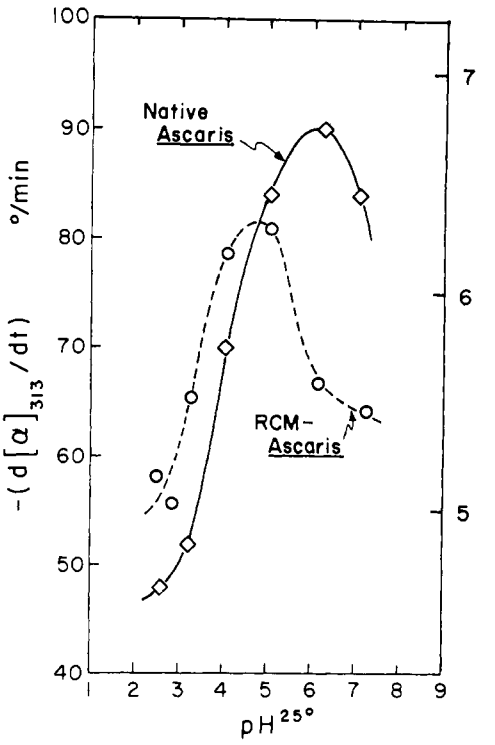

FIGURE 8: pH dependence of *Ascaris* gelatin initial refolding rates at constant undercooling: left ordinate, native *Ascaris* (c = 0.107 mg/ml) refolded at $T\Delta T$ = 6470 deg² ($\Delta T \sim 22°$); right ordinate, RCM-*Ascaris* (c = 0.140 mg/ml) refolded at $T\Delta T$ = 5300 deg² ($\Delta T \sim 19°$). Solvent is 0.2 M NaCl–0.009 M citrate. Collagens were melted at 60° (RCM) or 80° (native) for 10 min before refolding.

by the introduction of cross-links. Because these pH effects have been observed for *initial* rates of renaturation, it is evident that even the very earliest stages of the refolding process are sensitive to pH. That this sensitivity is almost totally eliminated by cross-linking reveals the involvement of cross-linked regions of the chain in these initial stages.

Refolding kinetics of *Ascaris* collagen were obtained for comparison with the ichthyocol results. For RCM-*Ascaris*, using Figure 4 and the 2.8° correction to equilibrium T_m, the undercooling (ΔT) was about 19° for all samples; actual refolding temperatures varied from 3.3° to 13.3°, with $T\Delta T$ = 5300 deg². Initial rates of mutarotation as a function of pH are presented in Figure 8. The total variation in rate is only 25%, yet the rate reaches a well-defined maximum at pH = 4.5. Native *Ascaris* initial refolding rates were measured at $\Delta T \sim 22°$ (T_r = 19.0° to 31.1°; $T\Delta T$ = 6500 deg²) and are included in Figure 8. In addition to the expected increase in rate for native *Ascaris* compared to its noncross-linked RCM derivative, the pH of maximum rate is elevated by about 1.5 units to pH 6.0. This difference is presumed to be directly related to the isoelectric points of the two proteins (see Discussion).

In order to test the hypothesis that the maximum refolding rate occurs at the isoelectric point, measurements of the gelatin isoelectric points were carried out by agarose gel electrophoresis. The experiments of Jackson and Neuberger (1957) and Veis *et al.* (1958) have clearly demonstrated the effects of ionic environment on the isoelectric point of gelatin.

TABLE I: Comparison of Collagen Properties.

Parameter	Native Ichthyocol	Cross-Linked Ichthyocol	RCM *Ascaris*	Native *Ascaris*
Thermal stability				
T_m (pH 6) − T_m (pH 2.5) (deg)	5.0	4.4	10.4	12.3
$\partial T_m/\partial$pH (max), (deg/pH unit)	2.5	2.2	5.0	5.0
ΔT_t, pH 6 (deg)	3.0	3.0	11.0	4.1 (6.9)[a]
ΔT_t, pH 2.5 (deg)	5.0	5.0	11.8	6.5 (7.2)[b]
Renaturation (initial rates)				
Rate (pH 6)/rate (pH 2)	9.2	1.5	1.1	1.9
Maximum observed ratio of rates at various pH	19.0	1.6	1.3	1.9
pH of maximum rate	4.6 ± 0.2	5.2 ± 0.4	4.5 ± 0.3	5.8 ± 0.5
Estimated isoelectric point	5.2	4.8	4.2	5.3

[a] ΔT_t at pH 7.15. Value in parentheses is ΔT_t for entire melt curve, while 4.1° represents the ΔT_t for the cooperative portion of the transition (Figure 5). [b] ΔT_t at pH 2.58. Value in parentheses is for entire melt curve; 6.5° is ΔT_t for cooperative portion only (Figure 5).

