The Renaturation of Soluble Collagen. Products Formed at Different Temperatures*

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ABSTRACT: The coil–helix transition of acid-soluble calfskin collagen dissolved in 0.25 M citrate buffer, pH 3.7, was studied isothermally over a temperature range of 4–30° at several concentrations. Recovery of negative optical rotation and viscosity and the re-formation of native molecules have been followed with time. The products formed at different temperatures have been examined by ultracentrifuge analysis, pepsin attack, and by their melting behavior. It was found that a re-formation of native molecules does occur under isothermal conditions. At low renaturation temperatures, however, this reaction is suppressed by a competing rapid formation of other stabilization forms. These are high molecular weight aggregates containing pepsin-resistant, helical regions connected by pepsin-digestible, nonhelical chain parts. Their formation is responsible for the high and fast recovery of optical rotation under those conditions. The helical regions seem to assume a collagen-like three-strand structure, but their thermal stability is low and shows a broad distribution. With increasing renaturation temperature an increasing fraction of these aggregates is no longer stable and thus the number of competing reactions is reduced. In accordance the yield of native molecules increases with increasing renaturation temperature. The lowest temperature at which native molecules are formed to the exclusion of all other stabilization forms is 26° (in 0.25 M citrate buffer, pH 3.7). This is, therefore, the optimum temperature for the investigation of the re-formation process. At this temperature the time dependence of the recovery of optical rotation corresponds to that of the re-formation of native collagen. The appreciable concentration dependence which is observed in the temperature range in which native molecules are re-formed indicates that the rate-limiting step of their formation is a multimolecular process. A simple explanation for the negative temperature coefficient for the recovery of optical rotation is discussed.

The monomeric collagen molecule in solution has a molecular weight of approximately 300,000 and a rigid rodlike shape with a length of 3000 A and a diameter of 14 A (for a summary, see Grassmann, 1965). Using X-ray diffraction data for solid collagen, Rich and Crick (1961) and Ramachandran and Sasisekharan (1961) have proposed various models for the tertiary structure of the molecule, which have the same basic structure, namely three polypeptide chains, $\alpha 1$, $\alpha 2$, $\alpha 3$ (Piez *et al.*, 1963; Piez, 1964), each having the polyproline II conformation (Katchalski *et al.*, 1963) twisted about each other to form a three-strand coiled coil. The molecule is stabilized by hydrogen bonds connecting the three chains. Their polyproline II type structure is responsible for the high negative optical rotation.

In aqueous solution the collagen molecule undergoes a sharp thermal transition (Doty and Nishihara, 1958). An abrupt decrease in the negative optical rotation and intrinsic viscosity is observed, indicating a profound loss of helical structure and a complete breakdown of the rodlike molecule. In a following, slower step (Engel, 1962b) the molecule dissociates into its three component polypeptide chains ($\alpha 1$, $\alpha 2$, $\alpha 3$). The molecular weights of these components, which have essentially the shape of random coils, are about 100,000 (Lewis and Piez, 1964). A β component (M_w = 200,000) consisting of two α chains and a γ component (M_w = 300,000) made up of all three α chains by intramolecular cross-linkage can also be observed (for a summary, see Grassmann, 1965).

The reversibility of this helix–coil transition has been studied by several investigators. It was found that a re-formation of native molecules is easily achieved by quenching solutions containing merely the γ component (Altgelt *et al.*, 1961; Drake and Veis, 1964). The same was found to be true for collagen, partially denatured so that its triple-helical structure had been destroyed but its chains had not completely dissociated (Engel, 1962a; Engel *et al.*, 1962). From these two results, it was concluded that all the information needed to build the native structure is contained in the amino acid sequence, at least in those cases in which the proper register between the chains is maintained after denaturation (Engel and Beier, 1964; Kühn *et al.*, 1964).

In solutions of completely separated chains (α or β) kept under conditions similar to those applied in the successful renaturation of the γ component (quenching to temperatures about 35° below the melting temperature) some but not all of the features which are characteristic for native collagen return. These are the high negative optical rotation and an increased resistance

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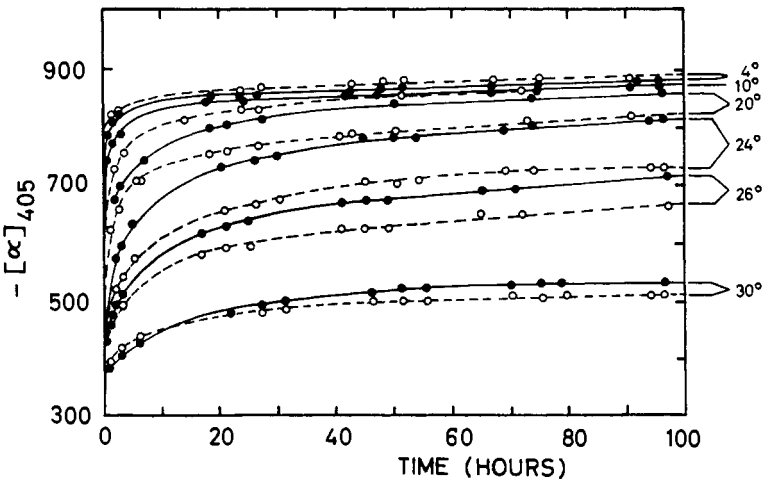

FIGURE 1: Recovery of specific optical rotation of denatured collagen with time at various temperatures (as indicated on the right). The samples were all quenched from 40°. Concentrations (g/100 ml): ○, 0.2; and ●, 0.4.

against collagenase attack (Von Hippel and Harrington, 1959; Von Hippel *et al.*, 1960). From these findings and from kinetic data two theories have been advanced for the mechanism of formation of the "collagen fold." Von Hippel and Harrington (1959) postulated a folding of the chains into loose polyproline II type single helices stabilized by water bridges. This step should be followed by a slower association between such helices. In an alternative proposal Flory and Weaver (1960) suggested a slow and rate-determining formation of the polyproline II type single helix as a transient intermediate, which in contrast to the first theory is not stable as such and associates rapidly with two others to form the collagen-type supercoil. Both mechanisms do account for the observed independence on concentration of the back mutarotation. The conclusions which may be drawn from these studies are limited, however, because no true re-formation of native molecules takes place under the conditions described above as shown by Engel (1962b).

It was, therefore, an important step forward when it was discovered that a reasonable yield of re-formed collagen could be obtained by an annealing procedure (maintaining the sample alternately at high and low temperatures or very gradual cooling) (Kühn *et al.*, 1964) and that even under isothermal conditions reformation does in fact take place, if the renaturation temperature chosen is not too far below the denaturation temperature (Beier *et al.*, 1964).

The purpose of this study was the systematic investigation of the products formed during isothermal renaturation at various temperatures over the whole temperature range of interest. The authors wished to find conditions under which a true reversal of the denaturation process, leading to collagen molecules, may be studied, and to obtain more information on other forms of stabilization.

