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Interchain Interactions in Collagen-Fold Formation. I. The Kinetics of Renaturation of γ-Gelatin*

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The recovery of optical rotation and viscosity was followed as a function of temperature and time for α-chain gelatin obtained by denaturation from buffalo fish–swim-bladder ichthyocol and for a γ-gelatin prepared by the denaturation of synthetically cross-linked monodisperse ichthyocol from the same source. Both gelatins had the same pyrrolidine ring content. The rotation recovery was found to be second order in both cases. The viscosity recovery of the γ-gelatin was first order but the kinetics of viscosity recovery were indeterminate in the case of the α-gelatin. The second-order rate constant for rotation recovery was about 30-fold greater for the γ-gelatin than for the α-gelatin at the same concentration and in the same solvent environment. The activation energy was −10 kcal for the γ-gelatin refolding reaction in contrast to −35 kcal for the α-gelatin. An intramolecular refolding mechanism involving the interaction of chain segments of the same molecule is proposed for both gelatins, with cooperative effects and specific interactions providing the driving force for complete renaturation in the case of the γ-gelatins.

When aqueous solutions of gelatin are cooled below 40° certain characteristic changes may be noted. The reduced viscosity increases, the specific optical rotation becomes more negative, and films dried from the cooled gelatin solutions develop the wide-angle X-ray diffraction pattern and infrared spectrum characteristic of collagen (Smith, 1919; Ferry and Eldridge, 1949; Katz *et al.*, 1931; Bradbury and Martin, 1952; Robinson, 1953). These changes have been related to the formation of a specific peptide chain configuration termed the collagen-fold (Ambrose and Elliott, 1951) and, indeed, serve as a set of operational criteria for the formation of this configuration. In the usual case all of these changes do not follow the same time course; e.g., optical rotatory changes are more rapid than the viscosity changes, indicating that more than one reaction may be involved.

All gelatins so far examined exhibit collagen-fold formation upon suitable treatment, yet it is now clear that there are several specific varieties of gelatin with different macromolecular structures (Veis *et al.*, 1958, 1960, 1961; Orekhovich and Shpikiter, 1955; Doty and Nishihara, 1958; Grassmann *et al.*, 1961; Courts and Stainsby, 1958). The principal difference between gelatin species of mammalian origin is their multichain character, i.e., the degree of covalent cross-linking between peptide strands (Veis and Cohen, 1957). The basic unit is the α chain, a single peptide strand with a molecular weight of approximately 10^5. Two α chains cross-link to form a β-gelatin, three a γ-gelatin. Higher polymers, such as a γ-tetramer, δ, also have been found (Veis *et al.*, 1961). Harrington and von Hippel (von Hippel and Harrington, 1959, 1960; Harrington and von Hippel, 1961) have made a detailed analysis of the formation and stabilization of the collagen-fold as it relates to the configuration of the peptide-chain backbone. The primary step was considered to be the establishment of the poly-L-proline-II trans-trans′ configuration in the pyrrolidine-rich peptide chain sequences, particularly at the prolyl-hydroxyproline bonds. This configuration is rapidly established and serves as a nucleation site for the second step, a slow folding of the remainder of the peptide chain into a loose poly-L-proline-II helix. Finally, association between individual helical segments takes place. In the initial step, Harrington and von Hippel (1961) consider that water plays an integral role in stabilizing the poly-L-proline-II helix by joining successive carbonyl oxygens with water bridges. Their single-chain refolding mechanism was based primarily on the observations that fold formation, as measured by optical rotation, appeared to be independent of the gelatin concentration and that gelatins with different pyrrolidine ring contents had different refolding rates. The effect of intramolecular interchain interactions on the rate of refolding was not considered, although carp ichthyocol, dogfish ichthyocol, and calfskin tropocollagens have been shown to differ in their relative α-, β-, and γ-component composition (Piez *et al.*, 1960, 1961, 1963).

An analysis of the kinetics of the refolding reaction led Flory and Weaver (1960) to propose an alternate mechanism in which the stabilization of an ordered

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segment required the interaction of three units transitorily in the trans-trans' configuration. First-order kinetics were derived by considering the initial single-chain isomerization step to be the rate-controlling reaction. That is, the interaction of the individual helices was taken to be more rapid than the initial helix-forming isomerization reaction. The interhelix interaction was thought to provide the stabilizing energy. Thus, while the steps in both the Harrington and von Hippel (1961) and Flory and Weaver (1960) mechanisms of fold formation are essentially identical, the stabilization factor in each case is clearly different.

Altgelt *et al.* (1961), Engel (1962), and Drake and Veis (1962) have examined the kinetics of renaturation in terms of the gelatin species present. Each of these studies clearly showed that the rate of increase of levorotation on cooling was more rapid for gelatins containing the multichain β and γ components than for those containing α chains at concentrations so low that intermolecular interactions could be discounted. These data appeared to confirm the Flory and Weaver mechanism but suffered from the common disadvantage that mixtures of gelatin components rather than pure components were examined.

Veis and Drake (1963) have shown that one can obtain a γ-gelatin preparation without α and β components by the introduction of a limited number of cross-linkages into monodisperse native tropocollagen molecules. We have taken advantage of this to compare the kinetics of renaturation of α- and γ-gelatin preparations. Both optical rotation and viscosity have been used for measuring the formation of collagen-fold units.

Experimental Procedures

Substrate Material.—The preparation of buffalo fish–swim-bladder ichthyocol (I), and ichthyocol cross-linked with formaldehyde in neutral solution and in acidic solution was previously described by Veis and Drake (1963). Preparation of ichthyocol cross-linked with formaldehyde in neutral solution has now been modified by using 0.2 M $CaCl_2$ solution at *p*H 8.2, adding only 0.1 ml of 37% formaldehyde per 100 ml solution, and reacting for 8 days. The higher *p*H is obtained by adding 1 g of sodium borate per liter. The use of a lower concentration of formaldehyde simplified its removal during isolation of the cross-linked material, particularly since the use of Dimedon as a formaldehyde scavenger is no longer required.

Renaturation Studies.—Viscosity.—Cannon-Ubbelohde viscosimeters with water-flow times of 100–400 seconds were used. The viscosity at low temperature was determined first; then the viscosimeter was placed in a 40° bath from 7 to 30 minutes and the viscosity at 40° was determined. For renaturation the samples were replaced in the low-temperature bath and a clock was used to measure the time of renaturation. The clock time was recorded at the start and end of each measurement. The mid-point of the clock time at each measurement minus the clock time at zero time was used as the time of renaturation. The viscosimeters were sealed with Parafilm and placed at 4–10° in those instances where a viscosity measurement was to be made on a second or third day.

Optical Rotation.—Optical rotation measurements were obtained using a Rudolph Model 80 precision polarimeter equipped with a photoelectric attachment and an oscillating polarizer prism. A mercury arc served as the light source; a filter was used for obtaining the 365-mμ spectral line used in early measurements, and a monochromator was used later. One-decimeter polarimeter tubes equipped with silica end plates were employed. The tubes were water jacketed for temperature control ($\pm 0.3°$).

Baths maintained at the temperatures desired were used to change the temperatures rapidly. Measurements of optical rotation were made at the low temperatures first, then at 40° until the rotation stabilized (about 7 minutes). For renaturation, the water flow through the polarimeter tube jackets was switched from one bath to the other, the change in temperature of the sample solution occurring within the first 2 minutes. Readings could not be taken during the period of rapid temperature change because of refractive-index inhomogenieties resulting from temperature differences within the polarimeter tube. The first readings were obtained only after about 1.5 minutes had elapsed. The stop watch for measuring times of renaturation was started, therefore, 1.5 minutes after the switch in water flow from one bath to the other.

