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Helix Formation by Single- and Double-Chain Gelatins from Rat Skin Collagen

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The formation of a polyproline-type helix by single-chain (α1 and α2) and double-chain (β_{12}) gelatins was followed by optical rotation, viscometry, and light scattering. It was found that α1 and α2 formed helices by a concentration-dependent reaction which was accompanied by an increase in viscosity and molecular weight. This was interpreted as evidence for stabilization of a helix by interchain association. β_{12} formed a helix by a concentration-independent reaction and, at concentrations below 0.2 mg/ml, there was no change in molecular weight. At low concentrations there was also a large concentration-independent increase in reduced viscosity to about 2.0 dl/g. These data were interpreted as evidence for an intramolecular configurational change associated with helix formation in double-chain molecules. Melting curves demonstrated a close similarity of the gelatin helix, at the local level, whether formed by inter- or intramolecular association. The hydrodynamic properties of the β_{12} helix formed at low concentration indicated a relatively compact molecule with high asymmetry. The kinetics of helix formation by β_{12} were consistent with an initial first-order reaction associated with the intramolecular change, followed by a concentration dependent second-order reaction. The results were interpreted in terms of a polyproline helix stabilized by interchain association to form a double-chain helix. There was no evidence for significant amounts of single-chain helices under the conditions employed. Triple-chain helices also appeared not to contribute to gelatin structure in these studies.

When solutions of gelatin are cooled, the polypeptide chains assume a configuration closely related to the poly-L-proline II helix characteristic of native collagen. The accompanying mutarotation provides a convenient means of following the process. The various factors involved have been reviewed in detail by Harrington and Von Hippel (1961a). It is clear that the pyrrolidine ring of proline and hydroxyproline plays a central role in the process (Harrington, 1958; Burge and Hynes, 1959; Piez, 1960; Von Hippel and Wong, 1963a; Josse and Harrington, 1964) and the available evidence has generally been interpreted as demonstrating that helices are initially propagated along single chains without a need for interchain stabilization (Harrington and Von Hippel, 1961b). However, it is not evident how such helices are stabilized since the content of pyrrolidine rings in gelatin from vertebrate collagens is less than one residue in four and may be as low as one residue in six (Piez and Gross, 1960). Flory and Weaver (1960) have suggested that triple-chain helices must be formed, citing the parallel to collagen structure, even though the kinetics are not readily compatible with this mechanism.

One of the difficulties in the interpretation of the studies which have so far been reported may be that denatured collagen is a heterogeneous system in two respects. First, vertebrate collagens consist of three chains, one of which, the α2 chain, differs in amino acid composition from the other two, the α1 chains (Piez *et al.*, 1961, 1963; Schleyer, 1962). Since the differences include proline and hydroxyproline content, it would be expected that the chains would have different rates of helix formation. Second, denatured collagen contains covalently linked double chains, β_{12} (α1–α2) and β_{11} (α1–α1),[1] the amount varying from 18 to 66%, depending on the source and method of preparation (Piez *et al.*, 1961, 1963). It is not known what effect this type of cross-linking has on helix formation and stabilization. Since α1, α2, and β_{12} can now be prepared in pure form (Piez *et al.*, 1961, 1963; Lewis and Piez, 1964), it should be possible to gain further insight into the problem of helix formation by studies on these single molecular species. This was the object of the study reported here.

METHODS

Samples.—The single-chain gelatins α1 and α2 and the double-chain gelatin β_{12} were isolated from salt- or acid-extracted rat skin collagen by chromatography as previously described (Piez *et al.*, 1961, 1963). Samples prepared in this way have been shown to be homogeneous by the criteria of chromatography, sedimentation velocity, amino acid composition (Piez *et al.*, 1961, 1963), gel electrophoresis (Nagai *et al.*, 1964), and molecular weight (Lewis and Piez, 1964). The only exception is that samples of α2 may contain a small amount of β_{12}, estimated to be less than 5%.

[1] The designations of the covalently linked double chains were previously β1 and β2. These have been changed to β_{12} and β_{11} for clarity. See Bornstein *et al.* (1964) for further discussion of these components and their significance.

Polarimetry.—Samples were dissolved in *p*H 4.8, 0.15 M potassium acetate buffer by warming to 45° for a few minutes. This salt was chosen since it is known to have very little effect on the rate of mutarotation in contrast to certain other salts; the reaction is also known to be *p*H independent (Von Hippel and Wong, 1962). The solutions were then dialyzed against solvent for 24 hours at 5°. Optical rotations were measured in a Rudolph spectropolarimeter at 313 mμ using a mercury lamp. Concentrations were determined from the optical rotation at 45° on solutions containing about 1.5 mg/ml. Lower concentrations were made by dilution. The specific optical rotation at 45° and 313 mμ was found to be −825° by measurements on solutions standardized by microKjeldahl. To follow the rate of helix formation the sample was warmed to 45° in a test tube for a few minutes and then transferred to a 10-cm polarimeter tube with water flowing through the jacket at the temperature of the experiment, usually 15.0° ± 0.02°. Readings were commenced as soon as possible after filling the cell, about 2 minutes. The initial rotation was determined by a graphic extrapolation of specific rotation to zero time. At 15.0° this value varied from −850° to −890°, a range within the experimental error. The data were then expressed as the change in negative specific rotation, $[\alpha]_0 - [\alpha]_t$.

