Mechanism of in Vitro Collagen Fibril Assembly
KINETIC AND MORPHOLOGICAL STUDIES*

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The kinetics of in vitro fibril assembly of Type I collagen preparations that contain different amounts of covalently cross-linked oligomers was studied with turbidimetry. Fibril formation showed a lag phase with no solution turbidity and a growth phase with a sigmoidal increase in the solution turbidity. The length of the lag phase was inversely related to both the total collagen concentration and the amount of covalently cross-linked oligomers in the solution. Double logarithmic plots of the amount of time it takes for ¼ of the collagen to assemble into fibrils, versus the total collagen concentration were linear but the slope decreased from −0.84 to −2.3 with decreasing amounts of covalently cross-linked oligomers in the samples. Electron microscopy showed the formation of unbanded microfibrils with diameters in the range of 3–15 nm early in the lag phase and larger diameter banded fibrils coexisting with the microfibrils near the end of the lag phase. Centrifugation of the solution at the lag phase prolonged the lag time, presumably by removal of microfibrils, but subsequent growth of the fibrils was unaffected. The results suggest a cooperative nucleation-growth mechanism for the in vitro assembly of collagen fibrils which is consistent with the results of an equilibrium study of the fibril assembly reaction we reported earlier (Nač, G. C., Butz, L. J., Bailey, D. G., and Carroll, R. J. (1986) Biochemistry 25, 958–966).

The interstitial collagens are synthesized inside cells and secreted into extracellular space in the form of procollagen. There the propeptides are cleaved enzymatically and the collagen molecules polymerize into fibrils (Kivirikko and Myllyla, 1984). Currently, little is known regarding either the mechanism of formation of the collagen fibril from the monomer or the regulatory mechanism of the process. In vitro, solubilized collagen can be reconstituted into fibrils if incubated at 25 to 30 °C in a buffer with an ionic strength of 0.15 to 0.30 and a pH near neutral. The kinetics of the in vitro collagen fibril assembly consist of two phases, initiation and growth (Wood and Keech, 1960; Wood, 1960; Comper and Veis, 1977a, 1977b; Williams et al., 1978; Gelman et al., 1979a, 1979b). During the fibril initiation period, the solution does not display any turbidity, indicating that the large fibril structures have not yet formed. However, unbanded microfibrils of 3- to 5-nm diameter have been observed during the lag phase of fibril assembly (Veis et al., 1979; Gelman et al., 1979a). Growth of the fibril follows the initiation period and is characterized by a sigmoidal increase of the solution turbidity as well as the appearance of collagen fibrils with a 67-nm repeat band pattern.

There are few reported equilibrium studies of in vitro collagen fibril assembly. Wood and Keech (1960) observed that solubilized collagen polymerized entirely into fibrils under the assembly conditions. Nonetheless, a nucleation-growth mechanism was proposed on the basis of the sigmoidal characteristics of the reaction kinetics (Wood, 1960). More recently, Piez and co-workers confirmed that the fibril assembly reaction entails no critical concentration, i.e. the amount of fibrils formed, when plotted against the total collagen concentration, extrapolated to near the origin (Williams et al., 1978; Gelman et al., 1979a). Moreover, they carried out kinetic studies which showed a linear plot with a slope of −1 for the logarithm of the time required for half of the collagen to assemble into fibrils versus the logarithm of the total collagen concentration. On the basis of these observations, they suggested that the fibril assembly reaction proceeds through an accretion mechanism and does not involve the Oosawa helical cooperative assembly mechanism which has been shown in actin filament and microtubule formations (Oosawa and Kasai, 1962; Gaskin et al., 1974). However, they indicated that their equilibrium data could not rule out the existence of a critical concentration of 7 μg/ml or less because of the limited sensitivity of the technique (Williams et al., 1978).

