Pull, S. P., Pueppke, S. G., Hymowitz, T., & Orf, J. H. (1978) *Science (Washington, D.C.) 200*, 1277.
Reisfeld, R. A., Lewis, U. J., & Willis, D. E. (1962) *Nature (London 195*, 281.
Strosburg, A. D., Lauwerys, M., & Forieis, A (1983) in *Chemical Taxonomy, Molecular Biology, and Function of Plant Lectins* (Goldstein, I. J., & Etzler, M. E., Eds.) pp 7-20, Alan R. Liss, New York.
Sumner, J. B., & Howell, S. F. (1936) *J. Biol. Chem. 115*, 583.
Thanh, V. H., & Shibaski, K. (1976) *J. Agric. Food Chem. 24*, 1117.
Tsien, H. C., Jack, M. A., Schmidt, E. L., & Wold, F. (1983) *Planta 158*, 128.

In Vitro Collagen Fibril Assembly in Glycerol Solution: Evidence for a Helical Cooperative Mechanism Involving Microfibrils

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ABSTRACT: Glycerol inhibits the in vitro self-association of monomeric collagen into fibrils and induces the dissociation of fibrils preassembled from $NaBH_4$-reduced collagen. These effects were investigated in an effort to understand the mechanism of fibril assembly of the protein. In PS buffer (0.03 M NaP_i and 0.1 M NaCl, pH 7.0) containing 0.1-1.0 M glycerol, the self-association of type I collagen from calf skin took place only if the protein concentration was above a critical value. This critical protein concentration increased with increasing glycerol concentration. Velocity sedimentation studies showed that below the critical protein concentration and under fibril assembly conditions, the collagen was predominantly in a monomeric state. Electron microscopic examinations revealed that the collagen aggregates formed above the critical concentration consisted mostly of microfibrils of 3-5-nm diameter along with some banded fibrils. Upon removal of glycerol from the solution through equilibrium dialysis, only banded fibrils were found. Collagen treated with pepsin to remove its nonhelical telopeptides also self-associated into microfibrils and fibrils in the presence of glycerol, but the reaction did not exhibit any critical concentration. These results are consistent with a mechanism of in vitro collagen fibril assembly which involves the initial formation of microfibrils through a helical cooperative mechanism. They also suggest that contacts of the nonhelical telopeptides of each collagen with its neighboring molecules provide the necessary negative free energy change for the cooperativity and that subsequent lateral association of the microfibrils leads to banded fibrils.

The interstitial collagens are fibrous proteins with a unique amino acid composition rich in glycine, proline, and hydroxyproline. Their secondary structures differ substantially from those of globular proteins in that they consist of three polypeptides, each of which is a left-handed helix, intertwined into a right-handed triple helix. In extracellular matrices, the triple-helical collagen exists in fibril form. Little is known regarding the mechanism of formation of collagen fibrils from the monomers. In vitro, monomeric collagen can be induced to self-associate into fibrils by incubation at 25-30 °C near neutral pH. The kinetics of the in vitro collagen fibril assembly can be divided into two stages, initiation and growth (Wood, 1960a,b; Wood & Keech, 1960; Comper & Veis, 1977a,b; Williams et al., 1978; Gelman et al., 1979a). During the fibril initiation period, the solution does not display any turbidity, indicating that large fibril structure has not yet formed. The growth of the fibrils follows the initiation period and is characterized by a sigmoidal increase of the solution turbidity and the appearance of collagen fibrils with a 67-nm repeat band pattern. In addition to the banded fibrils, unbanded microfibrils of 2-4-nm diameter have been observed during in vitro fibril assembly (Gelman et al., 1979a). The exact time of the formation of the microfibril and its structural relationship with the banded fibrils are not clear.

There are few reported equilibrium studies of in vitro collagen fibril assembly. Piez and co-workers showed that the in vitro collagen fibril assembly entails no critical concentration (Williams et al., 1978; Gelman et al., 1979a,b). In addition, their kinetic studies showed a linear plot with a slope of -1 of the logarithm of the time required for half of the collagen to assemble into fibrils vs. the logarithm of the total collagen concentration. On the basis of these observations, it was suggested that the fibril assembly reaction proceeds through an accretion mechanism and does not involve the Oosawa helical cooperative assembly mechanism as has been shown in the actin filament and microtubule formations (Oosawa & Kasei, 1962; Gaskin et al., 1974). However, it was pointed out that from their equilibrium data, a very low critical concentration of 7 μg/mL or less cannot be ruled out due to the limited sensitivity of the technique (Williams et al., 1978). As far as the kinetic evidence is concerned, the interpretation of the slope of the double logarithmic plot is uncertain because of the possible presence of collagen oligomers in the initial solution (Chandrakasan et al., 1976; Purich & Kristofferson, 1984). Consequently, the possibility remains that the assembly reaction proceeds through a cooperative mechanism but has a critical concentration which is too low to be detected by available techniques.

In an attempt to further understand the mechanism of collagen fibril assembly, we added glycerol to the assembly buffer to weaken the intercollagen interaction and examined its effect on the chemical equilibrium of in vitro collagen fibril

formation. The results are reported here together with a discussion of the merits of a helical cooperative mechanism for the fibril assembly involving microfibrils. In the following paper (Na, 1986), the effect of glycerol on the thermal stability of collagen was examined, and the preferential interactions of both native and denatured collagens in glycerol solutions were measured to provide a thermodynamic basis for understanding the inhibition of collagen self-association and stabilization of the collagen triple-helical structure by glycerol. The preliminary results of these two papers have been reported elsewhere (Na & Butz, 1983).

Materials and Methods

Spectranalyzed glycerol was from Fisher Co.[1] UV-grade guanidine hydrochloride (Gdn·HCl)[2] was from U.S. Biochemical Corp., and its solution was filtered through a sintered glass funnel before use. Pepsin (3200–3800 units/mg) was obtained from Sigma Chemical Co. All other chemicals used were reagent grade.

Preparation of Calf Skin Collagen. Type I collagen was extracted from fresh calf skins obtained from a local slaughterhouse. All steps of protein purification were carried out at 4 °C. The skin was first rinsed with cold distilled–deionized water to remove debris and then shaved with an animal clipper. Both the fat and the epidermal layer embedded with hair roots were removed thoroughly with a surgical scalpel.[3] The resulting corium layer of the skin, which weighed approximately 2 kg, was rinsed several times, first with 1% NaCl and then with water. After being cut into 1-in. squares, it was extracted by slow stirring in 16 L of 0.5 M HOAc for 16 h. After extraction, the solution was filtered through four layers of cheesecloth and centrifuged at 15000*g* for 30 min to remove insoluble material. From the acidic solvent, collagen was salted out by adding dropwise $^1/_5$th volume of 30% NaCl in 0.5 M HOAc. The protein was collected by centrifugation at 15000*g* for 30 min and subsequently dispersed in 4 L of 0.05 M NaP_i and 1 M NaCl, pH 7.0, buffer. The solution was stirred for 16 h, and the insoluble matter was removed by centrifugation at 15000*g* for 1 h. From this neutral solvent, collagen was again salted out by slowly adding solid NaCl to 3 *m* with constant stirring. The protein was collected by centrifugation at 15000*g* for 30 min, dispersed in 2 L of 0.5 M HOAc, and stirred for 16 h to dissolve. This solution was then centrifuged at 37000*g* for 1 h. From the supernatant of this centrifugation, the salting out of collagen was repeated by adding $^1/_5$th volume of 30% NaCl in 0.5 M HOAc and collecting the protein by centrifugation. The pellets were dissolved in 0.5 M HOAc and then dialyzed exhaustively against 1 mM HOAc. The final collagen solution at a concentration of 10–20 mg/mL was completely transparent and was stored under liquid nitrogen until use.