Melting curves were obtained on some of the same solutions used to follow helix formation while they were still in the polarimeter cell. After cooling for 24 hours the temperature was raised stepwise allowing 30 minutes at each temperature before recording the rotation. A melting curve was obtained in the same manner for native rat skin collagen dissolved in the acetate buffer at 5°.

Viscometry.—Solutions were prepared and concentrations were determined as described above. The solutions were filtered through glass filters and dilutions were made with filtered solvent. To follow changes in viscosity 2 ml of the sample which had been at 45° for a few minutes were pipetted into a viscometer supported in a bath at the temperature of the experiment, usually 15.0° ± 0.01°. Measurements of flow rate were commenced immediately. All data in this study were obtained with a single coiled capillary viscometer. The flow time for water at 15° was about 2 minutes.

Light Scattering.—Changes in molecular weight during helix formation were followed by turbidity measurements at 436 mμ in a Brice-Phoenix light-scattering photometer equipped with narrow slits and a water-jacketed cell compartment. Standard procedures were followed (see, for example, Doty and Edsall, 1951). The cell was a small volume dissymmetry cell (D-104). Scattered-light intensities relative to the incident beam were measured at 45°, 90°, and 135° Since the dissymmetry ratio was always less than 1.4, relative weight-average molecular weights could be calculated from the 90° scattered light after a small correction for dissymmetry. As has been reported by Engel (1962a), the dependence of apparent molecular weight on concentration was very small in the concentration range studied and could be ignored, particularly since only relative molecular weights were desired. This was true of both the random-coil and helical forms.

Solutions containing less than 0.5 mg/ml α1 or β_{12} were prepared in the acetate buffer as described above. At the start of an experiment the solution was warmed to 45° for a few minutes and then filtered directly into the cell through a 10-mμ Millipore filter in a syringe holder. The cell was previously rinsed with filtered solvent. Solutions prepared in this manner routinely had dissymmetry ratios between 1.1 and 1.2. This ratio increased slightly during helix formation in approximate relation to the increase in molecular weight. Two ml of solution was removed at this point for determination of concentration by polarimetry. The cell was then placed in a water bath at 5° for 1 minute, which lowered the temperature in the cell to about 15°. The cell was then dried and placed in the instrument with the cell compartment at 15°. In this manner temperature could be maintained within about 0.5° though there was some uncertainty as to how fast the temperature fell to 15°. After some experiments the solution was diluted 2-fold in the cell with filtered buffer, reheated to 45°, and cooled to 15°, and the experiment was repeated.

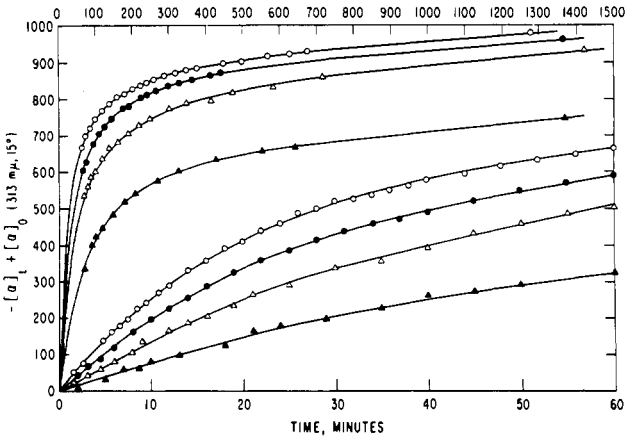

FIG. 1.—Helix formation at 15° by α1 single-chain gelatin as measured by optical rotation. ○, 1.61 mg/ml; ●, 1.06 mg/ml; △, 0.49 mg/ml; ▲, 0.25 mg/ml. The four lower curves (lower time scale) show the early stages of the overall reaction (upper time scale).

Ultracentrifugation.—The sedimentation coefficient of the β_{12} helix (see Results) was measured by ultracentrifugation in single-sector Kel-F cells at a speed of 59,780 rpm and a temperature of 15°. Schlieren optics were employed.

Kinetic Analysis.—The mutarotation data were plotted on a large scale and a smooth curve was drawn through the points. Corrected numerical data were then taken from the curve. The $d(-[\alpha])/dt$ was obtained by measuring the change in rotation in 2-minute intervals to 20 minutes and increasingly longer intervals thereafter. A plot of $\log (d(-[\alpha])/dt)$ against time provided a test for a first-order reaction. A second-order reaction was sought by plotting the reciprocal of $[\alpha]_t - [\alpha]_\infty$, trying several values of $[\alpha]_\infty$ for best fit.