Kinetics.—The transition involved in every case was that from the random-chain configuration to helix. Taking C_r to refer to the molar concentration of molecules in the random form at time t and C_0, to refer to the molar concentration of random chain molecules at zero time, the value of C_t was determined at any time t by the equation

$$C = \frac{(P)_\infty - (P)_t}{(P)_\infty - (P)_0} C_0 \qquad (1)$$

where $(P)_\infty$, $(P)_t$, and $(P)_0$ each refer to the values of the characteristic property, optical rotation or relative viscosity, determined at infinite time, at $t = t$ or at $t = 0$, respectively. The value taken for infinite time for optical rotation was the optical rotation of the native tropocollagen molecule. The value for infinite time for the relative viscosity was chosen as the value of the initial plateau in a plot of η_{rel} vs. t. The reason for this choice will be brought out in the discussion.

The kinetic data were tested in the usual rate equation for reactions involving a single component i.e.,

$$-\frac{dC_r}{dt} = kC_r^n \qquad (2)$$

where n is the order of the reaction. From equation (1) it can be seen that C_r/C_0 is x, the fraction of chains remaining in random-coil form at any time. Hence equation (2) can be rewritten

$$-\frac{d(xC_0)}{dt} = kx^nC_0^n \qquad (3)$$

which reduces to

$$-\frac{dx}{dt} = kC_0^{n-1}x^n \qquad (4)$$

for expressing each particular set of renaturation data. If first-order kinetics are followed then

$$-\ln x/x_0 = -\ln x = kt \qquad (5)$$

and a plot of ln x vs. t should be a straight line. For second-order kinetics,

$$\frac{1}{x} - \frac{1}{x_0} = kC_0(t) = \frac{1}{x} - 1 \qquad (6)$$

hence, a plot of $(1/x) - 1$ vs. t should give a straight line but each apparent constant (kC_0) should be different.

Results

In previous studies (Harrington and von Hippel, 1961; Flory and Weaver, 1960) collagen-fold formation

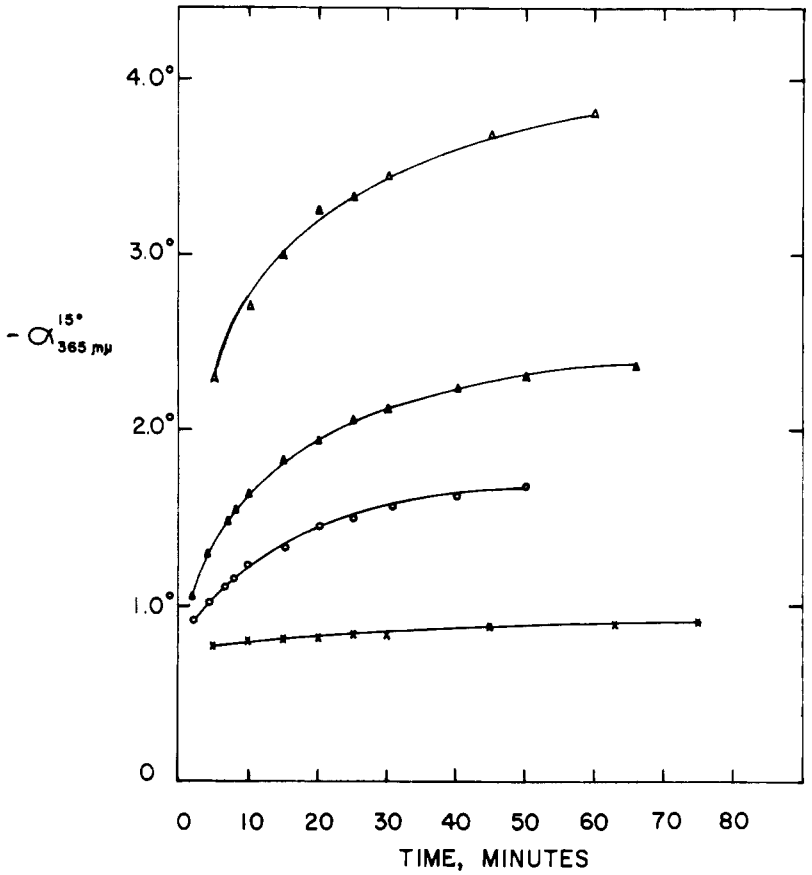

FIG. 1.—Recovery of rotation as a function of time for cross-linked ichthyocol γ-gelatin (○ = 0.15 g/100 ml, ▲ = 0.20 g/100 ml, △ = 0.31 g/100 ml) and for icthyocol α-gelatin (x = 0.16 g/100 ml). All measurements in *p*H 2.8, 0.1 M KCl, 0.1 M acetic acid at 15°.

has been induced by "quenching" a hot thoroughly denatured gelatin solution to a temperature well below the equilibrium melting temperature, T_m. Flory and Weaver (1960) showed that the over-all rate constant, k, for fold formation was related to the temperature difference ΔT ($\Delta T = T_m - T_q$) by the equation

$$k = ce^{-A/kT_q\Delta T} \qquad (7)$$

It is evident from this relationship that, while k would be reduced by raising the "quenching" temperature T_q, any differences between the gelatins would be accentuated by quenching closer to T_m. Cooling to very low temperatures, on the other hand, represents drastic supercooling which might tend to mask the differences between gelatins. In the present study quenching was carried out to only 15 or 20° (ΔT = 10–15°). The rates of fold formation for the α- and γ-gelatins were found to be markedly different.

Typical data are illustrated in Figures 1 and 2 which exhibit direct plots of the optical rotation and relative viscosity as a function of time at the indicated temperature after quenching from 40°. In view of the importance of the concentration-dependence question, rotation data are plotted in Figure 3 in terms of x, the fraction in random coil form, versus t. The α-gelatin rotation recovery has been reported to be independent of the gelatin concentration (Harrington and von Hippel, 1961) but it is evident that the γ-gelatin rotation recovery is concentration dependent to a small extent with renaturation being slowed at higher concentrations. The concentration dependence is also emphasized at higher renaturation temperatures. The same behavior is evident in Figure 4 in the plot of the reduced viscosity recovery. At the lowest concentration the initial viscosity recovery rate was greatest.

The denatured cross-linked ichthyocols, that is, the γ-gelatins, showed a very rapid recovery of collagen-fold character during the time in which the predominately single or α-chain ichthyocol gelatin (78% α, 22% β) recovered only slightly. Maximally rapid and complete recovery of a particular γ-gelatin preparation was observed during the first renaturation cycle only. The effect of repeated denaturation and renaturations of the cross-linked ichthyocols was to progressively decrease the initial rate of refolding and to lower the plateau value for specific rotation, Figure 5, or for the reduced viscosity.

Complete recovery of the optical rotation and viscosity values observed before the first denaturation was never achieved. Before the first denaturation $[\alpha]^{15}_{365}$ was −1350° and −1380° for ichthyocol and cross-linked ichthyocol, respectively. Denaturation lowered these values to −460° and −464° ($[\alpha]^{40}_{365}$), and subsequent renaturation brought them back to only −790° for ichthyocols and −1288° ($[\alpha]^{15}_{365}$) for cross-linked ichthyocols after 16 hours. These 16-hour values represent a 37% recovery of mutarotation for the α,β mixture of ichthyocols, and a 90% recovery for the γ-gelatin.