$NaBH_4$-reduced collagen was prepared by following the method of Gelman et al. (1979a). Pepsin digestion of collagen was carried out by using essentially the method of Gelman et al. (1979b), the only exception being that pepsin was added each time at a weight ratio of 1:100 collagen. After the digestion, the collagen was precipitated by adding $^1/_5$th volume of 30% NaCl in 0.5 M HOAc and collected through centrifugation. It was then dissolved in 0.5 M HOAc, dialyzed exhaustively against 1 mM HOAc, and stored under liquid nitrogen.

The protein concentration was determined by diluting a small aliquot of the sample with 6 M Gdn·HCl and measuring the solution absorbance at 218 nm. An extinction coefficient of 9.43 $mL \cdot mg^{-1} \cdot cm^{-1}$ was obtained through precision dry weight measurements following the protocol of Kupke and Dorrier (1978).

The tyrosine content of the protein was determined by near-UV spectroscopy following the method of Edelhoch (1967); 0.3 mL of the stock collagen was added to 0.6 mL of 9 M Gdn·HCl and 0.03 M NaP_i, pH 6.5. The solution showed an absorption peak at 276 nm. Since collagen does not contain any tryptophan residues, the molar extinction coefficient of 1500 $L \cdot mol^{-1} \cdot cm^{-1}$ was used. A collagen molecular weight of 285000 derived from the amino acid composition of the protein was used in the calculation (Miller, 1984).

Measurement of in Vitro Collagen Fibril Assembly. The in vitro self-association of solubilized collagen into fibrils was carried out in PS buffer (0.03 M NaP_i and 0.1 M NaCl, pH 7.0) with and without glycerol. A small aliquot of the stock collagen, usually 10–20 mg/mL in 1 mM acetic acid, and an equal volume of a double-strength PS buffer were added to PS buffer and dispersed through gentle shaking at 4 °C. A microman pipet (Rainen Co.) with a piston-type disposable tip was used to measure and deliver the highly viscous collagen stock solution. The formation of collagen fibrils was monitored two ways. With the first method, the solution was added to a jacketed cuvette maintained at 30 °C, and its turbidity at 350 nm was measured. In the second method, the sample was incubated in a 1.5-mL microfuge tube. The collagen fibrils assembled were then pelleted by centrifuging with an Eppendorf Model 5413 microfuge for 2 min. The difference in the solution protein concentration measured before the incubation and after the centrifugation was taken to be the amount of fibrils formed.

Analytical Ultracentrifugation. Velocity centrifugation of collagen was carried out with a Beckman Model-E analytical ultracentrifuge equipped with an electronic speed control and an RTIC temperature control. An AN-G six-hole titanium rotor was used. The cells were assembled from sapphire windows and Kel-F centerpieces. The sedimentation boundaries were measured with a UV–visible optical scanner; its analog signal was digitized and stored directly in a computer. The scanning wavelength used ranged from 230 to 235 nm, depending on the concentration of the protein. The weight-average sedimentation coefficients were calculated according to the established method (Schachman, 1959). The density and viscosity of the solvent used in correcting the sedimentation coefficient were measured with an Anton Parr DMA-2 precision densimeter and a Cannon flow viscometer, respectively.

Electron Microscopy. At various stages of in vitro fibril assembly carried out as described above, aliquots were removed from the solution and applied to 200-mesh carbon-coated grids. They were then negatively stained with 1% phosphotungstic acid and examined with a Zeiss Model 10-B electron microscope.

Results

Reversibility of in Vitro Self-Assembly of $NaBH_4$-Reduced Collagen. The reversibility of the fibril assembly reaction of $NaBH_4$-reduced collagen was verified prior to studying the glycerol effect. As shown in Figure 1, in PS buffer, the

[1] Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[2] Abbreviations: Gdn·HCl, guanidine hydrochloride; PS, 0.03 M NaP_i and 0.1 M NaCl, pH 7.0; AS, 0.01 M NaOAc and 0.02 M NaCl, pH 4.0.

[3] Our experience has shown that without thoroughly cleaning the skin, the protein solution subsequently obtained will be turbid and difficult to clear even with repetitive high-speed centrifugations.

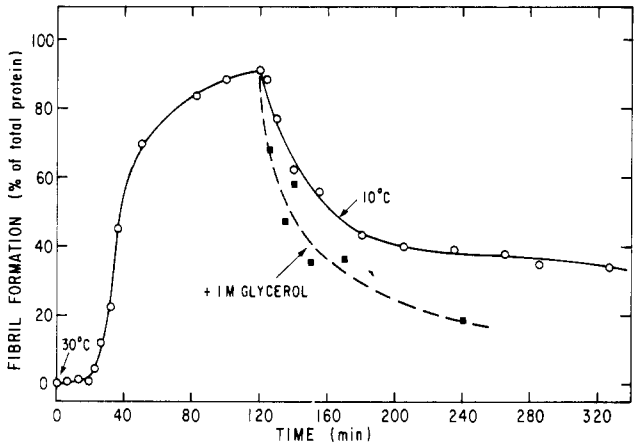

FIGURE 1: Association and dissociation of $NaBH_4$-reduced collagen. Collagen in PS buffer was heated to 30 °C to initiate assembly and then cooled to 10 °C 120 min afterward to induce disassembly (○). In several other aliquots, glycerol was added to a final concentration of 1 M, and the samples were maintained at 30 °C to follow the glycerol-induced disassembly of the fibrils (■). Fibril concentration was determined by the centrifugation method.

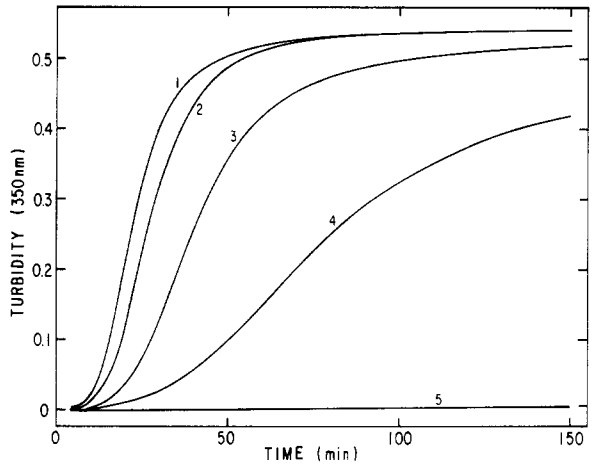

FIGURE 2: Effect of glycerol on the kinetics of collagen self-association. $NaBH_4$-reduced collagen (0.12 mg/mL) was added to PS buffer containing (1) 0, (2) 0.02, (3) 0.1, (4) 0.2, and (5) 0.4 M glycerol. The assembly reaction was initiated by bringing the solution to 30 °C, and its progress was monitored by measuring the solution turbidity at 350 nm.

self-assembly of the $NaBH_4$-reduced collagen proceeded in a sigmoidal manner. The fibrils formed were dissociated by lowering the temperature to 10 °C, and the dissociation was usually 90–95% complete after 12 h of cooling.