Materials and Methods

All measurements were performed on acid-soluble calfskin collagen dissolved in 0.25 M sodium citrate buffer at pH 3.7. The collagen was extracted and purified as described previously (Engel, 1962b). The physicochemical data of this particular preparation, designated "acid extracted collagen from skin III" in a previous paper, have already been published (Engel and Beier, 1963).

Concentrations were calculated from the optical rotation at 40° where the collagen is present in its denatured form, using a value of the specific optical rotation for denatured collagen ($[\alpha]_{405}^{40}$ −360°) based on a concentration determination by Kjeldahl nitrogen analysis. Optical rotation values were measured with an electric precision polarimeter made by Zeiss, Oberkochen, Germany, with an accuracy of ±0.0025°.

Melting curves (denaturation curves) were followed by raising the temperature in steps of 2° and reading the optical rotation 15 min after each change of temperature. The melting temperature (denaturation temperature, T_m), which is defined as the temperature of the midpoint of the transition, was read from these curves. For native calfskin collagen T_m was found to be 36° in the solvent used.

Measurements of viscosity were performed in capillary viscosimeters of the Ubbelohde type manufactured by Schott, Mainz, Germany. The temperature was held constant to ±0.1°. Before each experiment, the solutions were dialyzed against the solvent for 24 hr and the external liquid was used for the reference measurement.

The ultracentrifuge analyses were carried out either with an ultracentrifuge manufactured by Escher-Wyss, Zürich, Switzerland, or with a Spinco Model E made by Beckman Instruments, Palo Alto, Calif. Schlieren

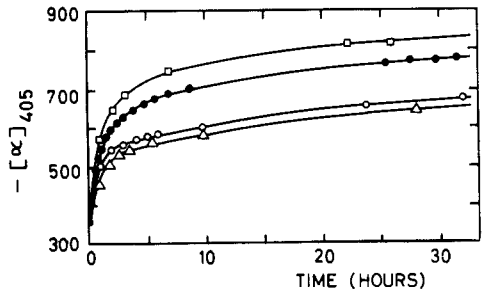

FIGURE 2: Recovery of specific optical rotation of denatured collagen at 22° for various concentrations as a function of time after cooling from 40°. Protein concentration (g/100 ml): Δ, 0.06; ○, 0.10; ●, 0.28; and □, 0.49.

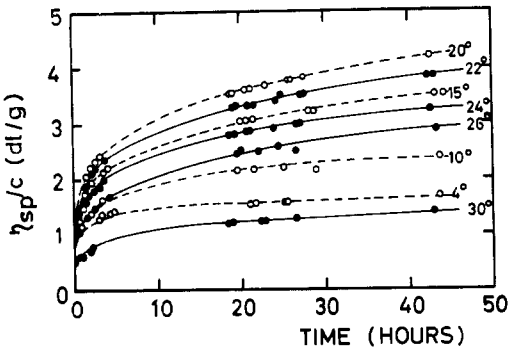

FIGURE 3: Recovery of reduced viscosity of denatured collagen at the indicated temperatures as a function of time after cooling from 40°. Protein concentration 0.06 g/100 ml.

optics were used throughout. For a quantitative evaluation of the amounts of sedimenting material, the concentrations were determined from the areas under the peaks and corrected for the dilution effect (Schachman, 1959). The weight-average molecular weight (M_w) was determined by a short-column equilibrium midpoint method (Yphantis, 1960), using a multichannel cell made by Beckman Instruments.

The light-scattering measurements were performed with an apparatus made by the Netheler & Hinz Co., Hamburg, Germany. The temperature in the cell was held constant to ±0.1°. All measurements were carried out at a wavelength of 436 mμ. The results were calculated in the usual manner (Doty and Edsall, 1951; Cantow, 1955), and the virial coefficients were taken to be zero (Engel, 1962b). The denatured solutions were purified by pressure filtration at 38° through sintered-glass filters Type G5, fine (Schott, Mainz, Germany).

Before every renaturation experiment, the collagen solution was completely denatured at 40° for a period of 30 min. Following denaturation, the solutions were quickly adjusted to the temperature at which the renaturation was performed. When optical rotation, light

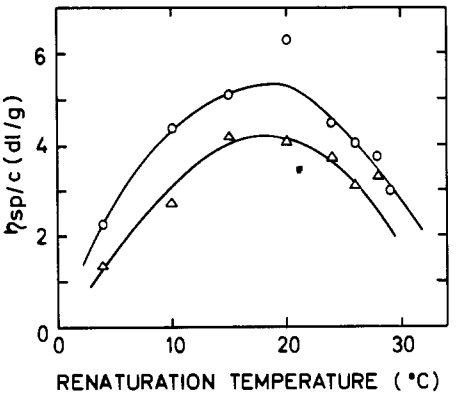

FIGURE 4: Reduced viscosities measured at a collagen concentration c = 0.06 g/100 ml after keeping solutions of denatured collagen of c = 0.12 g/100 ml (○) and c = 0.06 g/100 ml (Δ) at various temperatures for 20 hr.

scattering, or viscosity was to be followed during the course of renaturation, the denaturation procedure was applied to the solution in the polarimeter tube, light-scattering cell, or viscosimeter, respectively. In the pepsin treatment, pepsin (recrystallized three times, Serva, Heidelberg, Germany), dissolved in citrate buffer, was added in a weight ratio of pepsin:collagen, 1:10, and incubated for 18 or 24 hr at the given temperatures.

Results

1. Temperature Dependence of the Renaturation. When solutions of denatured collagen are cooled to temperatures below the denaturation temperature the specific levorotation increases with time (Figure 1). The rate of mutarotation increases with *decreasing* temperature. At renaturation temperatures lower than 15°, in accordance with an observation by Harrington and Von Hippel (1961), a fast increase of rotation within the first 10 hr is followed by a much slower increase, which continues over a period of more than 100 hr. At higher temperatures, however, no distinction can be made between these two phases; in fact, a smooth, continuous increase is recorded during the whole experiment.

As stated by Harrington and Von Hippel (1961) and Flory and Weaver (1960) the mutarotation is essentially independent of the protein concentration. It may be seen from Figure 1 that this is only true for low renaturation temperatures, at which their measurements had been performed. At temperatures above 15° a concentration dependence is observed, which is largest at approximately 24°. Figure 2 shows the back mutarotation for four concentrations at a temperature of 22°.