RESULTS

Mutarotation.—It was found that the rate of helix formation of the single chains α1 and α2 was concentration dependent as measured by the increase in negative rotation. This was unexpected since experiments done in several other laboratories on unfractionated gelatin demonstrated that there was essentially no concentration dependence within the experimental error. Possible reasons for this important difference will become apparent and are discussed later. Typical results obtained by cooling to 15° are shown in Figures 1 and 2. The concentration dependence was marked below about 1.5 mg/ml but became small above this concentration. A similar concentration dependence was observed at 5° for α1, and the rates were approximately four times as fast as at 15°. Most measurements were made at 15° since at lower temperatures the rate of mutarotation was too fast to obtain precise

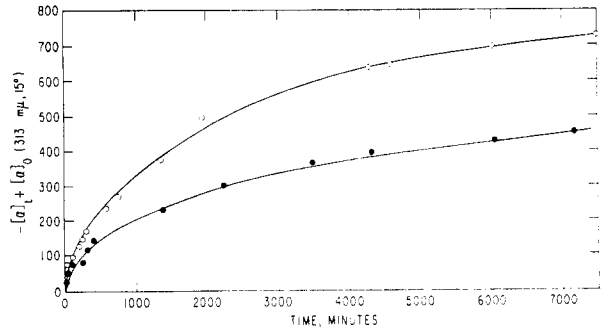

FIG. 2.—Helix formation at 15° by α2 single-chain gelatin as measured by optical rotation. O, 1.40 mg/ml; ●, 0.67 mg/ml.

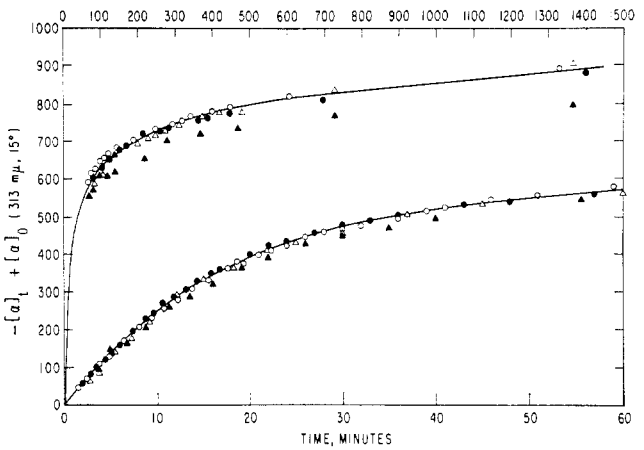

FIG. 3.—Helix formation at 15° by β_{12} double-chain gelatin as measured by optical rotation. O, 1.60 mg/ml; ●, 1.06 mg/ml; △, 0.54 mg/ml; ▲, 0.26 mg/ml. The lower curve (lower time scale) shows the early stages of the overall reaction (upper time scale).

data. A few experiments were done in calcium chloride solutions since much of the data in the literature was obtained in this solvent. Although the rates were about 50% slower in 0.2 M $CaCl_2$, there were no qualitative changes.

Helix formation by α2 was very much slower than by α1 (Fig. 2). This was expected since α1 from rat skin collagen has 226 residues containing the pyrrolidine ring (proline, 4-hydroxyproline, and 3-hydroxyproline), while α2 has only 196 (Piez *et al.*, 1963). At 15° the ratio of the rates was about 15:1 while at 5° it was about 3:1.

Unlike the result obtained with the single-chain components, the double-chain component β_{12} mutarotated at a rate which was independent of concentration. Data obtained at 15° are shown in Figure 3. At the lowest concentrations studied, 0.25 mg/ml, the rate appeared to be less near the end of the reaction than at higher concentrations. The initial rate of mutarotation was 31°/min, which was the same rate observed for α1 at a concentration of 1.6 mg/ml. It might be expected that β_{12} would form a helix at a rate equivalent to a 1:1 mixture of α1 and α2 since it has this composition. To test the possibility that there might be a specific interaction between α1 and α2, two experiments were performed. In one, a synthetic mixture of α1 and α2, each present at 0.8 mg/ml, was found to have a rate of mutarotation which was the average of α1 and α2 measured individually at 1.6 mg/ml. In the other, enriched samples of β_{11}, containing about 50% β_{11} and 50% α1, were consistently observed to mutarotate faster than α1 when both were measured at concen-

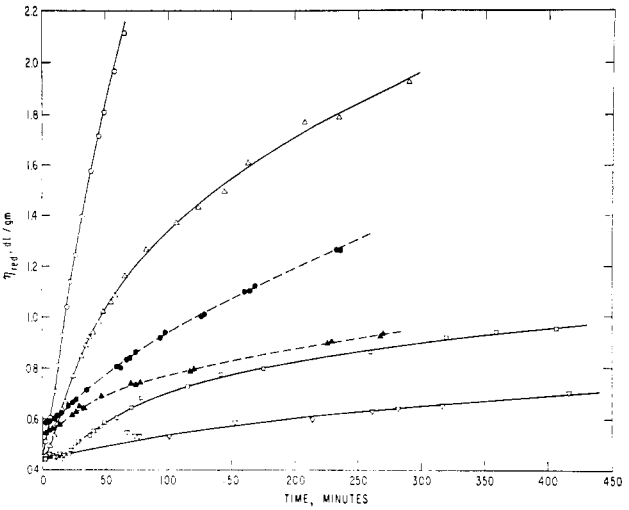

FIG. 4.—Reduced viscosity of α1 and α2 single-chain gelatins during helix formation at 15°. α1: O, 1.67 mg/ml; △, 1.11 mg/ml; □, 0.56 mg/ml; ▽, 0.25 mg/ml. α2: ●, 1.69 mg/ml; ▲, 0.84 mg/ml.

trations of 1.5 mg/ml. It was concluded that α1 and α2 interact randomly in mixtures and that cross-linking results in an increased efficiency.