As indicated by the time scales in Figures 1 and 2, the recovery of the reduced viscosity for γ-gelatin roughly parallels in time the recovery of mutarotation. However, the initial native ichthyocol had an intrinsic viscosity of 15.4 dl/g at 15° so the 80-minute recovery to η_{red} = 10.5 dl/g appears to indicate a recovery of only 68% of the original rodlike character of the tropocollagen. The reduced viscosity of the renaturing γ-gelatin continued to increase very slowly for at least 2 days after the renaturation was begun. Although, as just stated, the reduced viscosity at 80

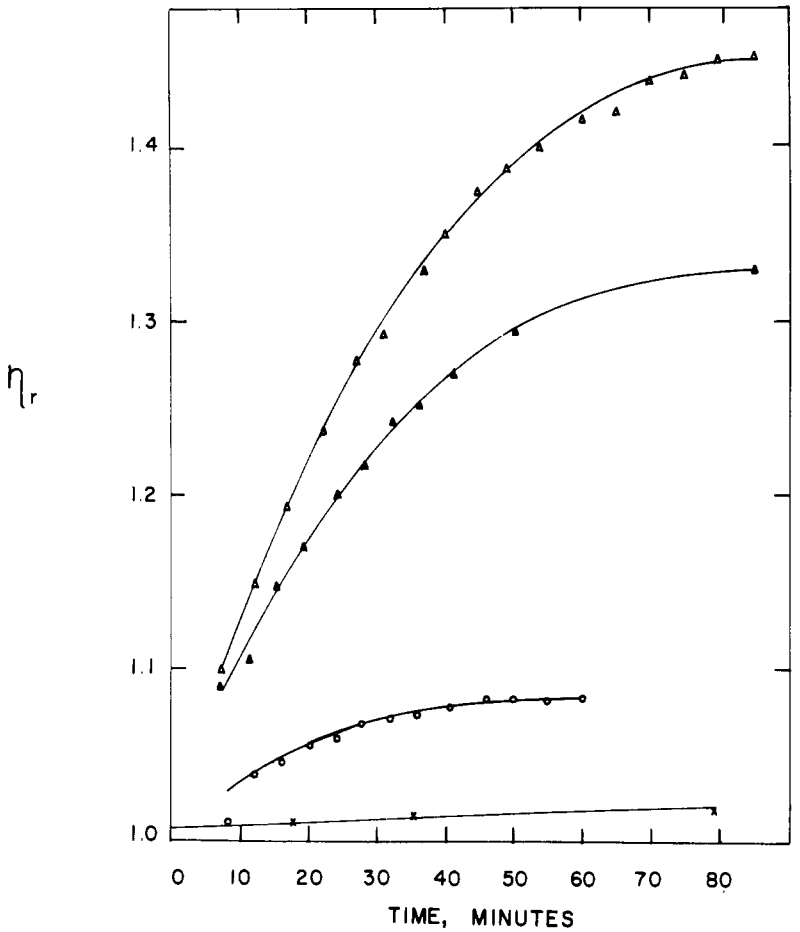

FIG. 2.—Recovery of relative viscosity with time for cross-linked ichthyocol γ-gelatin (○ = 0.009 g/100 ml, ▲ = 0.033 g/100 ml, △ = 0.042 g/100 ml) and ichthyocol α-gelatin (x = 0.17 g/100 ml). All measurements in *p*H 3.6, 0.15 M sodium citrate at 20°.

minutes was substantially less than the intrinsic viscosity of the native ichthyocol, it was readily apparent that completely rodlike tropocollagen molecules were formed at this stage in the renaturation. The renatured γ-gelatins would form both native-type fibers and segment-long-spacing aggregates under appropriate precipitation conditions (Veis and Drake, 1963).

In marked contrast, the α-gelatin recovery at 15° had made scarcely any progress within the 80 minute "renaturation" period. After one day at 15° the α-gelatin had reached optical rotation values only about 10% of the original value for the native ichthyocol solution. Similarly, the viscosity recovery was slight. Collagen fibers and segment-long-spacing aggregates could not be recovered from the α-gelatin. Clearly, the end product of renaturation, "recovered" or "collagen-fold" α-gelatin, was not in the tropocollagen structure.

The kinetics of renaturation were determined through the use of equation (2) in its various integrated forms (equations 5 and 6). The optical rotation data for both the multichain and the single-chain gelatins were found to fit a second-order plot. The term $(1/x) - 1$ was linear in t (Fig. 6). The second-order rate constants determined from renaturations at 15 and 20° are listed in Table I. It should be noted that the second-order constants varied inversely with the protein concentration even at the very low concentrations examined. This point is of particular significance in determining the mechanism of the renaturation reaction.

Analysis of the kinetics of the viscosity increase for the γ-gelatin did not produce reasonable fits for orders up to three as long as the value used for the

TABLE I
SECOND-ORDER RATE CONSTANTS FOR THE RECOVERY OF OPTICAL ROTATION

Gelatin Type	Concentration (g/100 ml)	kC_0 ($sec^{-1} \times 10^4$)	k (ml $g^{-1} \times$ $sec^{-1} \times 10^{-2}$)
I. Recovery at 15°, *p*H 2.8, 0.1 M KCl, 0.1 M HAc			
γ	0.107	12.7	118.7
	0.150	10.7	71.3
	0.199	10.3	51.7
α	0.162	0.44	2.7
II. Recovery at 15°, *p*H 3.6, 0.15 M citrate			
γ	0.05	14.4	238.0
III. Recovery at 20°, *p*H 3.6, 0.15 M citrate			
γ	0.05	10.5	210.0
	0.16	6.5	40.6

viscosity at infinite time was that of the starting material before the first denaturation. The parallel noted earlier between the plateau regions of the optical rotation and viscosity recoveries suggested that it might be more appropriate to take the viscosity at the time when the plateau value of $[\alpha]^{15}_{365}$ was reached as $(\eta_{rel})_\infty$. This takes into account the observation of Veis and Drake (1963) that the native ichthyocol solutions are not monomeric even at very low concentrations and that the true monomer tropocollagen intrinsic viscosity is 9.7 dl/g. Thus the choice of this value for $(\eta_{rel})_\infty$ should reflect the primary formation of the rod form of the individual tropocollagen mole-