Effects of Glycerol on in Vitro Collagen Fibril Assembly. The effects of glycerol on both the kinetics and the equilibrium of the in vitro collagen fibril assembly reaction were studied. As shown in Figure 1, introduction of 1 M glycerol to the collagen fibrils preassembled in vitro resulted in rapid disassembly of the fibrils. As depicted in Figure 2, if glycerol was added before the commencement of the assembly, it reduced both the rate and the total amount of fibrils formed. At a collagen concentration of 0.12 mg/mL, the reaction half-time increased from 22 min (curve 1) in PS buffer to 75 min (curve 4) in PS–0.2 M glycerol buffer. In PS–0.4 M glycerol buffer, no collagen self-association can be observed after 2.5 h of incubation (curve 5).

The data in Figure 3 reveal the effects of glycerol on the equilibrium of the in vitro fibril assembly reaction. The self-assembly of the $NaBH_4$-reduced collagen was brought to equilibrium by incubating the solution at 30 °C for 24–72 h. The amount of fibrils formed was plotted against the total

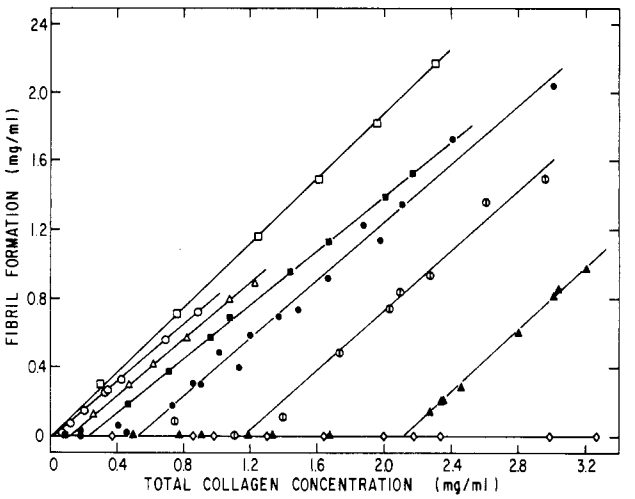

FIGURE 3: Self-association of $NaBH_4$-reduced collagen in glycerol solutions. PS buffer was used, and the samples were incubated at 30 °C for 24–72 h before the amount of aggregates formed was determined through centrifugation and protein concentration determination as described under Materials and Methods. The glycerol concentrations were 0 (□), 0.25 (○), 0.4 (△), 0.5 (■), 0.6 (●), 0.7 (⊕), 0.8 (▲), and 1 M (◇). The critical concentrations and slopes, obtained from linear least-squares fittings of the data, are listed in Table I.

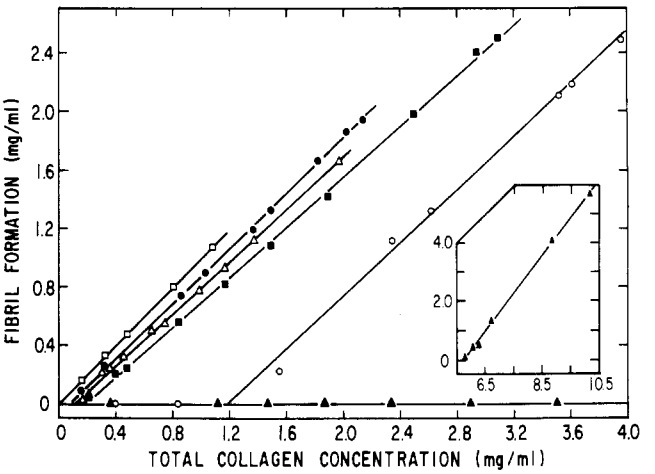

FIGURE 4: Self-association of native collagen in glycerol solutions. The experimental conditions were identical with those of Figure 3. The glycerol concentration were 0 (□), 0.4 (●), 0.5 (△), 0.6 (■), 0.8 (○), and 1 M (▲). The inset shows data collected at 1 M glycerol at high collagen concentrations. The critical concentrations and slopes of the data were obtained from linear least-squares fittings and are listed in Table I.

protein concentration. In PS buffer, the amount of collagen fibrils formed decreased linearly to essentially zero protein concentration (□). However, in PS buffers containing various concentrations of glycerol, the presence of a critical concentration for the fibril assembly reaction was clearly evident. Below the critical concentration, no self-association of the protein could be detected by either the turbidity or the centrifugation method. Above the critical concentration, the protein self-associated to form large visible aggregates. Linear least-squares fitting of the data of Figure 3 gave the critical concentration and the slope of the data above the critical concentration at each glycerol concentration as listed in Table I. The critical concentration of the association reaction increased with increasing glycerol concentration, from 0.036 mg/mL in 0.25 M glycerol to 2.128 mg/mL in 0.8 M glycerol. The slopes of the lines above the critical concentration ranged from 0.80 to 0.93.

Table I: In Vitro Self-Association of Native and $NaBH_4$-Reduced Collagens in Glycerol Solutions

[glycerol] (M)	$[collagen]_{critical}$ (mg/mL)	K_H[a] (M^{-1})	$\Delta G°$[b] (kcal/mol)	slope[c]	correlation coefficient[c]
		$NaBH_4$-Reduced Collagen			
0	0.005 ± 0.014	5.7×10^7	−10.8	0.93	0.999
0.25	0.036 ± 0.002	7.9×10^6	−9.6	0.85	0.999
0.40	0.092 ± 0.009	3.1×10^6	−9.0	0.82	0.998
0.50	0.237 ± 0.005	1.2×10^6	−8.4	0.80	0.999
0.60	0.528 ± 0.029	5.4×10^5	−8.0	0.85	0.999
0.70	1.167 ± 0.041	2.4×10^5	−7.5	0.88	0.999
0.80	2.128 ± 0.012	1.3×10^5	−7.1	0.92	0.999
1.0	>3.26	$<8.7 \times 10^4$	>−6.9		
		Native Collagen			
0	0.002 ± 0.004	1.4×10^8	−11.3	0.99	0.999
0.4	0.079 ± 0.008	3.6×10^6	−9.1	0.95	0.999
0.5	0.113 ± 0.007	2.5×10^6	−8.9	0.89	0.999
0.6	0.196 ± 0.012	1.5×10^6	−8.6	0.86	0.999
0.8	1.187 ± 0.068	2.4×10^5	−7.5	0.89	0.996
1.0	5.755 ± 0.068	5.0×10^4	−6.5	1.30	0.999

[a] $K_H = 1/[collagen]_{critical}$. [b] For polymer growth at 30 °C. [c] These are results of linear least-squares fittings of the data shown in Figures 3 and 4 above the critical concentrations.