The increase of reduced viscosity (η_{sp}/c), after quenching denatured collagen solutions to various temperatures, is given in Figure 3. At low temperatures, where the recovery of optical rotation occurs very quickly, the reduced viscosity rises slowly and reaches only a very

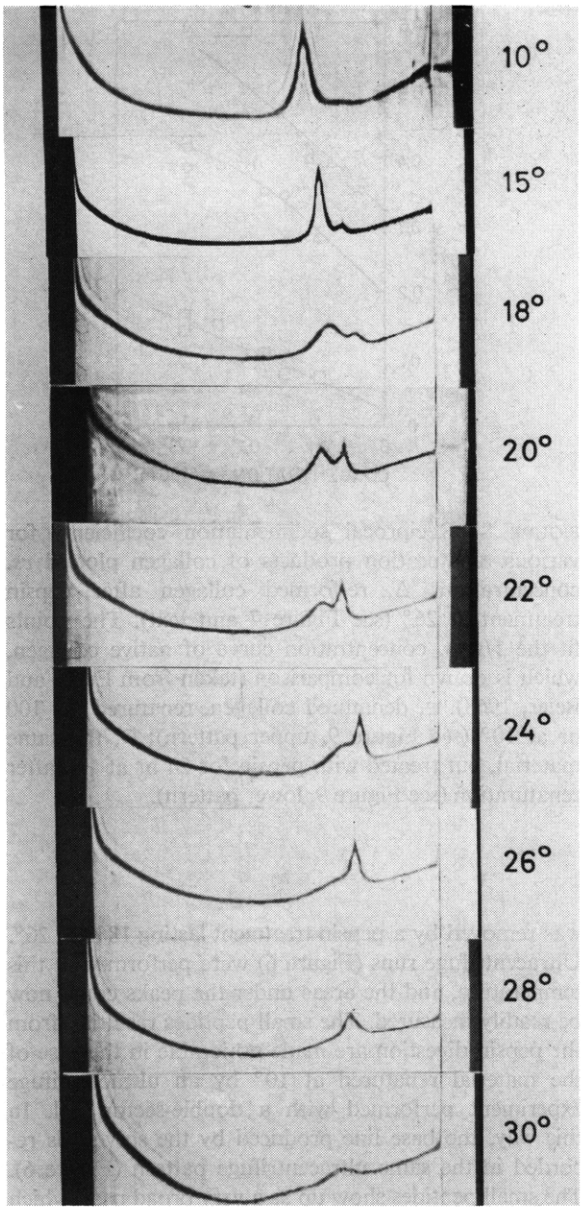

FIGURE 5: Sedimentation velocity patterns of denatured collagen solutions renatured for 100 hr at the temperatures indicated. Sedimentation takes place from right to left. In each case the sedimentation was performed at the renaturation temperature. The photographs were taken 80 min after reaching the final speed, *i.e.*, 59,780 rpm in the Spinco Model E centrifuge (10 and 15° samples) and 59,000 rpm in the Escher-Wyss centrifuge (remaining samples). The phase angle was 78° for the experiments from 15 to 28° and 66° for the experiments at 10 and 30°. The protein concentration was 0.30 g/100 ml for the experiments at 10 and 15° and 0.20 g/100 ml for all others.

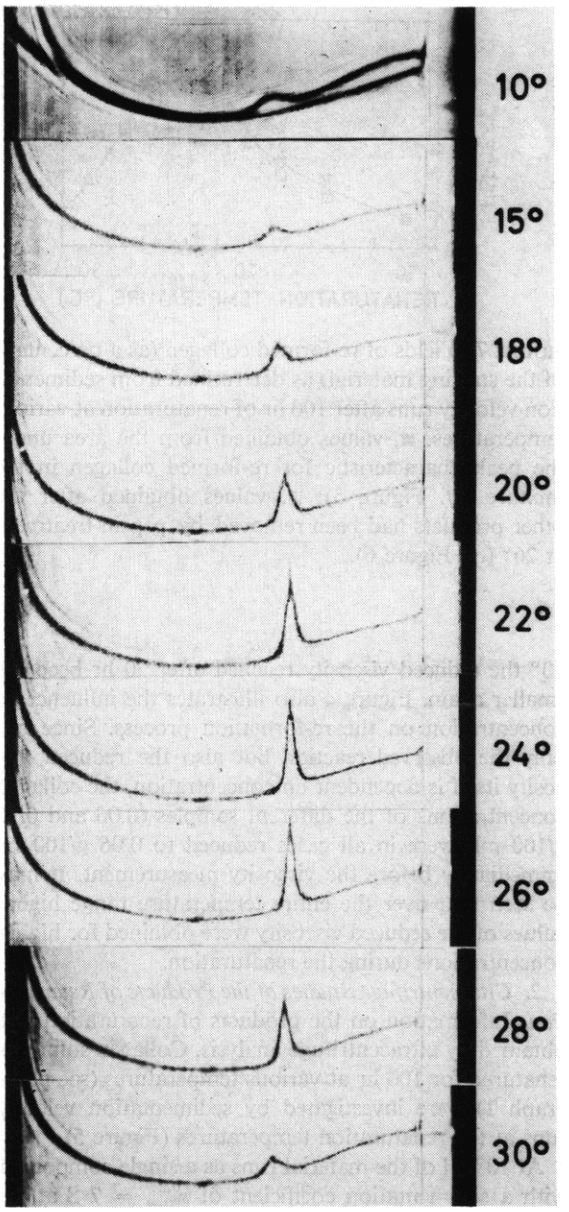

FIGURE 6: Sedimentation velocity patterns of denatured collagen solutions (c = 0.30 g/100 ml), renatured for 100 hr at the temperatures indicated and treated with pepsin for 18 hr at 26° (weight ratio enzyme:collagen 1:10). All sedimentation runs were performed at 26°. The photograph of the sample renatured at 10° was taken with a phase angle of 66° 160 min after reaching a final speed of 59,780 rpm in the Spinco Model E centrifuge. The photographs of all other samples were taken with a phase angle of 78° 120 min after reaching a final speed of 59,000 rpm in the Escher-Wyss centrifuge.

small fraction of the value characteristic for native collagen, which is η_{sp}/c = 26 dl/g for the concentration of 0.06 g/100 ml. In further contrast to the behavior of the optical rotation, the rate of increase of the reduced viscosity shows a *positive* temperature coefficient up to a renaturation temperature of approximately 20°. This is clearly shown in Figure 4, where the reduced viscosities after 20 hr are plotted against the temperature at which the denatured collagen solutions were kept. Above

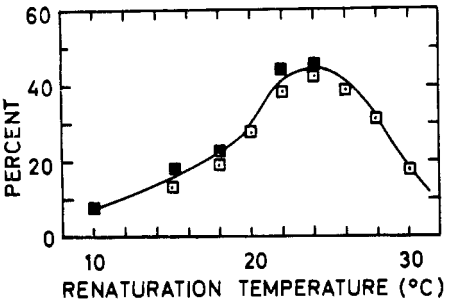

FIGURE 7: Yields of re-formed collagen (as a percentage of the starting material) as determined from sedimentation velocity runs after 100 hr of renaturation at various temperatures. ■, values obtained from the area under the peak characteristic for re-formed collagen in the mixture (*cf.* Figure 5); □, values obtained after the other products had been removed by pepsin treatment at 26° (*cf.* Figure 6).