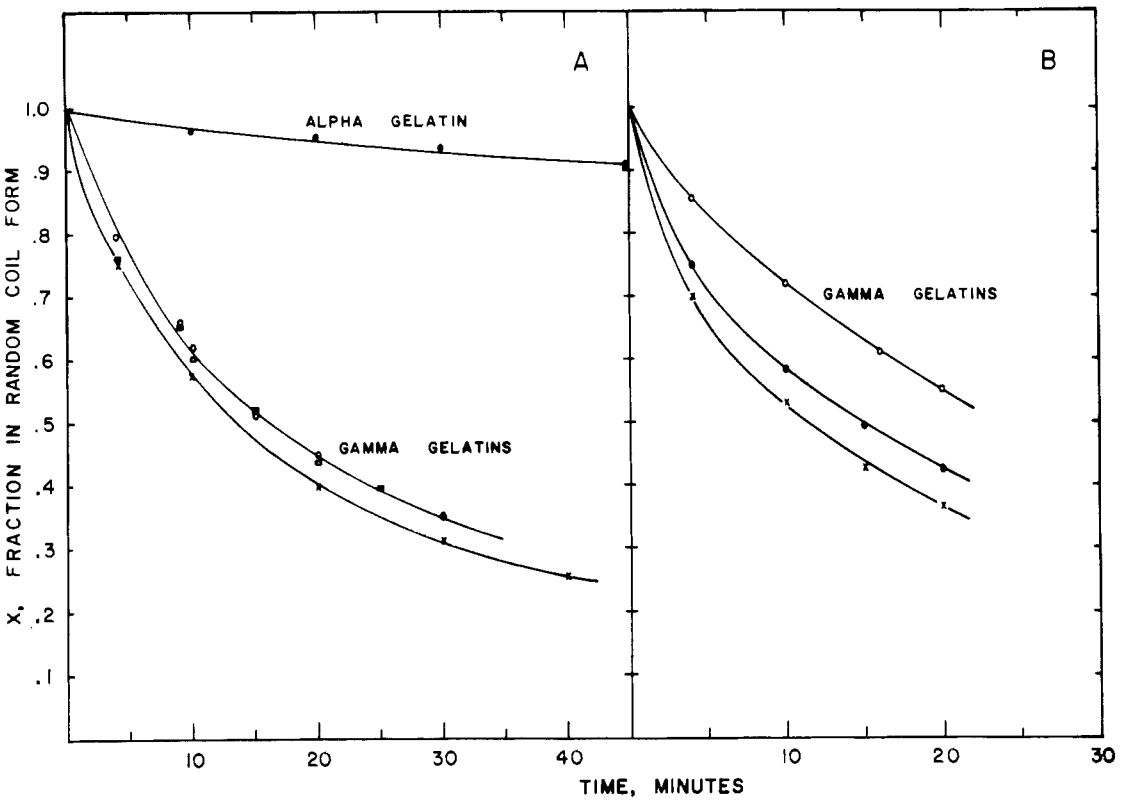

FIG. 3.—The fraction of gelatin in random-coil form as a function of time as determined from optical rotation. (A) Rotation recovery at 15° in *p*H 2.8, 0.1 M KCl, 0.1 M acetic acid. γ-Gelatins (x = 0.107 g/100 ml, O = 0.150 g/100 ml, □ = 0.199 g/100 ml); α-gelatin (● = 0.16 g/100 ml). (B) Rotation recovery in *p*H 3.6, 0.15 M citrate, all γ-gelatins (x = 15°, 0.05 g/100 ml; ● = 20°, 0.05 g/100 ml; O = 20°, 0.16 g/100 ml).

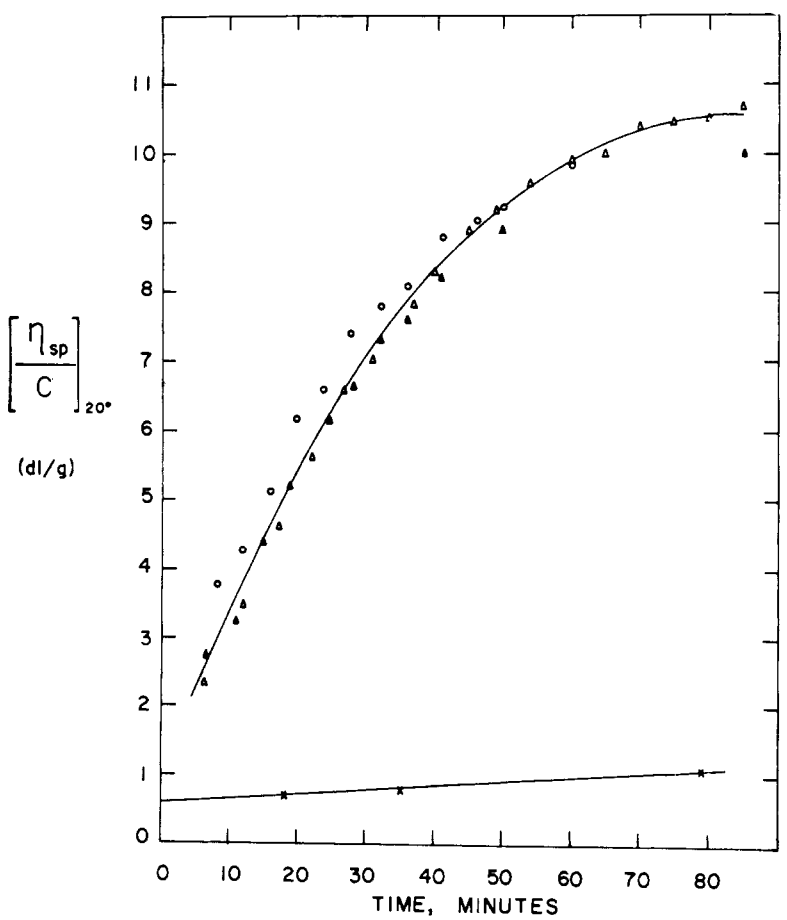

FIG. 4.—The recovery of reduced viscosity as a function of time at 20° in *p*H 3.6, 0.15 M citrate. γ-Gelatins (O = 0.009 g/100 ml, ▲ = 0.033 g/100 ml, Δ = 0.042 g/100 ml); α-gelatin (x = 0.17 g/100 ml).