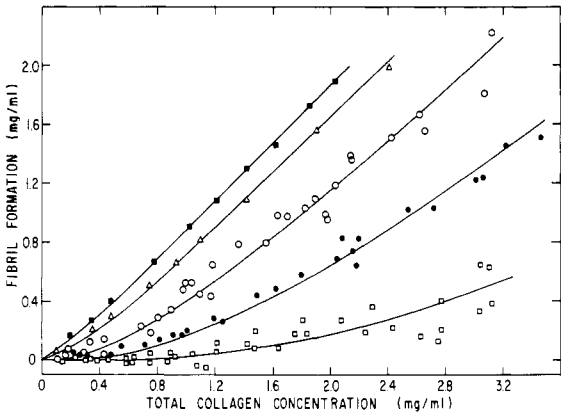

FIGURE 5: Self-association of pepsin-treated collagen in glycerol solutions. The experimental conditions were identical with those of Figure 3. The glycerol concentrations were 0 (■), 0.15 (△), 0.3 (○), 0.45 (●), and 0.6 M (□). The curves only indicate the trend of the data and have no theoretical meaning.

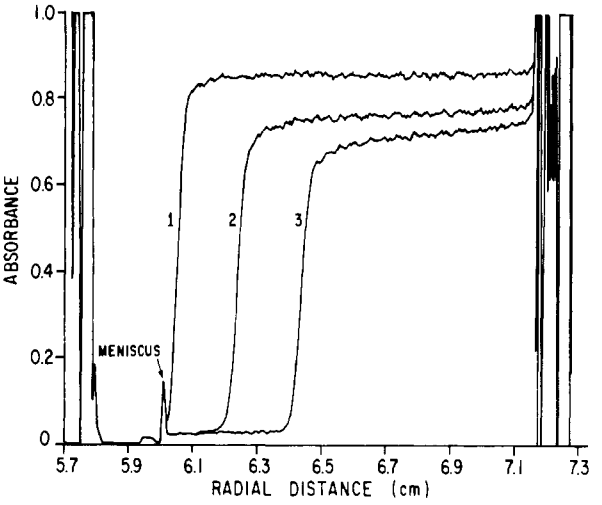

FIGURE 6: Velocity sedimentation boundaries of collagen. $NaBH_4$-reduced collagen (0.91 mg/mL) in PS–0.8 M glycerol buffer was incubated at 30 °C for 72 h before being analyzed with the analytical ultracentrifuge. The rotor speed was 44 000 rpm, and the temperature was 20 °C. The UV scans of the boundaries were obtained at 232 nm and at 24, 159, and 300 min after reaching speed.

Self-Assembly of Native Collagen. The self-association of native collagen was also examined by the pelleting method as described above. As shown in Figure 4, the self-association of this protein in PS–glycerol buffers, too, displayed critical concentrations, although they were slightly lower than the corresponding values of the $NaBH_4$-reduced collagen. The critical concentrations for the self-association of this protein are also listed in Table I.

Self-Assembly of Pepsin-Treated Collagen. The calf skin collagen isolated by the method described above contained 12 ± 1 tyrosine residues per collagen molecule, suggesting that the nonhelical telopeptides of the protein remained intact (Chandrakasan et al., 1976; Miller, 1984). This number was reduced to 4.5 tyrosines per collagen after pepsin digestion, reflecting the enzymatic removal of a portion of the nonhelical terminals (Gelman, 1979b). As shown in Figure 5, the self-association of the pepsin-treated collagen, too, was inhibited by glycerol. However, unlike the native and $NaBH_4$-reduced collagens, the association reaction did not display any critical concentration. Instead, in PS–glycerol buffers, the amount of fibrils formed appeared to increase curvilinearly with increasing protein concentration, and these curves converge at zero protein concentration. The effect of glycerol was manifested in a decrease of the slope of the data, from 0.99 in PS buffer to less than 0.2 in PS–0.6 M glycerol buffer.

Velocity Sedimentation of Collagen. The state of association of the $NaBH_4$-reduced collagen under the fibril assembly conditions but at and below the critical concentration of fibril formation was studied with velocity sedimentation. For the purpose of comparison, experiments were carried out at 20 °C in the pH 4.0 AS buffer (0.01 M NaOAc and 0.02 M NaCl) and at 20 and 30 °C in the pH 7.0 PS–0.8 M glycerol buffer. As represented by the results shown in Figure 6, under all of the conditions described, the protein exhibited single, symmetrical sedimentation boundaries. The shape of the sedimentation boundary was not affected by the presence of glycerol in the solution. The sedimentation coefficients obtained were converted to the condition of water at 20 °C and are shown in Figure 7. They decreased curvilinearly with increasing protein concentration and did not show any change in the presence of glycerol. Below 0.5 mg/mL, the data were linear and can be fitted by the equation (Schachman, 1959):

$$s_{20,w} = s^0_{20,w}(1 - gC) \quad (1)$$

where g is the hydrodynamic nonideality constant and C is the weight concentration of the protein. The fitting gave $s^0_{20,w}$ = 2.9 S and g = 0.346 mL/mg.

Electron Microscopic Examination of Collagen Aggregates. The morphologies of the collagen aggregates formed above the critical concentration were examined by electron mi-

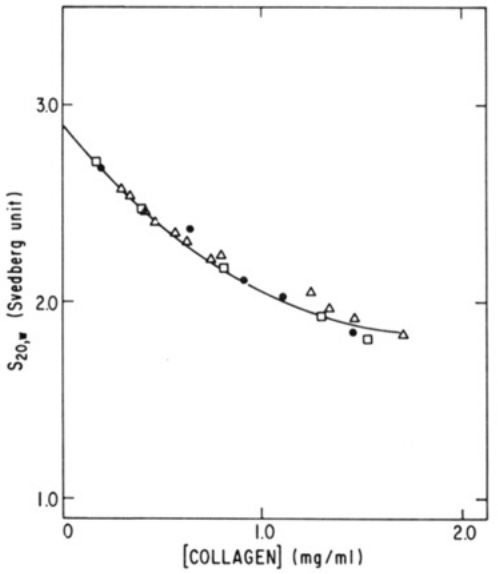

FIGURE 7: Sedimentation coefficient of calf skin collagen as a function of protein concentration. The velosity sedimentations were carried out in (1) AS buffer at 30 °C (●), (2) PS–0.8 M glycerol buffer at 30 °C (△), and (3) PS–0.8 M glycerol buffer at 20 °C (□). The samples in PS–0.8 M glycerol buffer at 30 °C were the supernatant from the in vitro fibril assembly reaction as shown in Figure 4; i.e., they have been incubated at 30 °C for 24–48 h before the velocity sedimentation. The rest of the samples were used right after preparation. The rotor speed was 44 000 rpm.

croscopy. As shown in Figure 8a, in PS buffer, normal fibrils with a band pattern approximately 67 nm long were formed. In the presence of 0.6 M glycerol, however, many of the aggregates formed had a microfibrillar structure with diameters in the range of 3–5 nm (Figure 8b). To understand the relationship between the microfibrils formed in the presence of glycerol and the banded fibrils, glycerol was removed from the solution after the self-association process had plateaued by dialyzing against PS buffer at 4 °C for 24 h. When the aggregates inside the dialysis sac were subsequently examined with the electron microscope, only normally banded fibrils were observed with no trace of microfibrils (Figure 8c).