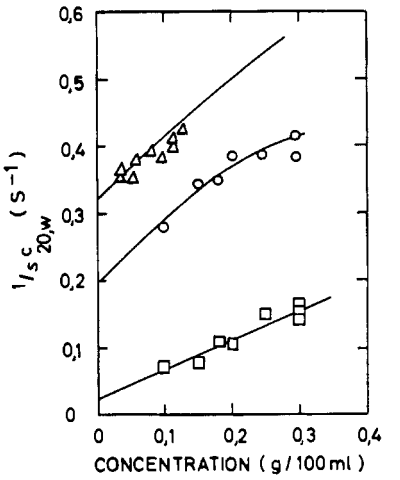

FIGURE 8: Reciprocal sedimentation coefficients for various renaturation products of collagen plotted *vs.* concentration: Δ, re-formed collagen after pepsin treatment at 26° (see Figure 7 and text). The points fit the $1/s$ *vs.* concentration curve of native collagen, which is drawn for comparison (taken from Engel and Beier, 1963). □, denatured collagen, renatured for 100 hr at 10° (see Figure 9, upper pattern); ○, the same material, but treated with pepsin for 24 hr at 10° after renaturation (see Figure 9, lower pattern).

20° the reduced viscosity reached after 20 hr becomes smaller again. Figure 4 also illustrates the influence of concentration on the re-formation process. Since not only the observed reaction but also the reduced viscosity itself is dependent on concentration, the collagen concentrations of the different samples (0.06 and 0.12 g/100 ml) were in all cases reduced to 0.06 g/100 ml immediately before the viscosity measurement. It may be seen that over the entire temperature range higher values of the reduced viscosity were obtained for higher concentrations during the renaturation.

2. Ultracentrifuge Studies of the Products of Renaturation. Information on the products of renaturation was obtained by ultracentrifuge analysis. Collagen solutions renatured for 100 hr at various temperatures (see paragraph 1) were investigated by sedimentation velocity runs at the renaturation temperatures (Figure 5).

At 10° all of the material runs as a single component with a sedimentation coefficient of $s^{0.3}_{20,w} = 7$ S at the concentration of 0.3 g/100 ml, at which the renaturation experiment was performed. In some runs a small, more slowly sedimenting peak could be detected, accounting for an estimated 10% of the sample. With increasing renaturation temperature, the fast peak gradually vanishes, and the slowly sedimenting component grows at the expense of the fast one.

3. The Slowly Sedimenting Component (Re-Formed Collagen). The amount of material present in the slower peak was determined from its area and plotted as a percentage of the initial material against the temperature of renaturation (Figure 7). Unfortunately, these values may be erroneous as a more slowly sedimenting peak may have a larger area in the presence of a faster one (Johnston–Ogston effect) (Schachman, 1959). In addition, the measurement of the areas becomes impossible for the runs performed beyond 24° because of the bad separation (see Figure 5). For these reasons and in order to separate the slowly sedimenting material for further studies, the fast-sedimenting one was removed by a pepsin treatment lasting 18 hr at 26°. Ultracentrifuge runs (Figure 6) were performed at this temperature, and the areas under the peaks could now be readily measured. The small peptides resulting from the pepsin digestion are made noticeable in the case of the material renatured at 10° by an ultracentrifuge experiment performed with a double-sector cell. In this way, the base line produced by the solvent is recorded in the same ultracentrifuge pattern (Figure 6). The small peptides show up as a very broad peak which has not separated from the meniscus, following the small but sharp peak of the pepsin-resistant material. The yield of the latter material is plotted in Figure 7 and almost fits the curve obtained from the areas of the slow peak before pepsin treatment. Considering the Johnston–Ogston effect, the observed small discrepancy is in the direction to be expected. Because of this agreement it was concluded that the faster component is completely digested by pepsin treatment at 26°, but that no digestion of the slower component occurs. (For further verification of this point see paragraph 5.)

The yield of this material shows a pronounced maximum at a renaturation temperature of 24° as shown in Figure 7. A plot of the reciprocal sedimentation coefficients against concentration fits the curve for native collagen reasonably well (Figure 8). The melting temperature as measured by optical rotation was found to be 35°. This is only 1° lower than the melting temperature of native collagen in the same solvent. Because of these findings, together with the fact that this material

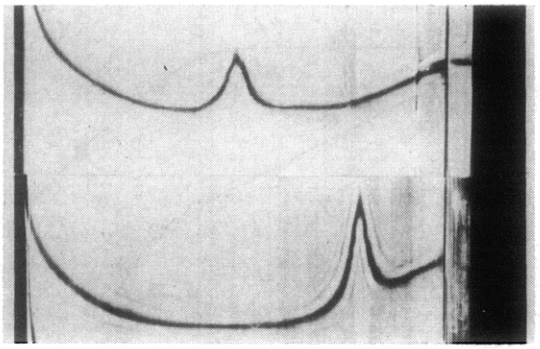

FIGURE 9: Upper pattern: sedimentation pattern of soluble collagen renatured for 100 hr at 10°. Lower pattern: same material treated with pepsin for 24 hr at 10° after the renaturation. Sedimentation is from right to left. Photographs were taken 96 min after reaching a final speed of 59,780 rpm with the Spinco Model E centrifuge. The phase angle was 66°.

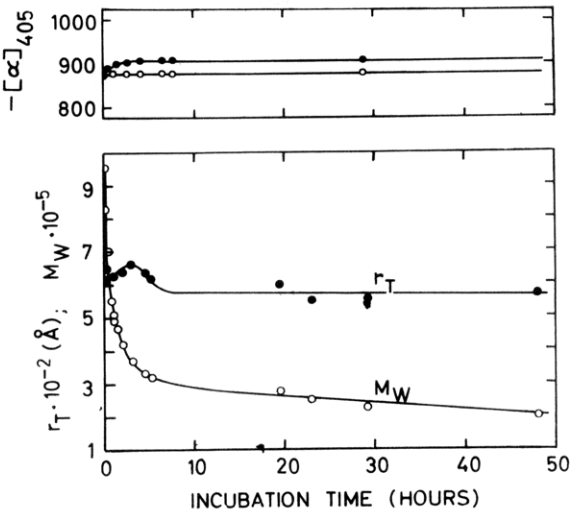

FIGURE 10: Behavior of molecular parameters during pepsin attack at 10° on denatured collagen renatured at this temperature. (Upper) Specific optical rotation at 405 mμ plotted *vs.* time of pepsin incubation after 100 hr of renaturation. (The optical rotation of the added pepsin is substracted.) (○) Control sample without pepsin. Protein concentration: 0.15 g/100 ml. (Lower) Weight-average molecular weight, M_w (○), and radius of gyration, r_T (●), as derived from light-scattering measurements, plotted *vs.* time. The pepsin was added after 22 hr of renaturation. Protein concentration: 0.10 g/100 ml.

is not attacked by pepsin at 26°, it was designated "re-formed collagen."