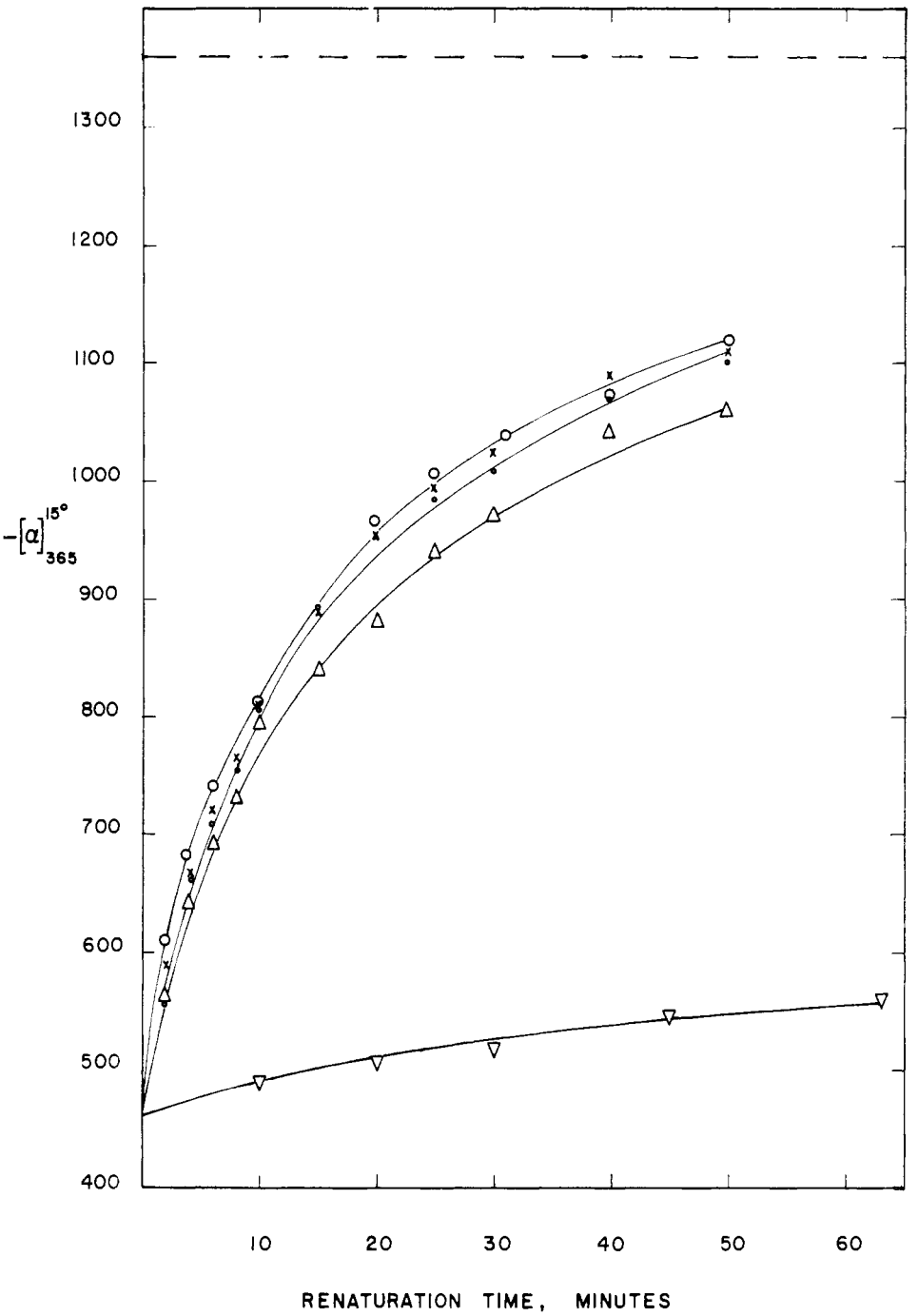

FIG. 5.—The effect of repeated denaturation and renaturation on the recovery of specific optical rotation for a γ-gelatin. Concentration 0.15 g/100 ml in *p*H 2.8, 0.1 M KCl, 0.1 M acetic acid at 15°. O, first renaturation; x, second; o, third, Δ, fourth. The lower curve, ∇, indicates the first renaturation of α-gelatin. The dashed line represents the initial specific rotation of the undenatured ichthyocol.

cules. It was gratifying to find that with this value a first-order reaction plot of ln x vs. t, as shown in Figure 7, gave an acceptable straight line in every case. As indicated by the tabulation of first-order viscosity recovery rate constants in Table II the γ-gelatin viscosity recovery was somewhat concentration dependent, slowing as the total concentration increased. This was much more evident at 20° than at 15°. Harrington and von Hippel (1961) have shown that the α-gelatin viscosity recovery is markedly concentration dependent. It was not possible to fit the α-gelatin viscosity recovery data to any integer-order rate equation.

The 15 and 20° *p*H 3.6 rotation and viscosity recovery data were used to estimate the activation parameters for the renaturation reaction at a concentration of 0.05% γ-gelatin. The values are compared in Table III. As anticipated from the work of Flory and Weaver (1960), ΔH^* was negative, ΔF^* was positive, and ΔS^* was large and negative. Both viscosity and rotation recoveries gave similar results. However, the value for ΔH^* of −5 to −10 kcal/mole for the γ-gelatin renaturation was very much less than the α-gelatin enthalpy of activation of ∼ −35 kcal/mole (Harrington and von Hippel, 1961) determined from refolding experiments at temperatures between 2 and 12°. As indicated by equation (7), ΔH^* depends on ΔT, becoming larger as T_q approaches T_m. Thus the difference of ∼ −25 kcal noted between $\Delta H_\alpha{}^*$ and $\Delta H_\gamma{}^*$ represents a minimum difference in the apparent enthalpy of activation for refolding of the two gelatins.

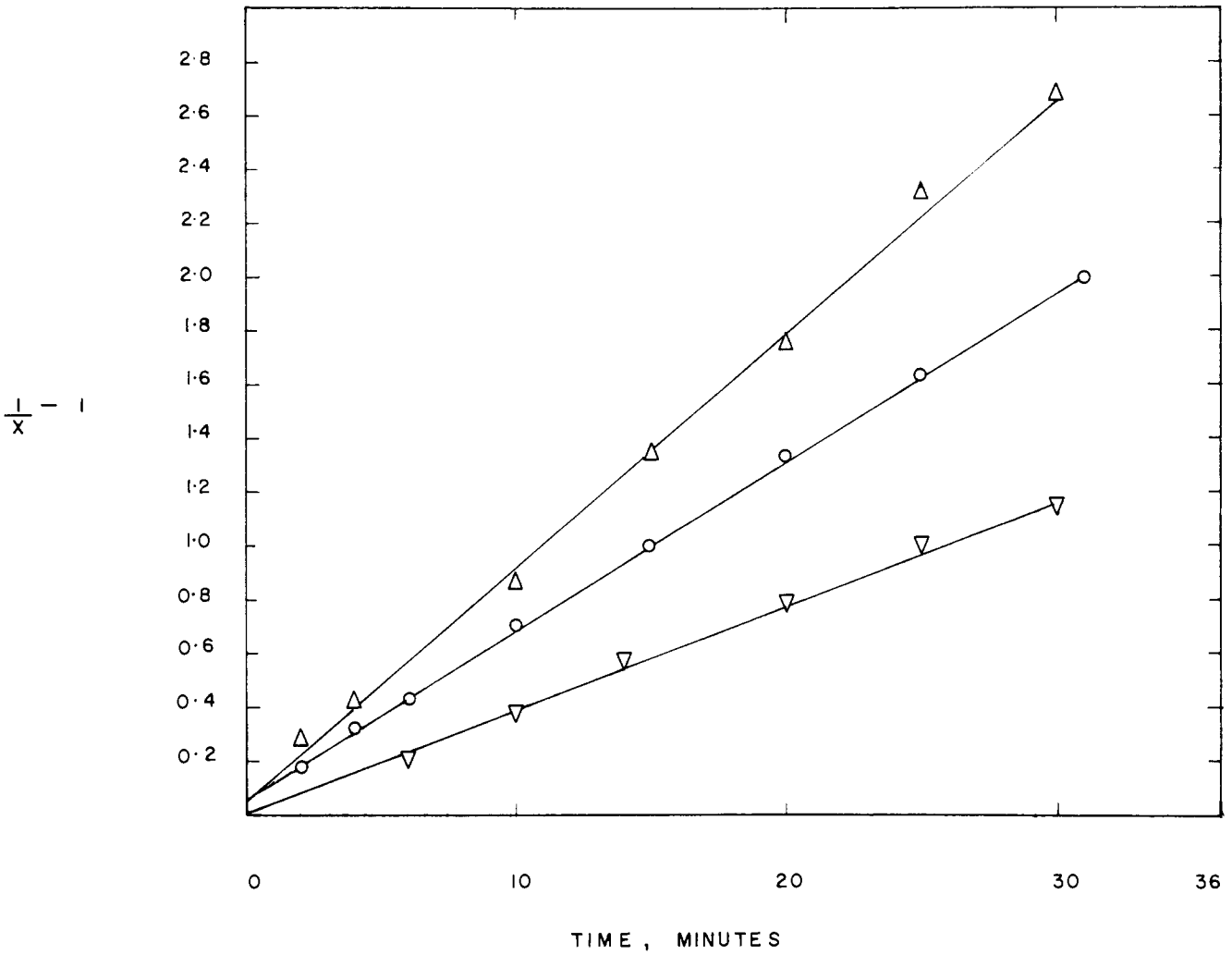

FIG. 6.—Kinetics of the rotation recovery plotted as a second-order reaction in terms of equation (6). Renaturation in 0.15 M citrate, *p*H 3.6. All γ-gelatins (△ — 15°, 0.05 g/100 ml; ○ — 20°, 0.05 g/100 ml; ▽ — 20°, 0.15 g/100 ml).

TABLE II

FIRST-ORDER RATE CONSTANTS FOR THE VISCOSITY RECOVERY OF VARIOUS GELATINS

Gelatin Type	Temperature (°C)	Concentration (g/100 ml)	Rate Constant ($sec^{-1} \times 10^4$)
I. Recovery at *p*H 2.8, 0.1 M KCl, 0.1 M HAc			
γ	15	0.074	5.4
		0.099	5.4
		0.12	5.4
α	15	0.162	0.25[a]
II. Recovery at *p*H 3.6, 0.15 M citrate			
γ	15	0.025	12.4
		0.030	12.4
		0.050	12.4
	20	0.025	11.7
		0.030	11.7
		0.050	8.4

[a] Although the α-recovery data do not fit a straight line in a plot of $-\ln x$ vs. t, this value is for the limiting slope and is included to indicate the magnitude of the rate constant relative to the γ-gelatin case under similar conditions.