Equilibrium Distribution of Different Size Polymers in Cooperative and Noncooperative Self-Associations. To analyze the chemical equilibria observed for in vitro collagen fibril assembly, the equilibrium distribution of monomer and polymers was calculated for a two-step helical cooperative self-association with different association parameters.

From the thermodynamic point of view, a helical cooperative self-association can be viewed as consisting of two isodesmic self-associations. The first step of the self-association, often referred to as initiation, involves an isodesmic, stepwise self-association of the monomers (Chun & Kim, 1969; Chun et al., 1969; Na & Timasheff, 1980):

$$\begin{aligned} A + A &\rightleftharpoons A_2 && K_L \\ A_2 + A &\rightleftharpoons A_3 && K_L \\ &\vdots && \vdots \\ A_{i-1} + A &\rightleftharpoons A_i && K_L \end{aligned} \qquad (2)$$

where A_i represents the i-mer and $[A_i]$ is its molar concentration. K_L is the equilibrium constant for each step of the self-association. As the stoichiometry of the linear polymer reaches a certain value n, it closes itself into a looped structure:

$$A_n(\text{linear}) \rightleftharpoons A_n(\text{circular}) \qquad K_C \qquad (3)$$

where K_C is the equilibrium constant between the linear n-mer and the circular n-mer. After the formation of the circular structure, the elongation stage sets in. The elongation or

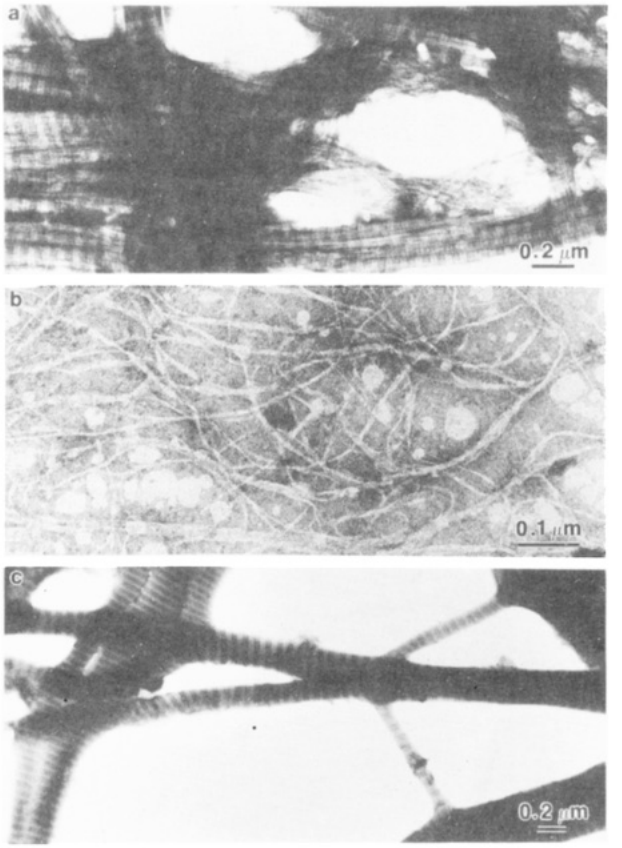

FIGURE 8: Electron micrographs of collagen aggregates. (a) Normal collagen fibrils formed in PS buffer; (b) microfibrils of 3–4-nm diameter formed in PS–0.6 M glycerol, pH 7.0, buffer after 24-h incubation at 30 °C; and (c) fibrils observed after the sample from (b) was dialyzed against PS buffer for 24 h at 4 °C.

growth of the polymer is similar to the initiation stage except each step of the association has a more negative free energy change. Its reaction equilibria can be expressed as

$$\begin{aligned} A_n + A &\rightleftharpoons A_{n+1} && K_H \\ A_{n+1} + A &\rightleftharpoons A_{n+2} && K_H \\ &\vdots && \vdots \\ A_{n+j-1} + A &\rightleftharpoons A_{n+j} && K_H \end{aligned} \qquad (4)$$

where K_H is the equilibrium constant for the incorporation of one monomer to the end of the helical polymer. Consequently, one can express the total concentration of the system in terms of [A], the monomer concentration, as (Oosawa & Kasai, 1962, 1971; Timasheff, 1981)

$$[A_t] = \sum_{i=1}^{n} iK_L{}^{i-1}[A]^i + \sum_{j=0}^{m}(n+j)K_C K_L{}^{n-1}K_H{}^j[A]^{n+j} =$$

$$\sum_{i=1}^{n} iK_L{}^{i-1}[A]^i + \sum_{j=0}^{m} K_C(K_L/K_H)^{n-1}[(n+j)K_H{}^{n+j-1}[A]^{n+j}] \qquad (5)$$

If the growth of the polymer proceeds indefinitely, eq 5 becomes

$$[A_t] = \sum_{i=1}^{n} iK_L{}^{i-1}[A]^i + K_C(K_L/K_H)^{n-1}\left\{\frac{[A]}{(1-K_H[A])^2} - \sum_{j=1}^{n-1} jK_H{}^{j-1}[A]^j\right\} \qquad (6)$$

Given values of [A], n, K_L, and K_H, one can calculate the concentration of each of the linear and helical polymers directly from eq 6. The only constraint that must be observed in such a calculation is that [A] must not be greater than $1/K_H$ in order for the infinite series of eq 5 to converge. According

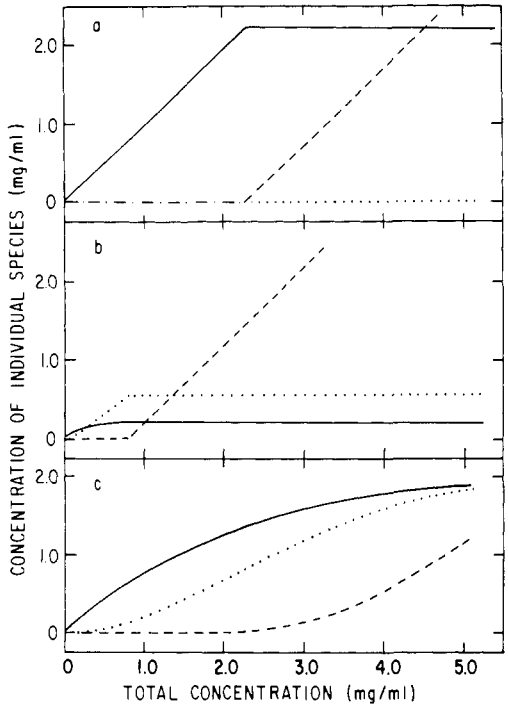

FIGURE 9: Weight distributions of macromolecule in helical cooperative and noncooperative self-associating systems. The curves reflect the concentrations of monomer (—), linear oligomers (···), and helical polymers (- - -). The details of the association mechanism were described under Results. (a) Cooperative helical self-association where $K_L = 1.28 \times 10^3\ M^{-1}$, $K_H = 1.28 \times 10^5\ M^{-1}$, $K_C = 100$, and $n = 5$. (b) Cooperative helical self-association arising from an unfavorable cyclization reaction where $K_L = 6.41 \times 10^5\ M^{-1}$, $K_H = 1.28 \times 10^6\ M^{-1}$, $K_C = 1 \times 10^{-6}$, and $n = 5$. (c) Noncooperative helical self-association where $K_L = 4.28 \times 10^4\ M^{-1}$, $K_H = 1.28 \times 10^5\ M^{-1}$, $K_C = 3$, and $n = 5$. A monomer molecular weight of 285000 was assumed in the calculation.