4. The Fast-Sedimenting Component. As shown in Figure 5, the faster sedimenting component may be investigated in an almost pure state after renaturation at 10°. Its sedimentation coefficient at 0.3 g/100 ml is $s^{0.3}_{20.w} = 7$ S. An unusually high dependence on concentration was observed (Figure 8). Extrapolation to zero concentration gives a sedimentation constant of 20–40 S. The weight-average molecular weight as determined by light-scattering measurement is $M_w > 10^6$. The melting temperature is about 27° and the material melts over a much broader temperature range than the re-formed collagen (see paragraph 7 and Figures 15 and 16).

ACTION OF PEPSIN AT THE TEMPERATURE OF RENATURATION. On incubation with pepsin for 24 hr at the temperature at which the renaturation was performed, namely 10°, the sedimentation behavior of this material changed drastically (Figure 9). The material is almost quantitatively transformed into a slowly sedimenting component ($s^{0.3}_{20,w} = 2.5$ S). The extrapolation of the sedimentation coefficients of the latter material to zero concentration yields a value of about 5 S (Figure 8). This material will be called 5S component. The weight-average molecular weight as determined by the Yphantis midpoint method was found to be 280,000 at 5784 rpm. At this rotor speed the assumption which is made for the calculation [*i.e.*, the concentration at the midpoint equals the initial concentration (Yphantis, 1960)] is valid to within 2%, if the molecular weight of the heaviest fraction present in the mixture does not exceed 400,000. The value of 280,000 is in good agreement with that obtained by light scattering (see Figure 10, lower) after an equal period of incubation. The melting temperature and the width of the transition are the same as before the treatment.

The changes in molecular weight and radius of gyration during the pepsin attack were followed by light scattering (Figure 10, lower). The fact that the radius of gyration remains approximately constant while the molecular weight drops to about one-fourth of its initial value within the first 10 hr suggests that the end products after pepsin treatment have a more asymmetric shape than the starting material. Assuming rodlike molecules for the end products, an average length of about 2000 A is calculated from the radius of gyration of 600 A. However, the numerical values from this experiment may deviate from the true values by a constant factor because no corrections were applied for the concentration dependence and the influence of pepsin itself. Further it should be born in mind that these values reflect averages over the probably broad distribution of products formed.

No decrease of optical rotation takes place during the reaction; in fact, a small increase is observed within the first 2 hr (Figure 10, upper). This observation seems to indicate that no helical regions which contribute to the high levorotation are digested by the pepsin at 10°.

ACTION OF PEPSIN ON DENATURED COLLAGEN AT LOW TEMPERATURES. That denatured, nonhelical collagen may well be attacked by the enzyme at the same low temperature was shown in the following experiment. In order to expose collagen to the enzyme in the denatured state before renaturation could take place, a collagen solution at 40° was quickly poured into a

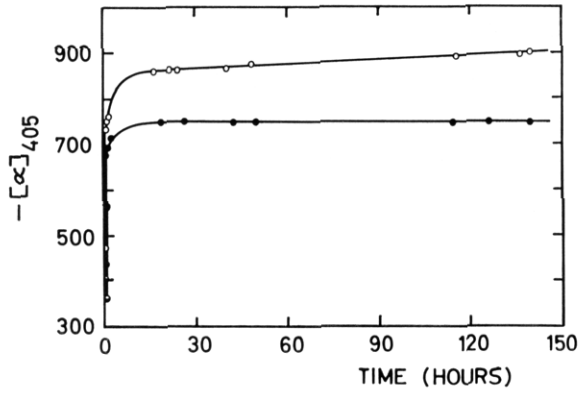

FIGURE 11: Recovery of optical rotation during renaturation of collagen at 10° with and without pepsin, showing the digestion of denatured collagen at 10°. ●, a hot solution of denatured collagen was mixed with a cold pepsin solution at zero time; ○, control without pepsin. For experimental details see text.

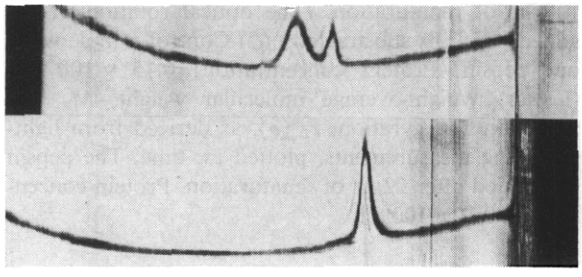

FIGURE 12: Upper pattern: sedimentation velocity pattern of denatured collagen, renatured for 100 hr at 18°. Lower pattern: the same material but treated with pepsin for 24 hr at the same temperature after renaturation. Sedimentation is from right to left. Photographs were taken 96 min after reaching a speed of 59,780 rpm with the Spinco Model E centrifuge. The phase angle was 78°.

pepsin solution kept at 4°. The relative volumes were calculated so that the temperature after mixing was 10°. The recovery of optical rotation at this temperature was plotted as a function of time (Figure 11). A control experiment was performed using plain buffer at 4° without pepsin. In this case, a normal regain of optical rotation is observed which reaches 80% of the difference between the optical rotation of native and denatured collagen after 140 hr and shows a slow but continuous further increase. In the presence of pepsin, a value of only 57% is reached after about 15 hr, and no further change is observed.

These findings show that there is a digestion of the denatured peptide chains at 10°. At this temperature, however, the action of pepsin is slow enough to allow part of the material to stabilize and become resistant to pepsin attack. The reaction comes to an end when all the peptide chains have either been

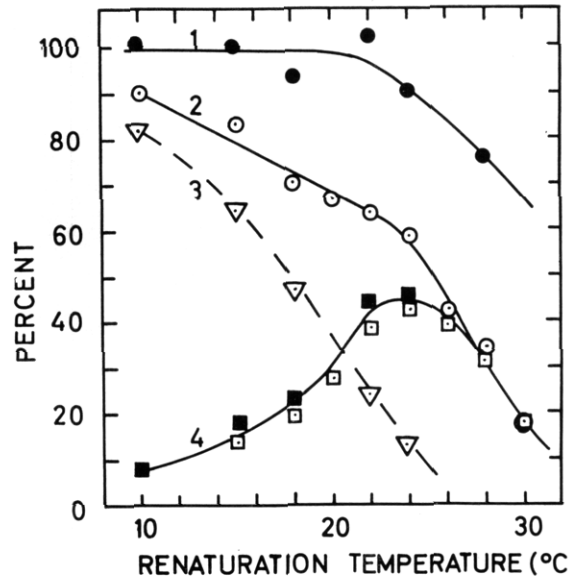

FIGURE 13: Products formed at different renaturation temperatures, as observed in the ultracentrifuge before and after pepsin treatment. Curve 1: total amounts of sedimenting material found after renaturation for 100 hr. Curve 2: total amounts of sedimenting material after pepsin treatment for 24 hr at the renaturation temperature. Curve 3: amounts of the 5S component calculated as described in the text. Curve 4: amounts of re-formed collagen as given in Figure 7. All values are given as a percentage of the initial collagen concentration which was taken for the renaturation experiments.

renatured or digested. This leads to the observed fast establishment of a true final value of optical rotation.