TABLE III

APPARENT ACTIVATION PROPERTIES FOR RENATURATION OF γ-GELATIN AT 15°, *p*H 3.6 IN 0.15 M CITRATE

Property	ΔH^* (cal/mole)	ΔF^* (cal/mole)	ΔS^* (ev/mole)
Optical rotation[a]	−10,500	+20,500	−107
Viscosity[b]	−600	+20,600	−74

[a] Concentration 0.05 g/100 ml. [b] Concentration 0.03 g/100 ml.

DISCUSSION

As indicated in Table I, the second-order rate constant for refolding of γ-gelatin was about 30-fold greater than the corresponding refolding rate constant for α-gelatin at the equivalent weight concentration and temperature. This difference cannot be ascribed to differences in the pyrrolidine ring contents of the α- and γ-gelatins since both were derived from the same collagen preparation. It appears from this comparison that the mechanism of refolding was different in each case. It is also clear that the final products, tropocollagen monomer units and collagen-fold-form gelatin, are different. In contrast to these differences, both γ- and α-refolding reactions are second order and the measurements which identify the collagen-fold indicate basic similarities in peptide chain arrangements and bonding in the end products of fold formation. Furthermore, Flory and Garrett (1958) demonstrated that the collagen-fold structures in α-gelatins and in native collagen have the same intrinsic stability as indicated by the identity of their equilibrium melting temperature, T_m. It is this seeming paradox that must be resolved by any proposed

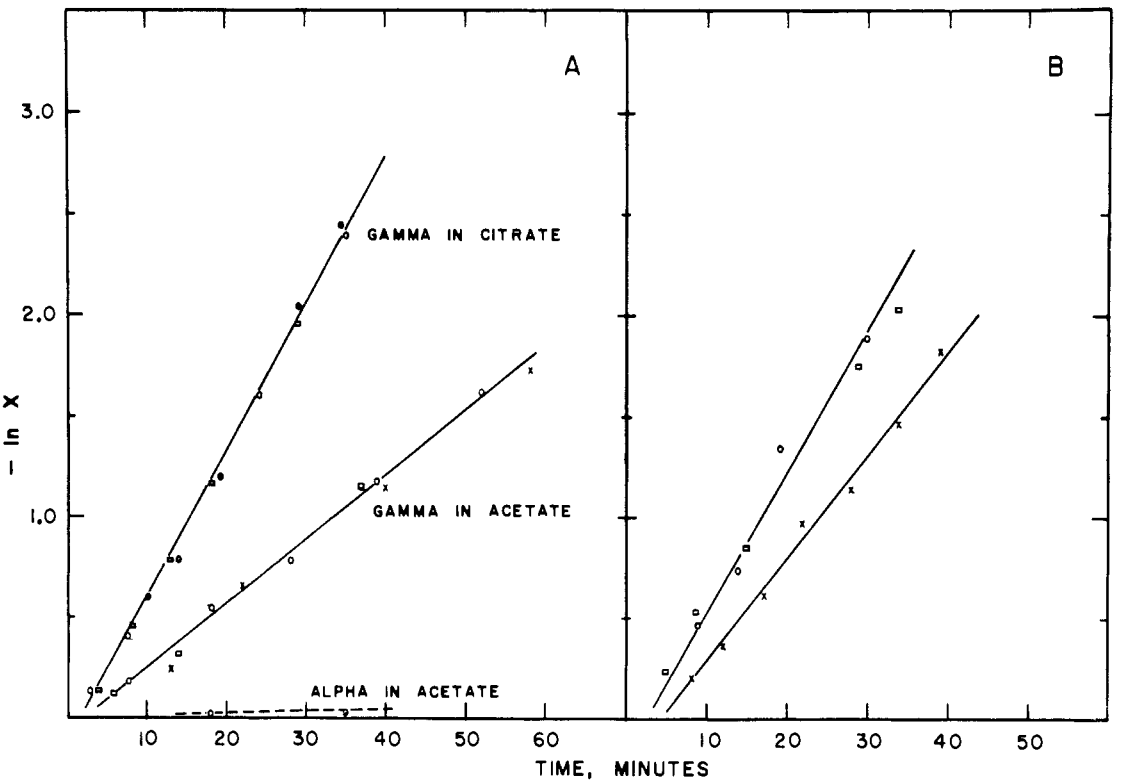

FIG. 7.—Kinetics of the viscosity recovery plotted as a first-order reaction in terms of equation (5). (A) Recovery at 15°. Upper line, γ-gelatin in 0.15 M citrate, *p*H 3.6 (○ = 0.025 g/100 ml, ● = 0.030 g/100 ml, □ = 0.050 g/100 ml). Middle line, γ-gelatin in 0.1 M KCl, 0.1 M acetic acid, *p*H 2.8 (○ = 0.074 g/100 ml, x = 0.099 g/100 ml, ▢ = 0.12 g/100 ml). Lower line, α-gelatin in 0.1 M KCl, 0.1 M acetic acid, *p*H 2.8 (0.16 g/100 ml). (B) Recovery at 20°. All γ-gelatins in 0.15 M citrate at *p*H 3.6 (□ = 0.025 g/100 ml, o = 0.03 g/100 ml, x = 0.05 g/100 ml).

mechanism of collagen-fold formation and any model of a collagen-fold unit.

Ferry and Eldridge (1949) showed that after a 24-hour quenching at 0° followed by heating to the measurement temperature the degree of fold-formation, as indicated by optical rotation, in the α-gelatins was independent of the concentration at concentrations both above and below that at which gel formation occurred. Harrington and von Hippel (1961) further found that the rate of refolding appeared to be independent of the concentration and that the kinetics of the refolding reaction were second order. They proposed that the configuration responsible for the mutarotation developed initially along individual chains and therefore suggested that collagen-fold formation was an intramolecular event involving only single-chain segments and the solvent. It does seem clear that the optical rotation is a reflection of the helix content of each individual chain and is not a reflection of the formation of lateral associations between chains.

Nevertheless, in spite of the fact that the property used to measure the helix content provides information only on the relative number of chain segments in the helix form, a multichain mechanism cannot be ruled out even if the reaction is entirely intramolecular. The second-order kinetics of the mutarotation suggests that two or more chain segments of a single peptide chain may be involved in forming a stable collagen-fold unit.

The principal objection to any multichain mechanism is the apparent concentration independence of the mutarotation rate as indicated above. This objection does not appear to be valid for the following reason. An α-gelatin chain behaves essentially like any other random-chain polymer. As shown by Boedtker and Doty (1956) the weight-average molecular weight of denatured ichthyocol gelatin is on the order of 135,000. For an even lower molecular weight gelatin (Knox P-111-20) with a weight-average molecular weight of only 90,000 the weight-average end-to-end chain extension at infinite dilution was found to be 258 A in good solvents at 40° (Boedtker and Doty, 1954) or in 2.0 M potassium thiocyanate at room temperature. The weight concentration within the domain of an "average" single strand molecule is thus on the order of 2×10^{-3} g/ml. Hence it is clear that it is meaningless to talk of eliminating chain segment interaction by dilution below this value. The effective chain segment concentration is always on the order of 2×10^{-3} g/ml in the vicinity of any particular chain segment. A similar computation shows that a solution of this gelatin is also effectively "filled" at a weight concentration of 2×10^{-3} g/ml so that interchain segment contacts are equally as probable as intrachain segment contacts at that concentration. Dilution thus lowers the probability of network- or gel-forming bonds but does not decrease the probability of what Ferry (1954) has called "cyclic" intramolecular bonds.