Because of salt binding the apparent isoelectric point drops as the ionic strength increases. It was important, therefore, to carry out measurements of the isoelectric points of the gelatins in solvents identical with those used for the refolding studies. Protein mobilities as a function of pH are plotted in Figure 9. The pH value of zero mobility is assumed to be the isoelectric point of the gelatin in this solvent system (0.2 M NaCl–0.009 M citrate). It was impossible to use 0.1 M citrate as a solvent for electrophoresis because of its high conductivity. As indicated in Methods, ichthyocol exhibits "heat precipitation" in solvents other than 0.1 M citrate. This precipitation is graphically illustrated in Figure 9, where there is no mobility of ichthyocol above pH 5.5. Extrapolation of the mobilities at lower pH was necessary for estimation of the isoelectric points of native and cross-linked ichthyocol. Sensibily, the isoelectric point of cross-linked ichthyocol (pH 4.8) is slightly lower than that of native ichthyocol (pH 5.2) because some free amino groups have been eliminated by reaction with formaldehyde in the formation of methylene cross-linkages. Native *Ascaris* gelatin does not move in the agarose gel at any pH, presumably because of its large size (900,000 daltons). However, its isoelectric point may be estimated. In the preparation of RCM-*Ascaris*, about 32 carboxymethyl groups/1000 residues were attached at the reduced disulfide bonds of native *Ascaris*. The resulting *S*-carboxymethylcysteine residues increased the total number of free carboxyl groups from 85/1000 to 117/1000 residues in RCM-*Ascaris*. Simple calculation shows that the effect of this modification is to lower the isoelectric point by about 1.1 pH units (*i.e.*, from 5.3 to 4.2), a change which is in reasonable agreement with the 1.5 pH unit difference between the renaturation rate maxima. In Table I, a good correlation is apparent between the pH of maximum refolding rate and the isoelectric point of the gelatin.

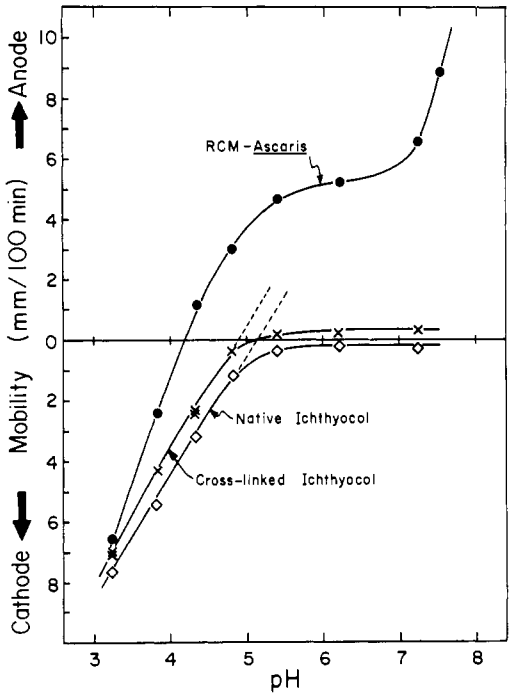

FIGURE 9: Electrophoretic mobility of several gelatins at 20° as a function of pH, supporting medium: 0.8% agarose gel in 0.2 M NaCl–0.009 M citrate. Protein samples were heated (60°, 10 min) before application. Dashed lines are extrapolations of ichthyocol mobilities into region where isoelectric precipitation occurred. Mobilities are corrected for endosmotic flow by subtraction of blue dextran mobility.

Discussion

The properties of the two cross-linked collagens under investigation show several interesting differences. Cross-linked ichthyocol has a sharper, more symmetrical melt curve than native *Ascaris* and possesses none of the high temperature, noncooperative structure exhibited by the latter material (Figure 5). Noncooperative structure is loosely defined

as that structure which melts *without* showing an inflection point in a plot of $[\alpha]$ *vs.* T. This structure is denatured only with difficulty in the 60–90° temperature range, or by 5 M Gd·HCl, and accounts for the steep slope of the native *Ascaris* melt curves above 60° in Figure 3. Asymptotic approach of the melt curves to the 5 M Gd·HCl base line is taken as evidence that most, if not all, of the noncooperative structure can be melted out at sufficiently high temperatures. It appears that the melting process might be completed at about 100–120°, although measurements were not extended beyond 90° in this aqueous system.