5. Analysis of the Renaturation Products Formed at Various Temperature. It has been shown that the fast-sedimenting component formed at 10° is almost completely transformed into the 5S component by pepsin attack at this temperature. At most, 10% of the material is digested by the pepsin to small peptides. This is calculated from the difference of the areas under the peaks in the ultracentrifuge patterns before and after incubation (Figure 9).

The method of enzyme treatment at renaturation temperature was now applied over the whole temperature range from 10 to 30°. This method has the advantage of showing the action of pepsin alone without the effect of temperature on the products characteristic for each renaturation temperature. This was not the case in the previous group of experiments where renaturation and incubation temperatures were not identical and temperature-induced structural changes could take place (see paragraph 3).

Collagen solutions (c = 0.30 g/100 ml) were denatured and isothermally renatured at various temperatures. After 100 hr, pepsin was added, and each solution was kept at its renaturation temperature for another 24 hr.

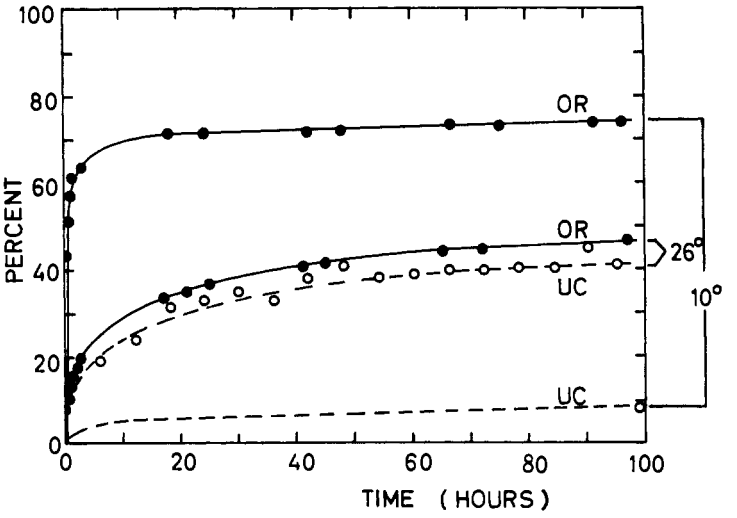

FIGURE 14: Course of the formation of re-formed collagen derived from ultracentrifuge studies (○), compared with the increase of levorotation (●) at 10 and 26°. Protein concentration: c = 0.30 g/100 ml. The amounts of re-formed collagen are plotted as a percentage of the starting material. The change of optical rotation is presented as a percentage of the difference between the values for native and denatured collagen.

Sedimentation velocity runs were performed before and after the addition of pepsin.

As an example, the patterns obtained at 18° are shown in Figure 12. The upper pattern, obtained before pepsin treatment, shows the fast- and the slowly sedimenting component, the latter previously designated re-formed collagen. After the incubation, only one slowly sedimenting peak is observed, which should contain both the re-formed collagen and that portion of the fast-sedimenting component which has been transformed by pepsin treatment (5S component). The components are apparently not resolved in the ultracentrifuge because of the similarity in their sedimentation coefficients (see Figure 8) which is enhanced by the retardation of the faster component in the presence of a slower one (Schachman, 1959).

For a quantitative analysis, the amounts of the components visible in the ultracentrifuge before and after pepsin incubation were determined from the areas under the peaks. They are plotted in Figure 13 as percentages of the total collagen which was taken for the renaturation experiment. The fraction of material present in the single peak after pepsin incubation (curve 2) was found to be larger than that of the re-formed collagen (curve 4) for all renaturation temperatures lower than 26°. The difference between these curves resembles the amount of the 5S component (curve 3). This decreases from 80% at 10° to 0% at 26°. The fact that the slow-sedimenting component obtained after pepsin treatment at 26° no longer contains the 5S component justifies our choice of this incubation temperature for the determination of the yield of re-formed collagen in section 3.

Also plotted in this diagram is the sum of the amount of re-formed collagen and the faster sedimenting component (curve 1). This sum should be equal to the total amount of collagen taken for the experiment, *i.e.*, 100%. The apparent loss of material above 25° may be explained by a partial hydrolysis of peptide chains during the 100 hr at elevated temperatures. This hydrolysis leads to small-chain fragments which are hardly detectable in the ultracentrifuge patterns (Engel and Beier, 1963; Lewis and Piez, 1964).

The difference between curves 1 and 2 gives the percentage of material which is digested by pepsin to small peptides. Their direct determination from ultracentrifuge patterns is difficult because their very broad peak is not resolved from the meniscus, as seen, *e.g.*, in the pattern of the double-sector cell run in Figure 6. In single-sector cell runs the broad peak remains hidden in the base line.

6. Kinetics of the Formation of Re-Formed Collagen. In the light of the results described in the last paragraph, it appeared of interest to follow the formation of re-formed collagen as a function of time at the temperature of 26°. This is the lowest temperature at which re-formed collagen is formed to the exclusion of all other forms of stabilization (see Figure 13). Denatured collagen was renatured at 26° and samples were taken at intervals of 6 hr. The amount of re-formed collagen was determined for each sample after pepsin treatment at this temperature from the ultracentrifuge patterns as described in section 3. In Figure 14 the results are plotted as a percentage of initial material. For comparison, the simultaneous increase in levorotation is also plotted. The same experiment was performed at a renaturation temperature of 10°. In striking contrast to the situation at 10° are the results obtained on renaturation at 26°. At 26° the curves of the optical rotation and of the amount of re-formed collagen (calculated from the area under the slowly sedimenting ultracentrifuge peak) are almost identical.

7. Thermal Stability of the Products of Renaturation Formed at Different Temperatures. The melting tem-

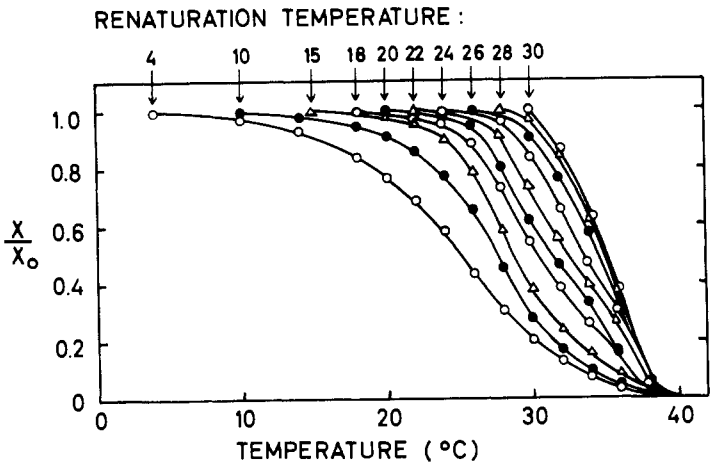

FIGURE 15: Melting curves for denatured soluble collagen renatured for 100 hr. Renaturation temperature (T_R) is indicated above each curve. The curves were normalized by setting the specific optical rotation at the end of the renaturation ($[\alpha]_{T_R}$) equal to unity and the value for the denatured state at 40° ($[\alpha]_{40}$), which was the same for all samples, equal to zero. Ordinate: $X/X_0 = ([\alpha]_T - [\alpha]_{40})/([\alpha]_{T_R} - [\alpha]_{40})$, where $[\alpha]_T$ is the optical rotation measured on heating the renatured collagen to temperature T. Protein concentration: 0.20 g/100 ml.