The total increase in negative rotation was about 1000° irrespective of the temperature, the component, or the concentration. However, in the case of α2 or low concentrations of α1, many days were required to reach this value at 15°. It was also found that the rotation continued to increase slowly for many weeks. Similar observations have been reported by Harrington and Von Hippel (1961b).

These results suggest that helix formation requires interaction between chains which can be satisfied intramolecularly in the case of double-chain molecules but not in the case of single chains.

Viscosity.—To provide more information about interaction, reduced viscosity was measured during helix formation. Results obtained for α1 and α2 under the same conditions as employed for polarimetry are shown in Figure 4. As expected from results obtained by others on unfractionated gelatin, there was a progressive increase in viscosity with time. However, the increase was concentration dependent in the same concentration range as observed by polarimetry. Further, α2 showed a much slower rate of change than α1. This again correlated with the results obtained by polarimetry. Therefore the viscosity data support the idea of a concurrent aggregation step in helix formation by single chains.

Although β_{12} showed changes in viscosity at high concentrations similar to α1, at concentrations below 0.3 mg/ml the change was independent of concentration. Typical data appear in Figure 5. The change was large, from 0.67 dl/g to about 2.0 dl/g in 24 hours, and followed a time course that was very similar except for an initial lag in the formation of helix as measured by polarimetry. A similar type of viscosity change was observed by Engel (1962a) working with unfractionated calfskin gelatin. At the low concentrations studied by Engel, the properties of the double-chain components, which represent the greates part of this type of gelatin, would predominate This is more readily interpreted as a change in intramolecular configuration than aggregation and the new configuration is presumably associated with the formation of helix.

Molecular-Weight Changes.—Since viscosity is a complex property reflecting both molecular weight and configurational changes, it was felt that an unequivocal

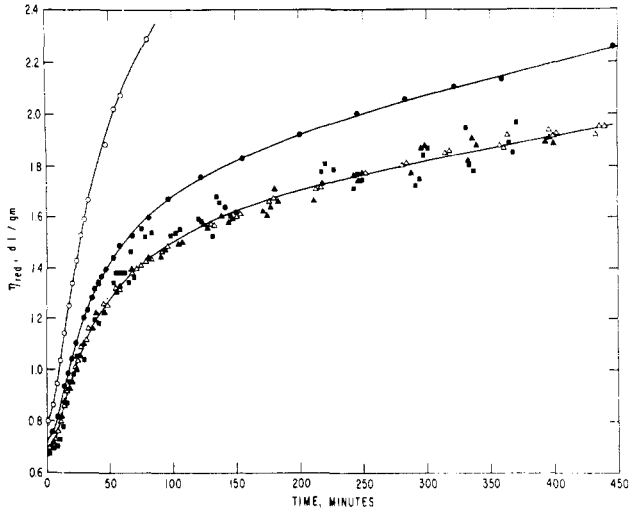

FIG. 5.—Reduced viscosity of β_{12} double-chain gelatin during helix formation at 15°. O, 1.01 mg/ml; ●, 0.49 mg/ml; △, 0.30 mg/ml; ▲, 0.20 mg/ml; ■, 0.10 mg/ml.

interpretation of the viscometry and polarimetry data required the measurement of molecular weights during helix formation. Light scattering was selected since it provides a continuous measure of weight-average molecular weight. In the present instance a relatively simple approach was sufficient since concentration dependence was negligible and, in the concentration range studied, dissymmetry ratios were always small (see Methods). Moreover, absolute molecular weights were not necessary since only the change in molecular weight was of interest. $\alpha 1$ and β_{12} have been studied under the same conditions employed here (45°, pH 4.8 acetate buffer) by sedimentation equilibrium and found to be homogeneous with molecular weights of 98,000 ($\alpha 1$ and $\alpha 2$) and 196,000 (β_{12}) (Lewis and Piez, 1964).

When samples of $\alpha 1$ were cooled to 15°, the weight-average molecular weight increased at a rate which was concentration dependent in much the same fashion as shown by the polarimetry and viscosity data. This is illustrated in Figure 6 for two concentrations. At the lowest concentration studied, 0.24 mg/ml, the molecular weight increased 2-fold in 24 hours. At higher concentrations large aggregates were formed. The initial small lag may be related to difficulty in reaching the desired temperature quickly and maintaining it constant (see Methods).

In contrast to $\alpha 1$, β_{12} showed only a very small increase in molecular weight in a period of time when helix formation, as measured by polarimetry, was nearly maximal (Fig. 6). At 0.33 mg/ml, the molecular weight increased about 30% in 24 hours. At 0.17 mg/ml the change was within the experimental error of the method. This demonstrated that aggregation is not necessary for helix formation where chain interaction can occur intramolecularly.