to eq 6, the concentration of the helical polymers in the solution depends on the value of $K_C(K_L/K_H)^{n-1}$. If the value of this term is small, the formation of the helical polymers will be cooperative and exhibit a critical concentration. It is evident that a small value of $K_C(K_L/K_H)^{n-1}$ can stem either from a small value of K_L/K_H (a stronger polymer elongation than initiation), from a small value of K_C (an unfavorable cyclization reaction), or from a large value of n (a large size of the linear oligomer required to form the nucleation center), or a combination of these factors. Figure 9a depicts the weight distribution of a self-association system whose cooperativity is derived from a low K_L/K_H ratio (10^{-2}). For such a system, the helical polymers are present in the solution only when the total protein concentration is above the critical value $[A_c]$, and all of the protein above $[A_c]$ exists in the helical polymer form. Furthermore, since $K_L << K_H$, below the critical concentration, the monomer will be the dominating species, and there will be so few linear oligomers present they will be most likely undetectable by available physical techniques. Also, since $K_L/K_H << 1$, according to eq 6, the only possibility of obtaining a significant amount of helical polymers is that $1 - K_H[A] \simeq 0$ or $K_H \simeq [A]^{-1}$. Since $[A_c] \simeq [A]$ at the total protein concentration above the critical concentration of helical polymer formation, $1/[A_c]$ can be taken as a good estimate of K_H.

If K_C has a very small value, one could also observe a critical concentration for the formation of the helical polymers even though the values of K_L and K_H are not very far apart from each other. K_C is not a completely independent parameter since $K_C \leq K_H$. The equal sign applies only if the circular structure results naturally from the geometric arrangement of the monomers as dictated by the intermonomer angle of contact, and consequently, no extra free energy is needed to bend the linear polymer into a circular shape before the cyclization can take place. Therefore, K_C could have a quite small value if a large amount of energy must be expended to bend the linear oligomers into circular ones. The molecular distribution of such a system is shown in Figure 9b. It differs from the system shown in Figure 9a in that a significant amount of linear oligomers is present near the critical concentration of the helical polymer formation. Consequently, $[A_c] > [A]$ at the total protein concentration above the critical concentration of helical polymer formation, and the value of $1/[A_c]$ can no longer be taken as K_H even though the formation of the helical polymers shows a critical concentration.

As the value of $K_C(K_L/K_H)^{n-1}$ increases, the system gradually loses its cooperativity, and the helical polymers will emerge gradually with increasing protein concentration without showing any critical concentration effect. The polymer weight distribution of such a noncooperative self-association system is shown in Figure 9c. It is evident that for such a self-association system, there should exist a substantial amount of linear oligomers in the solution that they ought to be detectable by available physicochemical techniques.

Discussion

The inhibition of in vitro collagen fibril assembly by glycerol has been observed previously (Hayashi & Nagai, 1972). In order to determine the effect of glycerol on the noncovalent intercollagen interactions within the fibrils and thus the chemical equilibrium of the reversible fibril assembly reaction, we used the $NaBH_4$-reduced collagen which has its allysine and hydroxyallysine side chains reduced. Since this protein is unable to form covalent intercollagen cross-links during its in vitro fibril assembly, the fibrils formed can be dissociated upon cooling to 10 °C as demonstrated by the results of Figure 1. Our results shown in Figure 2 confirmed that in vitro fibril formation of the $NaBH_4$-reduced collagen is also suppressed by the presence of 1 M glycerol. Furthermore, using this reversible system, we showed that glycerol can actually dissociate the collagen fibrils.

The results of the equilibrium study of collagen fibril assembly in PS buffer without glycerol shown in Figure 3 agree with earlier findings in that a critical concentration cannot be clearly identified (Williams et al., 1978; Gelman et al., 1979a). However, the rest of the results shown in the same figure indicate quite clearly that the self-association of collagen in the same buffer but containing 0.25–0.8 M glycerol required that the protein concentration be above a critical value. Since glycerol inhibits collagen fibril assembly, the gradual emergence of the critical concentration of fibril assembly with increasing glycerol concentration suggests that a cooperative step is indeed involved in the assembly reaction. The critical concentration of the cooperative self-association has not been detected in the absence of glycerol because it is too low, or the polymer elongation is so strong according to the model described by eq 2–4 that it cannot be measured by the technique used. Indeed, in studying the in vitro collagen fibril assembly, the methods used to measure the protein concentration are only sensitive to approximately 10 μg/mL. Consequently, a cooperative self-association with a critical concentration of less than 10 μg/mL or, for collagen, a standard free energy change more negative than −10 kcal/mol cannot possibly be discerned.

If the in vitro assembly of collagen fibrils indeed proceeds through a cooperative helical polymerization mechanism like

the one described by eq 2–4, what are the association parameters that endow the self-association such a cooperative nature? According to the results shown in Figure 7, the calf skin collagen showed essentially the same sedimentation coefficient as that of the collagen extracted from the skin of lathyritic rat (Öbrink, 1972). Gel electrophoresis of the latter protein showed only α bands, indicating that it consisted of only monomers in its native state. Therefore, the sedimentation results of Figures 6 and 7 suggest that in glycerol–PS buffer and below the critical concentration of fibril assembly, the collagen was predominantly in a monomeric state without undergoing any significant polymerization. According to the polymer weight distributions calculated for two-step cooperative self-associations shown in Figure 9a–c, the velocity sedimentation results suggest that the cooperativity of the collagen self-assembly is derived from a low ratio of K_L/K_H, i.e., a stronger collagen self-association in fibril growth than in initiation. However, the data do not rule out a small value of K_C and/or a large value of n cocontributing to the cooperativity of the self-association. In any event, according to eq 6, the fact that the protein existed predominantly as monomers below the critical concentration of fibril assembly indicates that the reciprocal of the critical concentration can be taken as the polymer growth constant.

Given the cooperative nature of the assembly reaction, one can further examine the morphology of the collagen aggregates formed under these conditions and determine whether an association mechanism can be postulated that conforms to the thermodynamic characteristics of the reaction. Electron microscopic examination of the aggregates formed above the critical concentration of association showed the presence of numerous microfibrils of 3–5-nm diameter coexisting with the banded fibrils. These types of collagen microfibrils have been observed by other researchers, either at an early stage of the fibril assembly or by cold-induced disassembly of fibrils assembled in vitro (Veis et al., 1979; Gelman et al., 1979a). On the basis of the morphology and X-ray diffraction pattern of the microfibrils and the 1-D banded pattern of collagen fibrils,[4] Smith (1968) has proposed a five-membered helical structure for the microfibrils. According to this model, collagen monomers within the microfibrils are aligned longitudinally and staggered by 1 D. Five collagen monomers form a closed loop, and further addition of collagen monomers in the same manner gives the microfibrils. Such a helical polymer structure is reminiscent of the helical structures of microtubules and actin filaments, both of which have been shown to be assembled from their respective monomers through the helical cooperative association mechanism (Gaskin et al., 1974; Oosawa & Kasai, 1962). Within the confinement of the five-membered microfibril model, one can devise a number of cooperative association schemes for the collagen microfibril assembly. However, the simplest one appears to be the stepwise cooperative helical association described by eq 2–4. In such a model, the initiation of the polymer involves an isodesmic stepwise association of parallel collagen monomers staggered by 1 D as illustrated in Figure 10. This reaction could be driven by the formation of the 3.4-D overlap between two adjacent monomers. If the pentamer forms a closed-loop structure, then the value of K_C, the equilibrium constant of the cyclization reaction, will depend on several factors. As stated earlier, the circular structure may result naturally from the geometric arrangement of the monomers dictated by the