peratures for some of the products of renaturation have already been given. In addition, the denaturation curves (optical rotation) for the over-all mixtures formed on renaturation at various temperatures were measured (Figure 15). The curves were normalized for easier comparison (see legend). The melting temperature increases with the temperature at which the material was renatured and approaches a limiting value of 35°, thus remaining only 1° below the denaturation temperature of native collagen. For renaturation temperatures of 18–24° the melting curves reveal a subsidiary shoulder, indicating that stabilized structures with different thermal stability are present in the solution. At renaturation temperatures above 26°, where according to the findings reported in section 5 only re-formed collagen is present, a constant T_m of 35° is observed independent of the renaturation temperature. More detailed evidence of the melting behavior is given by the differentiated melting curves (Figure 16) obtained from the original, unnormalized melting curves. The ordinate of the differentiated melting curves is proportional to the number of optically active units destroyed per unit temperature increase at the corresponding temperature. A maximum in the differentiated curves corresponds to a point of inflection in the original melting curves, and its position indicates the temperature at which the largest fraction of the material melts.

The curve for the material renatured at 4° shows only one maximum at about 26°. Its broadness indicates a range of components with different degrees of stabilization. The material renatured at 10° already shows a much narrower distribution, but still only one maximum is observed. For the collagen renatured at 15°, a first indication of a second peak at higher temperatures can be observed. This peak increases in size, until, for renaturation temperatures above 26°, a single, relatively narrow distribution around a maximum at 35° is observed. The differentiated melting curves for collagen renatured in this temperature range of 26–30° thus resemble very closely the curve for native collagen, which is plotted for comparison.

Discussion

The results have clearly shown that the renaturation process leads to different products depending on the temperature applied. We may differentiate between two components with the help of ultracentrifuge analysis.

As partially shown in this paper and in more detail in a previous study (Kühn *et al.*, 1964), the properties of the *slowly sedimenting component* are so close to those of native collagen that one is justified in looking upon it as re-formed collagen. Although the amount formed depends on the renaturation temperature, its nature is always the same. This is shown by the fact that, regardless of the temperature at which it was formed, the slowly sedimenting component is completely resistant against pepsin attack and has the same sedimentation behavior and the same thermal stability. The latter conclusion may be drawn from the melting curves obtained for the over-all mixtures of products formed during renaturation at different temperatures (Figures 15 and 16). The peak with the maximum at 35° apparently reflects the melting of the re-formed collagen, for which a melting temperature of 35° was found in the isolated state. The temperature of this maximum is independent of the renaturation temperature. The *fast-sedimenting component* on the other hand is not homogeneous, and its composition and nature depend on the temperature at which it was formed.

On pepsin treatment at the temperature of its formation part of the material is digested. The remaining 5S component has the same thermal stability and optical

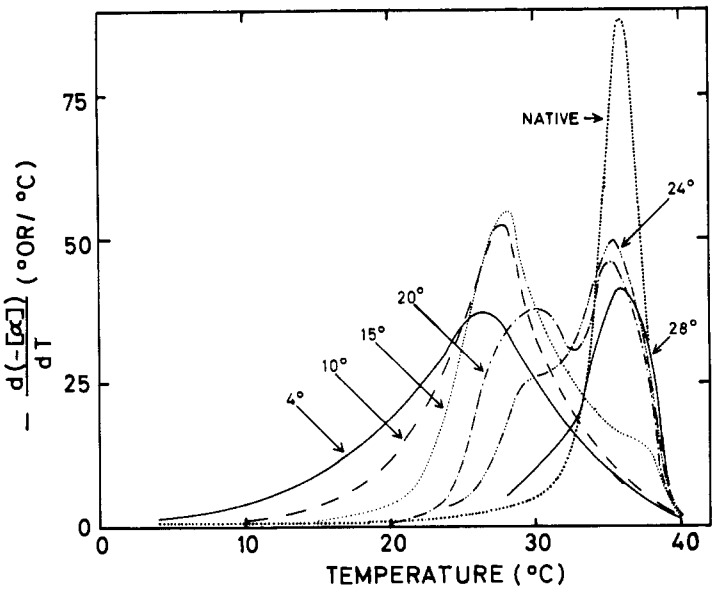

FIGURE 16: Differentiated melting curves of denatured collagen renatured for 100 hr at the indicated temperatures (4, 10, 15, 20, 24, and 28°) together with that of native collagen. For native collagen the scale on the ordinate was reduced by 0.5. Protein concentration: 0.20 g/100 ml.

rotation and thus the same helical content as the fast-sedimenting component, in spite of the drastic decrease in molecular weight from 10^6 to 200,000 which is induced by the enzyme. These findings may best be explained by interpreting the fast-sedimenting component as high molecular weight aggregates containing pepsin-resistant, helical regions connected by digestible, nonhelical chain sections.

The pepsin-resistant, helical regions are then identical with the 5S component and responsible for the high optical rotation which is obtained at low renaturation temperatures (see Figure 1). They must have a stiff, rodlike shape, because theirs must be the largest contribution to the value of the radius of gyration, which also remains unchanged on pepsin attack.

The fraction of the fast-sedimenting component, which is digested by pepsin during the incubation at the temperature of renaturation, increases with increasing renaturation temperature. Above 26°, where the fast-sedimenting component does not contain any helical parts which resist pepsin attack (5S component), the fast peak is certainly due to the denatured components α and β which are known to have a higher sedimentation constant than native collagen. It therefore seems highly probable that these separated random chains exist below 26° as well in addition to the aggregates containing helical and nonhelical regions.

There is evidence supporting the view that the helical regions discussed above consist of more than one polypeptide chain. Firstly, a molecular weight of 200,000 for the 5S component is too large to be explained by a single chain. Secondly, a preliminary experiment has shown that the weight-average molecular weight drops to about one-third of its original value if the 5S component is heat denatured after a careful inactivation of the pepsin. This supports the theory, previously put forward by a number of authors, that the stabilization of the polyproline II type structure within a peptide chain is always achieved by a mutual *association of several strands* (Flory and Weaver, 1960; Piez and Carillo, 1964; Bensusan and Nielsen, 1964; Drake and Veis, 1964). No final decision may be made as to whether two, three, or more chains are involved. To us, however, it appears that the triple-helical stabilization is the most probable one, as this structure permits maximum hydrogen bonding. In addition, our observation that all material which is in the helical form possesses a similar resistance against pepsin attack does support the idea of a triple-helical stabilization.