Because of this great thermal stability, it is believed that the noncooperative structure is stabilized by some of the abundant disulfide cross-links in the molecule. That disulfide cross-links are able to preserve regions of structure in apparently completely denatured proteins is known from the work of Tanford and associates (Aune *et al.*, 1967; Tanford *et al.*, 1967b). While low pH and heat are often unable to destroy such residual "beads" of structure, 6 M Gd·HCl apparently ruptures all noncovalent structure. All proteins lacking disulfide cross-links exhibit ideal random-coil behavior in the presence of 5–6 M Gd·HCl (Tanford *et al.*, 1967a). Native *Ascaris* collagen is not a random coil in 5 M Gd·HCl; the abundant disulfide cross-links obviate this possibility. Completely denatured native *Ascaris* gelatin is probably a loose network of cross-linked chains. Even in the presence of 5 M Gd·HCl, the opportunity arises at low temperature for the development of noncooperative levorotatory structure in the regions of the cross-links. The tenacious stability of these pockets of structure is evidenced by the remarkable observation that mutarotation occurred to a small extent (~10% helix) when the gelatin was kept at 2° in 5 M Gd·HCl after having been heated to 80° for 20 min (Figure 3).

Considering the differences between the cross-links in the ichthyocol and *Ascaris* systems, it is reasonable that only *Ascaris* exhibits cross-link mediated regions of noncooperative structure. Methylene bridges introduced into ichthyocol by formaldehyde treatment are thought to occur between the ϵ-NH_2 of lysine and the amide or guanidyl groups of other side chains. Because there is relatively unhindered rotation about most of the bonds in this cross-link, it is likely to be quite flexible, with possible distances between α-carbon atoms in the peptide backbone ranging from 3 to 16 Å (estimated from the structure: peptide-lysyl-$NH(CH_2)NH$-arginyl-peptide using the new Corey–Pauling–Koltun space-filling models). In conjunction with the loose nature of the methylene cross-linkages are (1) the belief that they are located in regions of high charge density (Veis and Drake, 1963) and (2) evidence that these charged regions are relatively structureless (Drake and Veis, 1964). Even if local regions of noncooperative structure were able to form, it is readily understood why the flexible cross-links would not endow this structure with unusual thermal stability, hence the normal melting profile exhibited by cross-linked ichthyocol.

The disulfide cross-link is notably inflexible by comparison. There are only four atoms connecting the two backbone α-carbons. The dihedral angle of the S–S bond is rigidly maintained at 90° so that the distance between the α-carbon atoms is virtually fixed at 5–7 Å. The presence of a disulfide cross-link thereby places severe restrictions on the mobility of the neighboring amino acid residues. In native *Ascaris* gelatin there are large numbers of disulfide cross-links (150/900,000 daltons). The rigidity of these bonds makes it likely that they could stabilize local regions of structure through entropic effects.

Quantitative interpretation of the present data on the pH dependence of the T_m of the collagens is difficult. The particular solvent systems chosen for this study, 0.1 M citrate buffer and 0.2 M NaCl–0.009 M citrate, show large differences in ionic strength (μ) over the pH range of interest. For 0.1 M citrate, μ varies from 0.023 at pH 2.5 to 0.53 at pH 6.0. On the other hand, μ for the sodium chloride solvent system only changes from 0.20 to 0.25 over this pH range. It would have been preferable to damp out such changes in μ by using only the latter solvent. However, the rapid heat precipitation of ichthyocol in this solvent precluded its use. A decreased electrostatic screening due to small μ at low pH could certainly participate in the observed destabilization of the collagens by amplification of charge repulsion effects. It is unlikely that μ plays more than a minor role in the pH dependence of T_m. In fact the observations of Dick and Nordwig (1966), which are very similar to the results in Figures 1 and 4, were made at constant μ. Furthermore, a 20% decrease in μ is accompanied by a 12° drop in T_m for native *Ascaris* collagen, while a 2000% decrease in μ for the ichthyocol system parallels a mere 5° decrease in T_m. Alteration of thermal stability by variation in the activity of dissolved salts is another factor which can be eliminated from the present discussion. The data of von Hippel and Wong (1963a), when taken in conjunction with the findings of Woodlock and Harrap (1968) that salt effects are magnified at low pH, predicts no more than a 2° shift in collagen T_m for either of the two solvent systems. It is emphasized that pH changes alone can markedly alter the thermal stability of the collagen molecule. Presumably this is a result of simple electrostatic repulsion.