Both Flory and Weaver (1960) and Harrington and von Hippel (1961) have associated the collagen-fold with the poly-L-proline-II structure which can form no intrahelix hydrogen bonds at the pyrrolidine rings and hence is "locked" into its helix form at high dilutions by restricted rotation about the peptide bonds (Harrington and Sela, 1958; Steinberg *et al.*, 1960). It may be equally as profitable to liken the collagen-fold configuration to the polyglycine-II structure, which is isomorphous with that of poly-L-proline-II. In polyglycine-II, however, the stabilization is achieved entirely through a three-dimensional network of inter-peptide strand hydrogen bonds. Peptide sequence analyses of collagen are much more readily compatible

with a polyglycine-II-like stabilization mechanism. Abundant evidence indicates that glycine is distributed uniformly throughout the structure (Grassmann *et al.*, 1959), occupying every third residue position. Proline generally occurs following glycine in sequences such as –gly–pro–X–gly–. Although in mammalain collagens the hydroxyproline content is almost equivalent to the proline content, the sequence –gly–pro–hypro–gly– accounts for only 23% of the hydroxyproline content (Kroner *et al.*, 1955; Schrohenloher *et al.*, 1959). Proline and hydroxyproline are thus primarily located in different regions of the molecule (Mandl, 1961). The sequence –pro–pro– has not been reported.

Thus, while isomerizations about the prolyl-hydroxyproline bonds (related of course to the total pyrrolidine ring content) may indeed nucleate collagen-fold formation (Josse and Harrington, 1963), the stabilization of the residues adjacent to these sequences is probably accomplished by a polyglycine-II-like arrangement of bonding with neighboring chain segments. A key factor in this proposal is that the second or "neighbor" segments need not contain the prolyl-hydroxyproline sequence. Since it is likely that any –gly–pro–X–gly– sequence could participate in fold formation, only the highly polar chain regions, containing the acidic and basic functional groups and relatively little proline and hydroxyproline, would be excluded from random intersegment collagen-fold formation. Grassmann *et al.* (1960) have shown that the collagen peptides are made up of alternating regions of polar and apolar character. Glycine represents approximately one-third of both regions but the proline and hydroxyproline are accumulated in the apolar regions. The regions which can form the collagen-fold are thus separated, in single peptide strands, by chain regions which can form the fold only with difficulty. These nonfolded regions provide the configurational mobility which permits the folded segments to achieve a suitable juxtaposition.

There are three lines of evidence that support the multichain hypothesis for fold formation.

We shall consider first the available data on fold formation in α-chain type gelatins. Optical rotatory studies by Smith (1919), Ferry and Eldridge (1949), Harrington and von Hippel (1961), and Flory and Weaver (1960) have all shown that the maximal recovery of specific rotation of α-type gelatins is on the order of 70%, whereas at high dilutions the maximal recovery of the reduced viscosity is between 30 and 50%. Furthermore, the recovery of viscosity was found to proceed at a much slower pace than the recovery of the optical rotation. The rotatory power recovery following 4° quenching was essentially complete before the reduced viscosity had achieved less than 50% of its total recovery. Assuming that the rotatory change does indeed reflect helix formation these data indicate that the viscosity increase in the single-chain gelatins takes place *after* the helical segments are formed. This is not consistent with a single-chain helix growth mechanism to form random structures with greater chain stiffness. In terms of the multichain mechanism these data are more readily explained. Fold formation stabilizes the single-chain molecule in a compact form with a reduced viscosity only slightly increased over the random-coil viscosity. The slow viscosity increase is probably the result of intermolecular aggregation. Engel (1962) measured the changes in light scattering following the quenching of an α-type gelatin to 4°. Even at concentrations as low as 1×10^{-4} g/ml the molecular weight was found to increase. At concentrations of only 6×10^{-4} g/ml the values of the weight-average molecular weight were found to exceed the molecular weight of the original tropocollagen. Engel found the reduced viscosity changes to follow the changes in light-scattering intensity. At the higher concentrations where the molecular weight was greater by a factor of 2 or 3 than the original tropocollagen weight the reduced viscosity was still a small fraction of its "original" native value. It is also interesting to note that the viscosity increase may take place without any appreciable increase in the specific rotation. This suggests that, at least at temperatures relatively close to T_m, a dynamic equilibrium exists in which the units in the less stable collagen-fold segments break down and re-form more stable units with more appropriately matched interchain interactions. This same process is seen in the "tempering" or "aging" of concentrated gels during which the gels increase in rigidity and melting point without any change in their specific rotation.

Finally, Ferry and Eldridge (1949) showed that the degree of folding after 4° quenching and 24-hour aging was dependent on the gelatin molecular weight, in the range from M_w = 72,000 to 33,000, decreasing with decreasing particle weight. This behavior is not consistent with a single-chain refolding mechanism unless very large chain segments at the ends of each chain are excluded from fold formation.

We now turn our attention to the renaturation of the γ-gelatins. Characterization studies (Veis *et al.*, 1962) show that γ-gelatin has no secondary or organized structure in solution at 40° but that it is not typically a single-chain random coil. It is rather a loose network of randomly contorted peptide-chain segments constrained only at the occasional chain-junction points or cross-link sites. The chain-segment density is probably higher within the molecular domain of the γ-gelatin than within the molecular domain of an α chain at the equivalent weight concentration. It is unlikely, however, that the role of the cross-linkages in increasing the rate of mutarotation is solely an effect of increasing the chain concentration. With nearly a 30-fold increase in k being observed at 15° for γ-gelatin, two possibilities are that additional nucleation sites for fold formation are being provided in the regions of restricted chain motion near the cross-linkages, or that the cross-linkages restrain the chains so that the proper alignment of adjacent segments is enhanced. Evidence to indicate that it is the latter proposal can be seen by a comparison of the kinetic data of Altgelt *et al.* (1961) on natural calfskin γ-gelatin with the present data on the cross-linked ichthyocol γ-gelatins. Altgelt *et al.* (1961) carried out their γ-renaturation at 25° at 0.067% and in the *p*H 3.6, 0.15 M sodium citrate buffer. This corresponds to a ΔT of 11° which is thus comparable to the cross-linked ichthyocol renaturation at 20° (ΔT = 11°). The value of (kC_0) for the calfskin γ system was 4.3×10^{-4} sec^{-1}, which is quite reasonably close to the value of 10.5×10^{-4} sec^{-1} indicated in Table I for the ichthyocol. We would expect (Veis and Drake, 1963) that the synthetically cross-linked γ-gelatin would contain the maximum number of possible cross-linkages along the body of the molecule whereas the calfskin γ-gelatin is probably less highly cross-linked and possibly cross-linked only at one end (Rubin *et al.*, 1963). This comparison, therefore, suggests that the relative positioning of the chains is of the utmost importance.

A lowering of the plateau values for both mutarotation and viscosity upon repeated denaturation and renaturation was observed in the present study, as shown in Figure 5, as well as in the studies of Altgelt *et al.* (1961). Veis and Drake (1963) demonstrated

that the cross-links in the synthetic ichthyocol γ-gelatin were heat labile so that the γ-gelatin slowly converted to the α-chain form. Natural γ-gelatin is likewise degraded by prolonged heating. In line with the idea that a specific intramolecular interaction of the three chains of the γ unit is required to reproduce a tropocollagen rod, any formation of α chains would have the double effect of reducing the number of intact potential tropocollagen units and of competing with the intramolecular association. Veis *et al.* (1961) showed that collagen-fold formation in mixtures of α- and γ-gelatins could lead to the precipitation of fibers without evident substructure due to the incorporation of the α-gelatin into the partly renatured γ-gelatin fibers.