It may be of interest to note that the principle of a stabilization by interchain association seems to be maintained even under conditions which do not favor the participation of several chains. Thus, in the case of *Ascaris* cuticle collagen, experimental evidence, *e.g.*, the fact that back mutarotation occurs without an increase in the molecular weight, supports the view that different segments of the same chain interact by backbending to form a collagen-type structure (Harrington, 1965; McBride and Harrington, 1965). A similar mode of stabilization has been discussed for relatively low molecular weight fractions of the polytripeptide $(Pro\text{-}Gly\text{-}Pro)_n$ (Engel *et al.*, 1966). As yet no direct evidence has been obtained for the existence of a stable single polyproline II type helix as postulated by Von Hippel and Harrington (1959).

Apart from a basic similarity in their mode of stabilization, there must be a significant difference between the stabilized regions of the fast-sedimenting component

and the native or re-formed collagen. This is shown by their different thermal stability. The low-temperature aggregates melt in a much lower and broader temperature range, and their temperature of maximum melting depends on the applied renaturation temperature (*cf.* Figure 15 and 16).[1]

The lower stability of the aggregates may be explained by a more or less erroneous association of chain portions as compared to the native structure, in which the chains are optimally aligned in a quite characteristic manner. The wide temperature range in which the melting of the aggregates takes place suggests that at low temperatures very many different combinations of the chains are stable. This means that at low renaturation temperatures a given sequence may combine with almost any parts of other chains or even of its own chain. Of the many competing reactions only one leads to the re-formation of the native alignment and therefore very little native collagen will be formed under these conditions.

At higher renaturation temperatures the low-melting aggregates will no longer be stable and therefore no longer be produced. This reduces the number of competing reactions which would lead to wrong associations and increases the probability of the peptide chains associating in higher melting aggregates and especially in the most stable, native alignment. In this way the first increase of the yield of re-formed collagen with increasing renaturation temperature up to 24° is easily explained.

Several explanations may be put forward for the observed falloff in the yield above 24°. One of them is loss of material due to a partial hydrolysis of peptide chains, which becomes increasingly pronounced at temperatures above 24° (see Figure 13, curve 1). Further, a considerable amount of denatured collagen is in equilibrium with native collagen itself at 30°. Finally, as the reported yields have been determined after 100 hr of renaturation, they are influenced by the rate of the process, and a negative temperature coefficient could contribute to the decreased yield above 24°.

Starting from the assumption that the recovery of optical rotation is a true reflection of the re-formation of collagen, Flory and Weaver (1960) have concluded that this re-formation does indeed possess a negative temperature coefficient and they derived a mechanism which offers a reasonable thermodynamic explanation. However, in the light of the present data a different and simple explanation for the experimentally observed increase of the rate of back mutarotation with decreasing temperature may be given. The rate of formation of the polyproline II type helix in a particular part of the peptide chain certainly depends on the number of possible ways of stabilizing it. As pointed out above, this number decreases with increasing temperature and consequently decreases the rate of formation, thus simulating a negative temperature coefficient. Therefore the question of the temperature coefficient of the true collagen re-formation remains open for a reinvestigation under conditions under which no other stabilization processes take place. In such experiments effects such as partial hydrolysis which play a role above 24° will have to be accounted for.

Knowing the products formed at different renaturation temperatures, some insight may also be gained into the complex kinetic measurements of the recovery of viscosity. The reduced viscosity mainly reflects the asymmetry in the shape of the products formed. Therefore the temperature dependence of its rate of recovery with maximum rate near the temperature of maximum formation of native collagen may be easily understood if one remembers that native collagen has a very high axial ratio relative to that of the aggregates as can be deduced by comparing their radii of gyration with their molecular weights. It remains to summarize the influence of concentration on the recovery of optical levorotation for the complete temperature range considered.

As already pointed out, no dependence on concentration had been observed in earlier studies which had been performed at low renaturation temperatures, and it was this observation which led to the postulate that the formation of a single-chain helical intermediate must be the rate-determining step in the renaturation process. The existence of this single-chain intermediate need, however, not be postulated; in fact, it appears that an association of chains is the essential step in the stabilization process.

A. Low Temperatures. Piez and Carillo (1964) reached this conclusion for low-temperature renaturation. They observed that the recovery of optical levorotation accompanying the stabilization of $\alpha 1$ and $\alpha 2$ chains is strongly concentration dependent for a wide concentration range even at temperatures below 15°. They further reported that the stabilization of β_{12} double chains is concentration independent and therefore deduced that the reaction necessary to stabilize the molecule must occur between different molecules in the α case but can take place as an intramolecular association in the β component. Piez and Carillo con-

[1] As a first approximation we have regarded the melting curves of samples renatured at certain temperatures as representing the successive melting of the stabilization products which were formed during the renaturation experiment. However, one serious limitation has to be noted. The elevation of temperature not only leads to the melting of existing stabilization forms but must also induce new renaturation processes leading to products with higher stability including re-formed collagen as was shown by the isothermal renaturation experiments at different temperatures. This causes a shift of the denaturation curves of those samples toward higher temperatures. The effect can be minimized, but it will still be considerable, if a fresh solution is used for each point on the denaturation curve and the optical rotation is followed and the minimum recorded, *i.e.* the value reached before the new renaturation processes set in (Engel, 1962a; Küehn *et al.*, 1964). Obviously, the equilibrium method of measuring denaturation curves as it is profitably applied to native collagen (Von Hippel and Wong, 1963) must lead to completely misleading results for renatured solutions. Equilibrium values, if established in reasonable times at all, should be those for native collagen which one assumes to be the thermodynamically most stable alignment. For practical reasons, we have chosen the procedure described in the section on Materials and Methods which allows at least a qualitative comparison of the thermal stability of the renaturation products investigated.

cluded that the stabilization into these low-temperature aggregates takes place by association into double helices. It appears to us that the observations do not rule out the stabilization in triple-helical form as this could be performed fairly simply by a single β molecule folding back on itself (explaining the concentration-independent renaturation), but would be very difficult for a single α molecule (explaining the concentration-dependent renaturation). In either case it appears clear that only an association of chains can explain the influence of concentration on the back mutarotation at low temperatures.

The present authors observed no concentration dependence of the back-mutarotation at low temperatures, which is in agreement with the above results as the acid-soluble calfskin collagen contains about 60% β component which stabilizes more quickly than the α component (Piez and Carillo, 1964) and therefore contributes most to the initial back mutarotation.

B. Around 24°, however, that temperature at which most native collagen molecules are re-formed and at which the aggregates have become unstable, the concentration dependence of the back mutarotation is very marked indeed. This effect merely results from the self-evident fact that the re-formation of the complete native triple helix requires the association of more than one molecule.

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