Melting Curves.—Information about the properties of the helix formed in gelatin solutions can be gained from the melting curves (see the discussion by Von Hippel and Wong, 1963b). A typical set of curves for $\alpha 1$, $\alpha 2$, and β_{12} obtained by a slow stepwise increase of temperature appear in Figure 7. A melting curve for native rat skin collagen is included for comparison. The melting points, taken as the temperature at which the rotational change was half completed, were 26.1° for β_{12}, 26.6° for $\alpha 1$, 22.5° for $\alpha 2$, and 38.0° for collagen. The lower melting point for $\alpha 2$ was expected since the pyrrolidine-ring content is lower than in $\alpha 1$. The β_{12}

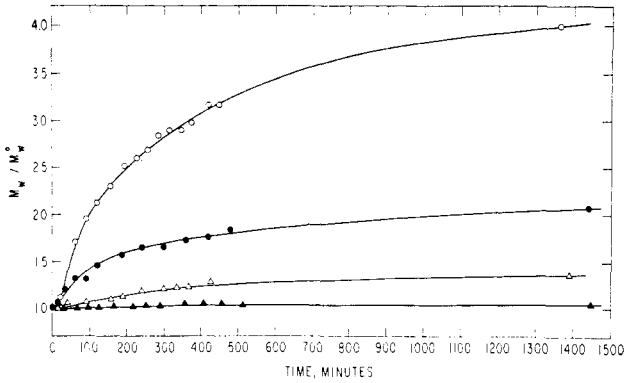

FIG. 6.—Relative weight-average molecular weight as measured by light scattering of $\alpha 1$ single-chain and β_{12} double-chain gelatins during helix formation at 15°. $\alpha 1$: O, 0.48 mg/ml; ●, 0.24 mg/ml. β_{12}: △, 0.33 mg/ml; ▲, 0.17 mg/ml.

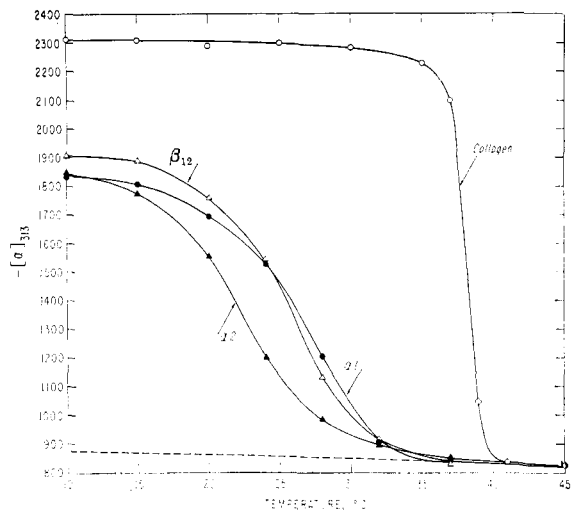

FIG. 7.—Melting curves of $\alpha 1$ (●) and $\alpha 2$ (▲) single-chain and β_{12} (△) double-chain gelatins and native rat skin collagen (O). Samples were kept at 10° for 24 hours prior to melting. Concentrations were approximately 1.5 mg/ml. The dashed line represents the residue rotation.

had a higher melting point than was obtained for a mixture of $\alpha 1$ and $\alpha 2$. This undoubtedly reflects a more efficient formation of helix as a result of the cross-linking, as was concluded from the rates of mutarotation.

The width of the transition, as indicated by the number of degrees required to change the rotation from one-fourth to three-fourths of the total, was 7.2° for $\alpha 1$ and $\alpha 2$, and 6.1° for β_{12}. The possibility that the transition might be sharper and the melting point might be higher at very low concentrations of β_{12} was checked, but found not to be the case. This observation, together with the similarity of the melting curves for single and double chains, indicates that the same type of local structure is obtained whether the interaction is inter- or intramolecular.

Another characteristic of the melting curves of gelatin which has been emphasized by Flory and Weaver (1960) is the temperature at which all structure has been melted. They pointed out that this temperature (measured by viscosity) was the same, about 41°, for native rat-tail-tendon collagen and cooled gelatin, and cited this as evidence of the essential equivalence of the two structures. However, it can be seen from Figure 7 that this was not true for $\alpha 1$, $\alpha 2$, or β_{12}. There is a

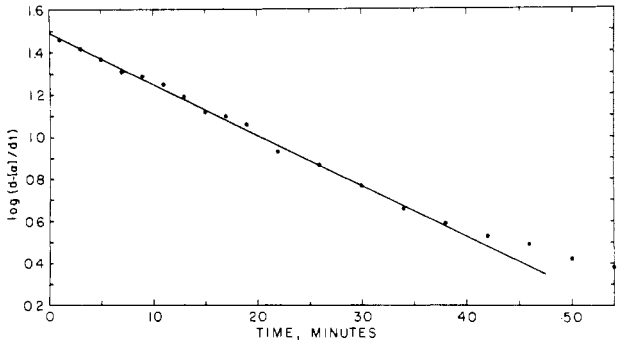

FIG. 8.—Early stages of helix formation by β_{12} double-chain gelatin at 15° plotted as a first-order reaction. The concentration was 1.6 mg/ml.