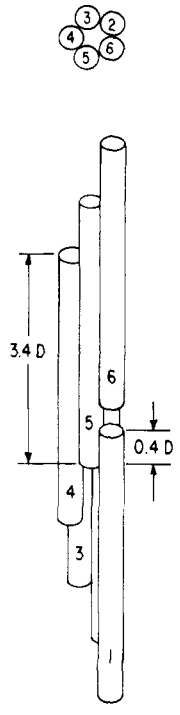

FIGURE 10: Schematic illustration of the top and side views of a five-membered helical structure model of collagen microfibrils. The monomers depicted by the cylinders are 4.4 D long where 1 D = 67 nm. Neighboring monomers are staggered by 1 D and overlapped by 3.4 D; the latter is indicated between the fourth and fifth monomers. Also shown is the 0.4-D contact between the first and fifth monomers which facilitates the formation of the closed-loop structure. A right-handed helix was assumed.

intersubunit bond angle within the pentamer. As shown by the schematic of Figure 10, the cyclization reaction can be driven by the negative free energy change derived from the 0.4-D overlap between the first and the fifth monomer. The formation of this bond should have a favorable entropy change because it is intramolecular instead of intermolecular and thus does not require the loss of translational freedom of the involved monomers (Frigon & Timasheff, 1975). From the pentamer on, the elongation step may follow. During the elongation, the addition of each monomer is accompanied by a more negative free energy change than that for the initiation reaction. An obvious possibility for this to happen is that, according to the five-stranded microfibril model, the incorporation of the n-th monomer in polymer growth is accompanied by the formation of two subunit contacts; one results from the 3.4-D overlap with the $(n - 1)$-th monomer and another from the 0.4-D overlap with the $(n - 4)$-th monomer. The latter interaction may allow $K_H >> K_L$. It is interesting to note that this scheme of endowing cooperativity to the assembly reaction is somewhat different from those found in the assemblies of microtubules and actin filaments. In the latter cases, the additional bond can be derived from the head to tail contact between two coaxial monomers within the helix. This cannot be achieved by the collagen within the microfibrils because of the presence of the 0.6-D gap between two neighboring coaxial monomers.

Several intercollagen covalent cross-links were found within the fibrils that suggest the existence of parallel packing of collagen molecules staggered by 4 D and overlapped by 0.4 D (Zimmerman et al., 1970; Kang, 1972; Henkel et al., 1976; Bailey et al., 1980; Scott, 1980). The 0.4-D overlap appears to play an important role in the fibril assembly reaction. A β-sheet structure was proposed for the N-telopeptide of the α1 chain which can account for the hydrophobic interactions

[4] The unit D is equal to 67 nm, the combined length of the dark band and light band of the collagen fibril.

between two tyrosine side chains on the N-telopeptide and phenylalanine-934 of a 4-*D* staggered neighboring collagen as well as electrostatic interactions between charged side chains (Helseth et al., 1979; Helseth & Veis, 1981). Similar interactions have been proposed by Capaldi and Chapman (1982, 1984) between the C-telopeptide and the helical region of its neighboring 4-*D* staggered collagen. It is evident that the 0.4-*D* overlap, spanning approximately 100 amino acids, is occupied in a greater proportion by the nonhelical telopeptides of the protein than is the 3.4-*D* overlap. Previous studies have shown that the nonhelical telopeptides of the protein, particularly the N-telopeptide, play a significant role in the assembly reaction. The assembly of collagen with either or both of these telopeptides deleted requires an increased initiation time and often results in fibrils of abnormal morphology (Leibovich & Weiss, 1970; Comper & Veis, 1977a,b; Gelman et al., 1979b; Capaldi & Chapman, 1982, 1984). According to the helical microfibril model described by eq 2–4 and illustrated by Figure 10, the interactions of the telopeptides with the helical region of their neighboring molecules are expected to contribute significantly to the free energy change necessary for the formation of the nucleation center and thus the cooperativity of the polymerization reaction. Collagen treated with pepsin has its N-telopeptide and a portion of its C-telopeptides removed, but its helical region remained intact (Comper & Veis, 1977b; Gelman et al., 1979b; Capaldi & Chapman, 1982, 1984). As shown in Figure 5, the fibril formation of the pepsin-treated collagen did not exhibit any critical concentration. The amount of fibrils formed increased curvilinearly with increasing protein concentration. One would expect the pepsin-treated collagen to self-associate with weaker K_L, K_H, and K_C. However, percent-wise, one expects K_H to be reduced more than K_L because the 0.4-*D* overlap is occupied to a greater extent by the nonhelical telopeptides than does the 3.4-*D* overlap. Consequently, the ratio K_L/K_H may no longer be small enough, and this may attribute to the loss of cooperativity by the assembly reaction.

The electron microscopic results of Figure 8 show that the microfibrils are present in abundance in the glycerol solvent after the self-association reaction has reached an equilibrium. The microfibrils existed as a kinetically stable species in the solution probably because they are prevented by glycerol from further self-association into fibrils. Once glycerol was removed from the aggregates by equilibrium dialysis, the microfibrils disappeared, and only the banded fibrils were observed under the electron microscope, suggesting that the microfibrils are an intermediate in the in vitro fibril assembly. In this regard, glycerol could become a useful agent in future studies of microfibril self-association into fibrils.

The absence of a significant amount of oligomers from the collagen solution coexisting with the fibrils, as suggested by the velocity sedimentation results, is consistent with the results of electric birefringence and quasi-elastic laser light-scattering studies of Bernengo et al. (1983). They concluded that no intermediate species, between the monomer and the very large microfibrils and fibrils, can be detected at either the nucleation stage or the growth stage of collagen fibril assembly. The collagen seems to change directly from a monomer state to very large structures. On the other hand, light-scattering studies from two other groups suggested that a significant amount of oligomers was present during collagen fibril initiation (Silver et al., 1979; Silver & Trelstad, 1980; Silver, 1981; Gelman & Piez, 1980). The former group proposed that these oligomers are mainly dimers and trimers staggered by either 4 or 4.4 *D* (Silver & Trelstad, 1980; Silver, 1981). If the suggested amounts of such dimers and trimers, with predicted sedimentation coefficients of 3.65 and 4.05 S (Silver et al., 1979), were present in our solution, they should have been detected in the analytical ultracentrifugation. Bernengo et al. (1983) attributed the disparities among the results of light-scattering studies at least partially to the differences in the homodyne and heterodyne techniques used. Further studies will be required to fully resolve the differences.