It is in the effect of cross-linking on the pH dependence of refolding rate that an important difference is observed between the ichthyocol and *Ascaris* systems. Disregarding the locations of the renaturation rate maxima which are correlated with the isoelectric pH values, the magnitude of the rate dependence on pH is very similar for the three gelatins, cross-linked ichthyocol, RCM-*Ascaris*, and native *Ascaris*. Normal noncross-linked ichthyocol gelatin differs from these three in that it displays a 10- to 15-fold greater sensitivity to pH. It is postulated that this is due to differences in the distribution of polar amino acid residues in ichthyocol and *Ascaris* collagen. Ichthyocol fibrils stained with polar, electron-opaque compounds show characteristic banding patterns which are caused by neighboring regions of high polarity and high nonpolarity (Schmitt *et al.*, 1955). Electron microscopy of *Ascaris* collagen fibrils has revealed no banding pattern (Reed and Rudall, 1948; Bird and Deutsch, 1957; Josse and Harrington, 1964). This lack of banding does not result from an absence of oriented periodic structure in the material examined. X-Ray diffraction photographs of whole *Ascaris* cuticle and fibrils prepared from the native collagen (W. Traub, personal communication) reveal a sharp collagen pattern with a 2.9-Å meridional arc, diagnostic of the triple-helical collagen structure (Rich and Crick, 1961; Ramachandran, 1967). Also, *Ascaris* collagen is not deficient in ionizable polar amino acids, of which there are 150/1000 residues compared with 208/1000 residues for ichthyocol. It would seem

that the lack of banding in *Ascaris* collagen is a consequence of the even distribution of these polar residues throughout the chain.

The observations that protonation punctuates helical segments in native *Ascaris* collagen (Figure 5) and that T_m is more than twice as sensitive to pH as the ichthyocol T_m (Table I) corroborate the homogeneous charge distribution of *Ascaris* collagen. Electrostatic repulsion between charges which are distributed evenly along the helical structure would cause a general destabilization of that structure. In ichthyocol, where the charged residues occur in clusters, repulsive forces would be concentrated in regions which, according to Drake and Veis (1964), are already devoid of collagen structure. Hence the effects of pH on T_m are diminished.

Related to the uneven distribution of polar residues in ichthyocol, Drake and Veis (1964) and von Hippel and Wong (1963b) have suggested that highly polar regions would not easily fold into the collagen conformation. It should also be expected that the polar regions would determine the ability of the nonpolar regions to enter the collagen fold. Because of their propinquity, the electrostatic interactions of polar chain segments are partially expressed in the nonpolar regions by simple mechanical coupling. The chemical nature of the methylene cross-links requires that they be located in regions of polarity. Chemical evidence suggests that all types of cross-links (ester, aldehyde, etc.) are in the highly polar regions of the collagen molecule (Veis and Drake, 1963; Blumenfeld and Gallop, 1962). Because the cross-links would counteract the normally large electrostatic repulsion of the polar regions by covalent connection, it is readily seen how they might decrease the magnitude of the pH dependence of refolding rate, as was observed for the ichthyocol system (Figure 7).

Two possible explanations exist for the lack of effect of cross-linking in *Ascaris* collagen in reducing the already small magnitude of the pH dependence of renaturation rate. First, the probable even distribution of polar residues throughout the *Ascaris* polypeptide chains makes it unlikely that various segments of the chain would exhibit strong differences in electrostatic repulsion. Thus at pH values below the isoelectric point there would not be any large chain segments which are so highly charged that the cooperative interaction required for collagen-fold formation is discouraged. Second, the positioning of the disulfide cross-links is undoubtedly subject to genetic control rather than to differences in chemical reactivity. Even if highly polar regions do exist in the chain, they probably are not selectively joined by cross-links, as is the case for ichthyocol.

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

Peter V. Hauschka† and William F. Harrington‡

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

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

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

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

Cross-linked collagen can be renatured under conditions

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