The first-order kinetics of the γ-gelatin viscosity recovery is another clear indication of the intra-γ-gelatin nature of the renaturation reaction. In the highly cross-linked synthetic γ-gelatin the recovery of rotation and viscosity proceed at nearly the same rate, particularly in the initial stages of the renaturation. In the less highly cross-linked natural calfskin γ-gelatin the viscosity increase was slower than the rotatory power recovery (Altgelt *et al.*, 1961). The first-order viscosity recovery rate constants for the two γ-gelatins, again calculated at equivalent ΔT, were $k_{\text{synthetic ichthyocol }\gamma} = 10 \times 10^{-4}$ sec^{-1} and $k_{\text{natural calfskin }\gamma} = 5 \times 10^{-4}$ sec^{-1}.

X-ray diffraction studies (Rich and Crick, 1961; Ramachandran *et al.*, 1961) have indicated that most of the native collagen molecule must be in the polyglycine-II or poly-L-proline-II type of configuration. The nearly complete recovery of monomer viscosity and of the rotation of the γ-gelatin signifies that the renatured gelatins are likewise in this form. The greater value of k for the viscosity recovery of the more highly cross-linked ichthyocol γ-gelatin than for the calfskin γ-gelatin suggests that the cross-linkages aid in the chain-stiffening reaction by helping to force collagen-fold formation through the regions of highly polar character. Regardless of the nature of the cross-linkages (ester or aldehyde), all available evidence indicates that they are located in the highly polar chain regions (Veis and Drake, 1963; Blumenfeld and Gallop, 1962). As mentioned earlier, it is probable that these chain regions always remain amorphous in the α-chain refolding.

The nature of the driving force and the fact of complete renaturation can be explained best in terms of the collagen structure recently proposed by Ramachandran *et al.* (1961). In this structure two interchain hydrogen bonds are normally formed for every three residues. The basic requirement is that glycine occupy every third residue position in each chain. While the –gly–pro–hypro– sequence can be accommodated in this doubly hydrogen-bonded structure forming five hydrogen bonds per nine residues it can occur in only one of the three chains at any particular point. The other two adjacent chain segments must not contain this sequence. If this is a correct picture of the collagen structure, then during renaturation a prolyl-hydroxyproline sequence is juxtaposed with two chain segments which do not contain this sequence. The nucleating isomerization at each prolyl-hydroxyproline sequence can then encourage the formation of a stable polyglycine-II-like hydrogen-bonded structure on the two adjacent segments. The H–D exchange data of Bensusan and Nielsen (1963) are consistent with this interpretation.

The slight decrease in the apparent rate constant (kC_0) for the rotation recovery of the γ-gelatin with increasing concentration can be explained in terms of increased chances for random intermolecular interaction. The randomization of the intersegment interaction inhibits development of the fully native structure. The same argument applies here as in the case of the addition of α-gelatin to a γ-gelatin system. If, instead of comparing the apparent rotation recovery constants kC_0, one compares k as in Table I, the effect of increasing concentration is seen to be very great indeed. On the other hand, in the highly cross-linked γ-gelatin systems the expected decrease in chain stiffening with increasing concentration may be reduced because of a counterbalancing effect of enhanced molecular weight upon formation of an intermolecular collagen-fold junction point. This observation is in line with the data of Veis *et al.* (1961) and Engel (1962). Both these studies indicated that fiber formation in neutral renaturation systems occurred most efficiently at the lower protein concentrations and was improved more by temperature cycling (tempering) than by keeping the system at a low quenching temperature.

A most surprising result was that the enthalpy of activation for fold formation in the γ-gelatin was much smaller than for fold formation in the α-gelatins. This result implies that the restrictions to configurational mobility in the vicinity of the interchain cross-linkages promote other interchain interactions which may reduce the randomness of the γ-gelatin in the denatured state. Nevertheless the denatured state ought to be considered as containing no helically wound segments. An enhancement in the salt-binding properties of the multichain gelatins was previously interpreted in this same fashion and is consistent with the present data (Veis *et al.*, 1958).

In summary, it appears that collagen-fold formation in the gelatins can be accounted for as an intramolecular reaction in which different segments of the same molecular chain must interact to form stable collagen-fold units. The basic differences between α-gelatin fold formation and γ-gelatin renaturation are those of specificity and cooperativeness. The α-gelatin intrachain associations are random and force a substantial fraction of the chains ($\sim 30\%$) to remain amorphous. The stability of the collagen-fold-form α chain can be improved by tempering which permits a progressive rearrangement of the collagen-fold units. The individual chain segments in the γ-gelatins, on the other hand, find the specific neighboring chain partners which permit the establishment of a polyglycine-II-like hydrogen-bond network throughout the molecule and by a cooperative effect force fold formation through the proline and hydroxyproline–poor peptide-chain regions. These chain regions may be juxtaposed with a sequence containing a prolyl-hydroxyproline segment. The γ-gelatin renaturation is slowed at higher concentrations, and in the presence of α-chain material, because these conditions lead to some random-chain interaction. These random collagen-fold units may be removed by tempering at elevated temperatures.

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Further Synthetic Studies with Phosphorylated Peptides*

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Synthetic carbobenzoxy-β-benzyl-L-aspartyl-L-serylglycine benzyl ester and carbobenzoxy-β-benzyl-L-aspartyl-L-seryl-L-glutamic dibenzyl ester were phosphorylated by means of di-*p*-nitrobenzylphosphorochloridate in the presence of imidazole in 90% and 50% yield, respectively. The crude phosphate triesters, carbobenzoxy-β-benzyl-L-aspartyl-*O*-(*O,O*-di-*p*-nitrobenzylphospho)-L-serylglycine benzyl ester and carbobenzoxy-β-benzyl-L-aspartyl-*O*-(*O,O*-di-*p*-nitrobenzylphospho)-L-seryl-L-glutamic dibenzyl ester could not be purified by fractional precipitation from usual solvents; however, countercurrent distribution has been found to be a suitable technique for isolating these and related substances in analytically pure form. Preliminary experiments on the synthesis of phosphodiesters of serine are also described. In this connection, *O*-butylphospho-D,L-serine was synthesized and its isolation from by-products, *e.g.*, phosphoserine and serine, was effected by countercurrent distribution.

The study of phosphoproteins, with special reference to the modes of linkage of the phosphoryl groups, has been hampered by the experimental difficulties in the available methods for the synthesis of phosphopeptides needed as model compounds.

In our laboratory we are investigating the problem of synthetic methods leading to the desired model phosphopeptides containing phosphomonoester, phosphodiester, mixed-amide ester, and pyrophosphate bonds. Diphenyl- or dibenzylphosphorochloridate has been used for the phosphorylation of the hydroxyl function of *N*-carbobenzoxy peptide esters of serine (Riley *et al.*, 1957; Fölsch, 1959), but the limitations of these reagents are particularly evident. Phosphorylation by these reagents does not proceed to completion and, since no method is available for the purification of the oily phosphotriesters usually obtained, the homogeneity of the products remains in doubt. In fact, hydrogenolysis of oily diphenylphosphate esters proceeds very slowly and the end products are contaminated with monophenyl derivatives, unphosphorylated products, and, in certain cases, with traces of the respective amino acids (Fölsch, 1959; Theodoropoulos *et al.*, 1960a). Isolation of the desired phosphopeptides from these by-products requires a laborious fractionation on ion-exchange columns (Strid, 1959).

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