The aggregates assembled in vitro from native collagen are usually less that 10% cold-reversible or glycerol-dissociable. The irreversibility is presumably due to the nonenzymatic formation of covalent intercollagen cross-links within the fibrils, between the allysine and hydroxyallysine residues. Nevertheless, the results of Figure 4 indicate that the self-association of native collagen in glycerol solutions, too, showed critical concentrations. The absence of self-assembly of this protein below the critical concentration can be understood on the basis of a weakened initiation reaction in the glycerol solution that provides the protein with no nuclei to grow upon. However, above the critical protein concentration, once the nucleation centers have formed, one would expect all of the protein to become trapped in the polymer state should the intercollagen covalent cross-links prevent the dissociation of monomer from the aggregates. Currently, it is not clear at what stage of in vitro fibril assembly, and to what extent, collagen becomes cross-linked covalently to its neighboring molecules within the fibril. Comper and Veis (1979b) have shown that the kinetics of in vitro fibril assembly are not affected by the potential of the protein to form covalent cross-links, suggesting that the cross-linking takes place at a later stage of the reaction. Our observation of a constant concentration of monomer coexisting with the microfibrils and fibrils above the critical concentration of self-assembly suggests that an equilibrium remains between the native monomer and the assembled structures in the glycerol solutions. One possible explanation of this observation is that the covalent cross-links take place only at the internal region and not at the terminals of the assembled structures. Consequently, monomers can associate to and dissociate from the ends but not the main body of the aggregates. Under this hypothesis, as long as the monomer remains as the only species in equilibrium with the helical polymer, one can take the reciprocal of the solution monomer concentration as the polymer growth constant despite the formation of covalent intercollagen cross-links within the aggregates. Since

$$d[A]/dt = k_r[P] - k_f[A][P] \quad (7)$$

where [A] and [P] denote the concentrations of monomer and polymer, respectively, and k_f and k_r are the forward and reverse rate constants of polymer growth, respectively. At equilibrium, the net assembly is zero and $K_H = k_f/k_r = 1/[A]$.

In summary, the glycerol inhibition of in vitro collagen fibril assembly was used as a tool to probe the mechanism of the assembly reaction. The equilibrium studies of collagen fibril formation in the presence of glycerol suggest that fibril assembly can be described by a helical cooperative mechanism using the currently accepted five-membered microfibril model. Intact nonhelical telopeptides of the protein are essential for the cooperative mechanism of association. The establishment of a helical cooperative mechanism permits future equilibrium studies of fibril assembly to complement the kinetic approaches which have predominated in the past.

Registry No. Glycerol, 56-81-5.

REFERENCES

Bailey, A. J., Light, N. D., & Atkins, E. D. T. (1980) *Nature (London) 288*, 408–410.

Bernengo, J. C., Ronziere, M. C., Bezot, P., Bezot, C., Herbage, D., & Veis, A. (1983) *J. Biol. Chem. 258*, 1001–1006.
Capaldi, M. J., & Chapman, J. A. (1982) *Biopolymers 21*, 2291–2313.
Capaldi, M. J., & Chapman, J. A. (1984) *Biopolymers 23*, 313–323.
Chandrakasan, G., Torchia, D. A., & Piez, K. A. (1976) *J. Biol. Chem. 251*, 6062–6067.
Chun, P. W., & Kim, S. J. (1969) *Biochemistry 8*, 1633–1643.
Chun, P. W., Kim, S. J., Stanley, C. A., & Ackers, G. K. (1969) *Biochemistry 8*, 1625–1632.
Comper, W. D., & Veis, A. (1977a) *Biopolymers 16*, 2113–2131.
Comper, W. D., & Veis, A. (1977b) *Biopolymers 16*, 2133–2142.
Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.
Frigon, R. P., & Timasheff, S. N. (1975) *Biochemistry 14*, 4567–4573.
Gaskin, F., Cantor, C. R., & Shelanski, M. L. (1974) *J. Mol. Biol. 89*, 737–758.
Gelman, R. A., & Piez, K. A. (1980) *J. Biol. Chem. 255*, 8098–8102.
Gelman, R. A., Williams, B. R., & Piez, K. A. (1979a) *J. Biol. Chem. 254*, 180–186.
Gelman, R. A., Poppke, D. C., & Piez, K. A. (1979b) *J. Biol. Chem. 254*, 11741–11745.
Hayashi, T., & Nagai, Y. (1972) *J. Biochem. (Tokyo) 72*, 749–758.
Helseth, D. L., Jr., & Veis, A. (1981) *J. Biol. Chem. 256*, 7118–7128.
Helseth, D. L., Jr., Lechner, J., & Veis, A. (1979) *Biopolymers 18*, 3005–3014.
Henkel, W., Rauterberg, J., & Stirtz, T. (1976) *Eur. J. Biochem. 69*, 223–231.
Kang, A. H. (1972) *Biochemistry 11*, 1828–1835.
Kupke, D. W., & Dorrier, T. E. (1978) *Methods Enzymol. 48*, 155–162.
Leibovich, S. J., & Weiss, J. B. (1970) *Biochim. Biophys. Acta 214*, 445–454.
Light, N. D., & Bailey, A. J. (1980) *Biochem. J. 189*, 111–124.
Miller, E. J. (1984) in *Extracellular Matrix Biochemistry* (Piez, K. A., & Reddi, A. H., Eds.) pp 41–81, Elsevier, New York.
Na, G. C. (1986) *Biochemistry* (following paper in this issue).
Na, G. C., & Timasheff, S. N. (1980) *Biochemistry 19*, 1347–1354.
Na, G. C., & Butz, L. J. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1886.
Öbrink, B. (1972) *Eur. J. Biochem. 25*, 563–572.
Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10–21.
Oosawa, F., & Kasai, M. (1971) in *Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) Vol. 5, pp 261–322, Marcel Dekker, New York.
Purich, D. L., & Kristofferson, D. (1984) *Adv. Protein Chem. 36*, 133–212.
Schachman, H. (1959) in *Ultracentrifugation in Biochemistry*, Academic Press, New York and London.
Scott, P. G. (1980) *Biochemistry 19*, 6118–6124.
Silver, F. H. (1981) *J. Biol. Chem. 256*, 4973–4977.
Silver, F. H., & Trelstad, R. L. (1980) *J. Biol. Chem. 255*, 9427–9433.
Silver, F. H., Langley, K. H., & Trelstad, R. L. (1979) *Biopolymers 18*, 2523–2535.
Smith, J. W. (1968) *Nature (London) 219*, 157–158.
Timasheff, S. N. (1981) in *Protein–Protein Interactions* (Frieden, C., & Nichol, L. W., Eds.) pp 315–336, Wiley, New York.
Veis, A., Miller, A., Leibovich, S. J., & Traub, W. (1979) *Biochim. Biophys. Acta 576*, 8898.
Williams, B. R., Gelman, R. A., Poppke, D. C., & Piez, K. A. (1978) *J. Biol. Chem. 253*, 6578–6585.
Wood, G. C. (1960a) *Biochem. J. 75*, 598–605.
Wood, G. C. (1960b) *Biochem. J. 75*, 605–612.
Wood, G. C., & Keech, M. K. (1960) *Biochem. J. 75*, 588–598.
Zimmerman, B. K., Pikkarainen, J., Fietzek, P. P., & Kühn, K. (1970) *Eur. J. Biochem. 16*, 217–225.