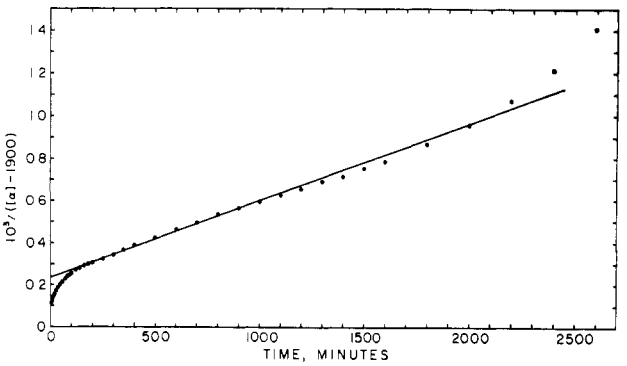

FIG. 9.—Late stages of helix formation by β_{12} double-chain gelatin at 15° plotted as a second-order reaction. The concentration was 1.6 mg/ml.

difference of at least 4° between the points where the three gelatins and collagen reach the residue rotation. It seems likely that high-molecular-weight aggregates which occur in unfractionated gelatin and can renature to triple-stranded structure are responsible for higher values. It should also be noted that the width of the transitions of the separate components is at the lower end of the 6–10° range which has been reported for a variety of unfractionated gelatins (Von Hippel and Wong, 1963b). The width of the transition must be a function of the chemical as well as the physical heterogeneity of the system.

Hydrodynamic Properties of the β_{12} Helix.—Since the helical form of β_{12} appeared to represent a single molecular species at low concentrations, it seemed worthwhile to determine some of its properties by standard hydrodynamic treatment. The approach outlined by Scheraga (1961) was followed. For this it was necessary to have, in addition to the data described, the sedimentation coefficient. This was obtained on solutions containing 0.1–0.3 mg/ml β_{12} which had been kept at 15° for 24 hours. Although only a single boundary could be seen in the ultracentrifuge, at the very low concentrations required to avoid aggregation it could be observed for only a short time because of diffusion. The sedimentation coefficients showed considerable scatter and no concentration dependence could be seen. The average and standard error of eight values was 3.4 ± 0.1 S. The value corrected to standard conditions ($s^\circ_{20,w}$) was 4.0 S, which is slightly less than the value (4.28 S) obtained for β_{12} in the random-coil configuration (Lewis and Piez, 1964). The intrinsic viscosity was taken as 2.0 dl/g. This is an extrapolated value from three concentrations of β_{12} kept at 15° for 24 hours. The reduced viscosities were 2.29, 2.21, and 2.10 dl/g at 0.3, 0.2 and 0.1 mg/ml, respectively. From the sedimentation coefficient, intrinsic viscosity, a molecular weight of 196,000, and a partial specific volume of 0.705, the Scheraga-Mandelkern β value was found to be 3.08. This is a large value indicating high asymmetry. The equivalent prolate ellipsoid has an axial ratio of 65, semiaxes a = 600 A and b = 9 A, and a volume of 2.9×10^5 A^3. This result may be compared to the native collagen molecule, which is a stiff rod about 3000 A long and 14 A in diameter. The calculated volume is 4.6×10^5 A^3. Remembering that β_{12} represents two-thirds of the collagen molecule, it appears that the two have approximately the same effective hydrodynamic volume per unit weight. The β_{12} helix is certainly unlike the β_{12} random coil, which has a volume, calculated in the same way and employing known parameters (Lewis and Piez, 1964), of 31×10^5 A^3.

These calculated properties of the β_{12} helix represent only rough approximations. The results are highly sensitive to errors in the intrinsic viscosity and sedimentation coefficient which are not known with high precision. However, it can be concluded that the hydrodynamic properties are consistent with a model which is asymmetric and relatively compact.

Mutarotation Kinetics.—Since the increase in negative rotation can be interpreted as a direct measure of the amount of polyproline helix formed (Von Hippel and Wong, 1963b), it might be expected that the data from mutorotation of homogeneous single- or double-chain gelatins would be susceptible to kinetic analysis Some initial attempts have been made.

It was found that the reaction kinetics for single-chain mutarotation were neither first nor second order. Although it was often possible to analyze the data in terms of three concurrent first-order reactions, there was no reason to think that this was other than a formal fit. If it is considered that gelatin is a heteropolymer and that aggregation and helix formation probably involve regions of the polypeptide chain that are selected at least partly at random, a heterogeneous reaction could readily result. That is, the structures involved in helix formation are not necessarily identical and may mutarotate at different rates.

The data for β_{12} were found to fit first-order kinetics for about the first 40 minutes. This is illustrated in Figure 8. The half-time of this reaction was 12.6 minutes and the velocity constant was 0.055 min^{-1} However, the total rotational change that this reaction could contribute was found by calculation to be −560°, slightly more than half the total change, indicating that other processes must also be taking place. The latter part of the mutarotation data was not first order but gave a reasonably good fit to second-order kinetics between about 100 and 1200 minutes. This is illustrated in Figure 9. The value of $[\alpha]_\infty$ found to give the best fit was −1900°. This second-order reaction contributed about −470° for a total change of −1030°. The initial rotation of the amino acid residues, −870°, makes the total −1900°.

If it was assumed that the second-order reaction began at time zero and the calculated contribution before 100 minutes was subtracted from the observed rotation, the difference was no longer first order. Therefore it was necessary to assume that there was a lag in the second-order reaction. However, a lag would be expected if intramolecular helix formation is initially preferred over intermolecular helix formation and both processes compete for the same reactive sites.

If the second-order reaction represents a real process, concentration dependence of the formation of the β_{12} helix should have been observed, though not in the initial stages of the reaction. This apparently was seen at the lowest concentration which was studied in

the polarimeter. The specific rotation at concentrations near 0.2 mg/ml was consistently lower between 100 and 1400 minutes than that observed at higher concentrations (Fig. 3). That aggregation was occurring was also indicated by the small concentration-dependent increase in molecular weight of β_{12} seen by light scattering (Fig. 6).

The fact that the data do not fit second-order kinetics as well as might be hoped, particularly beyond 1200 minutes (Fig. 9), may be attributed to heterogeneity of this part of the reaction, as pointed out previously for single chains. Otherwise, the kinetics are entirely consistent with an intramolecular formation of helix followed by a slow concentration-dependent interaction leading to additional helix formation. The latter phase presumably involves elements which do not readily react in the earlier phase, perhaps because they are remote from the site of cross-linking.

Discussion

It is necessary first to reconcile our data which demonstrate a concentration dependence of helix formation in single-chain gelatin with several other contrary reports. Flory and Weaver (1960) worked with rat-tail-tendon collagen which contains about 60% β (Piez *et al.*, 1963) and perhaps some higher aggregates. Therefore it would be expected that the contribution of single-chain components would be relatively small and concentration dependence would be difficult to detect. Similarly, Engel (1962a) worked with calfskin collagen which is rich in cross-linked components. In addition, the experimental conditions were such that rotation increased very rapidly preventing precise measurements. The study by Harrington and Von Hippel (1961b) is more difficult to explain in this fashion. They used carp-swim-bladder collagen which contains only about 30% β (Piez *et al.*, 1963) at concentration between 0.1 and 0.6 mg/ml but still did not observe any concentration dependence. At these very low concentrations β mutarotates very much faster than α and, by contributing most of the initial change in rotation, would tend to make the process appear concentration independent. However, some differences should have been seen. Therefore the possibility of intrachain stabilization cannot be completely discarded. Though no evidence was seen for it in the studies reported here, it might occur under some conditions. If this is the case, chain-folding rather than a single-chain helix, as suggested by Harrington and Von Hippel (1961b), seems the more likely mechanism.

A crucial question in assessing the importance of aggregation is whether the molecular weight increases fast enough to explain the rate of helix formation by single chains. This can best be answered by examining the data at low concentrations, since at high concentrations aggregation is rapid and extensive. At 0.24 mg/ml the increase in negative rotation was about 700° in 1400 minutes (Fig. 1) while the weight-average molecular weight doubled (Fig. 6). Since the minimal aggregate for helix formation is presumably a dimer (see below) and the total change in rotation is about 1000°, this is apparently sufficient to explain the amount of helix formed. At earlier times the two measurements followed a similar time course. The fraction of aggregate, calculated as dimer, was always equal to or greater than the fraction of helix under the conditions employed. Additional studies are necessary to determine the generality of this conclusion. In any case, the present experiments establish the importance of chain interaction in helix formation and indicate that it is probably necessary for helix stabilization.

This raises the question as to the type of structure resulting from this interaction. First of all, it has become clear that in gelatins containing high-molecular-weight aggregates essentially complete renaturation to the triple-chain collagen structure can occur. This is particularly evident from the studies on the covalently linked triple-chain γ-component which occurs naturally (Veis and Cohen, 1960; Rice, 1960; Grassman *et al.*, 1961; Altgelt *et al.*, 1961; Veis and Anesey, 1961) and can be made synthetically (Veis and Drake, 1963). This component has the same molecular weight as the collagen monomer and can be renatured to regain essentially all the properties of native collagen. It is also probable that gelatin can regain a triple-chain structure over at least short distances along associated peptide chains. Engel (1962b) has demonstrated that an annealing process involving alternate cooling and partial denaturation by warming of a gelatin solution sharpens the melting curve and produces a viscosity higher than that obtained by a single cooling. The gelatin structure becomes more collagenlike

In spite of this evidence it may not be necessary to assume that helix stabilization in gelatin is possible only through triple-chain associations. Every study which has been done has shown that the amount of helix regained in gelatin solutions as measured by optical rotation never exceeds two-thirds of the amount present in collagen, except in the special case of the γ-component. The annealing experiments of Engel (1962b) are particularly revealing on this point. Even though the melting transition could be sharpened, there was no accompanying increase in optical rotation. That is, the change was not toward more helix but from a less stable to a more stable helix. A reasonable explanation for this is a partial transition from double-chain helices to triple-chain collagenlike structures.

A second point, already mentioned, is that the melting of a helix formed from single- and double-chain gelatins is complete at a temperature where collagen has only begun to melt (Fig. 7). If partial coincidence of the melting curves is evidence of equivalence (Flory and Weaver, 1960) then complete separation is evidence of a fundamental difference. Here, also, the simplest explanation is that gelatin initially consists of double-chain helices.

An additional argument for this structure lies in the properties of the β_{12} helix formed by cooling at a low concentration. Under these conditions the helix forms intramolecularly in a molecule for which there is strong evidence of two chains, but it has an identical helical structure at the local level to that formed by aggregation (of either α or β). If β_{12} were to form a triple-chain helix it would presumably have to do so by folding over on itself. It would seem that if this were possible $\alpha 1$ and $\alpha 2$ could also do it. It is difficult to see how β_{12} could form anything but a double-chain helix by an intramolecular configurational change.

A model of the type of interaction being proposed would be obtained by the removal of a single chain from collagen to leave a double-chain helix. This would break two-thirds of the interchain bonds which are generally described as hydrogen bonds and are believed to be an important stabilizing element. To maintain the structure, it might be necessary to alter the configuration so that some of the bonds could re-form. Alternatively the bonds left may be sufficient since gelatin has both less structure and a less stable structure than collagen.

This same model, a double-chain collagen, may actually be the structure formed by β_{12} at low concentrations. Such a molecule would be expected to have the hydrodynamic properties found for the β_{12} helix. It would

lose the rigidity of a rod but should retain some stiffness and therefore asymmetry as a result of the helical regions. It would also be considerably more compact than the random-coil β_{12}.

With this model in mind, we can speculate on the manner by which interchain stabilization is achieved in collagen and gelatin. If we assume that each turn of the polyproline helix (three residues) requires a minimum of two pyrrolidine rings to stabilize the structure as a single chain (see Harrington and Von Hippel, 1961a), then mammalian collagens, with about 22 residue % total pyrrolidine, have only one-third of the necessary total. However, this is sufficient so that one of the three chains in collagen could have the necessary minimum at every level along the triple-chain collagen molecule. That is, it is possible that in any given region of the collagen molecule two of the three chains are pyrrolidine-poor and one is pyrrolidine-rich. In this manner the one chain, by virtue of the stability of the polyproline helix, could act as the "template" to direct the two adjacent chains into helical form. Overall stability could then be achieved by a regular array of interchain bonds, presumably hydrogen bonds, producing the cooperative structure characteristic of collagen. This structure also has the advantage of permitting a regular distribution of interchain bonds since the pyrrolidine rings, which have no amide hydrogen available for hydrogen bonding, are not grouped at the same levels.

For this arrangement of pyrrolidine rings to be achieved, the three collagen chains would have to be different. It is known that one, the $\alpha 2$ chain, is unlike the other two. The two like chains, the $\alpha 1$ chains, may acquire dissimilarity by being antiparallel. An alternate possibility is that the two like chains are staggered. But this is unlikely since the characteristic banding pattern of collagen aggregates indicates that alternate polar and nonpolar regions occur at the same level in the three chains.

This arrangement of polar and nonpolar residues, if exact, would require that proline and hydroxyproline be distributed throughout both polar and nonpolar regions. Although the present sequence data (Grassman *et al.*, 1960) show less of these amino acids in polar regions, the distribution is not absolute. Further, the distribution need not be equal since the polar and nonpolar regions are probably not aligned exactly, particularly if the two $\alpha 1$ chains are antiparallel. It is also very possible that parts of the collagen molecule have structures other than polyproline helix.

In the case of gelatin, it is quickly evident that the double-chain structure proposed here could produce only two-thirds as much helix as collagen by interaction of pyrrolidine-rich with pyrrolidine-poor regions. However, this is exactly the amount of helix observed in numerous studies. Also, the orderly array of interchain bonds would be disturbed resulting in a less cooperative structure than collagen. This again is consistent with observation.

Carrying this argument further, we can suggest that helix formation by gelatin is initiated by pyrrolidine-rich regions which are able to form a helix that is stable at least as a transient intermediate and that the helix is propagated by association with a pyrrolidine-poor region of another chain (or perhaps a fold of the same chain). The structure is completed by the formation of interchain bonds. In the case of double-chain β-components this process is more efficient than with single chains, which must depend on random collisions, since the appropriate regions are held in proper orientation by the crosslink.

A paper by Drake and Veis (1964) has recently appeared which reports studies on the renaturation of synthetic γ-component. Because this process must involve chain interaction, and by reinterpretation of data in the literature, they suggest that all gelatin structure arises from some type of chain interaction. They ascribe observations of concentration independence of helix formation by unfractionated gelatins to chain-folding.

Acknowledgment

We are indebted to Dr. William Harrington for helpful discussions.

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