If the fibril assembly does proceed through a cooperative mechanism with the initial formation of nucleation centers as suggested by Wood (1960), then there should be a critical concentration for the reaction, and its value should be inversely proportional to the polymer growth constant (Oosawa and Kasai, 1971; Timasheff, 1981). The reason that a critical concentration was not observed could be because the polymer growth was too strong. With this understanding, we added glycerol, which is known to inhibit the fibril assembly reaction, to the assembly buffer and demonstrated the presence of a critical concentration for the in vitro collagen fibril formation with its value inversely related to the glycerol concentration in the buffer (Na et al., 1986). Therefore, we proposed a helical cooperative mechanism for the fibril assembly reaction (Na et al., 1986), adopting as an intermediate the five-stranded microfibrils originally conceived by Smith (1968).

While the results of our equilibrium study support the helical cooperative mechanism for collagen fibril assembly, the kinetic evidence reported by Piez and co-workers remained contradictory (Williams et al., 1978; Gelman et al., 1979a, 1979b). It is true that a slope of −1 in the above-mentioned double logarithmic plot is inconsistent with a cooperative nucleation-growth association mechanism. However, the kinetic equation was derived under the assumption...
that the polymerization reaction starts exclusively from the monomer (Oosawa and Kasai, 1971; Purich and Kristofferson, 1984). Because of the possible presence of covalently cross-linked oligomers in the collagen solutions used in the earlier kinetic studies (Chandrakasan et al., 1976; Williams et al., 1978; Gelman et al., 1979a), their interpretation may not be valid. In order to further substantiate the cooperative spontaneous nucleation-growth mechanism of collagen fibril assembly and to determine the stoichiometry of the nucleation center, calf skin collagens that contain different amounts of oligomer were purified and the kinetics of their fibril formation was examined. The results are reported here.

MATERIALS AND METHODS

UV-grade guanidine hydrochloride (GdnHCl) was purchased from United States Biochemical Corp., and solutions were filtered through a sintered glass funnel before use. Spectroanalyzed glycerol was from Fisher. p-Chloromercuribenzoate and phenylmethanesulfonyl fluoride were from Sigma. All other chemicals used were reagent-grade.

Preparation and Concentration Determination of Calf Skin Collagen—Type I collagen was extracted from fresh calf skin obtained from a local slaughter house. The purification procedure was essentially the same as described in a previous publication (Na et al., 1986), except that 1 M glycerol was added to the extraction buffer to stabilize the collagen against denaturation and to facilitate the solubilization of the protein (Na, 1986). In several preparations, three protease inhibitors (0.02 M sodium EDTA, 1 mM p-chloromercuribenzoate, and 10 mM phenylmethanesulfonyl fluoride) were also added to the extraction buffer to prevent endogenous enzymatic digestion of the nonhelical telopeptides (Miller and Rhodes, 1982). The collagen extract in the absence of the protease inhibitors is called "noninhibitor collagen" whereas the one extracted in the presence of the protease inhibitors is called "inhibitor collagen" hereafter.

The protein concentration was determined by diluting a small aliquot of the sample with 3 volumes of 8 M GdnHCl and an appropriate volume of 6 M GdnHCl and measuring the solution absorbance at 218 nm. An absorption coefficient of 9.43 ml·mg⁻¹·cm⁻¹ was used (Na et al., 1986).

The tyrosine content of the protein was determined by UV spectroscopy (Na et al., 1986). 0.3 ml of the stock collagen was diluted with 0.6 ml of 9 M GdnHCl, 0.03 M NaP, pH 6.5. The solution showed an absorption peak at 276 nm. Since collagen does not contain any tryptophan residues, a molar absorption coefficient of 1500 liters·mol⁻¹·cm⁻¹ at 276 nm was used for the tyrosine residue (Edelhoch, 1967). A collagen molecular weight of 285,000 based on the amino acid composition of the protein was used in the calculation (Miller, 1984).

Preparation of Monomeric and Oligomeric Collagens—Calf skin collagens as described above is called "crude collagen." SDS-polyacrylamide gel electrophoresis showed that this preparation contained components with molecular weights greater than that of the γ band (M, = 285,000), reflecting the presence of covalently cross-linked oligomers. To remove the collagen oligomers, the protein was dissolved in PS buffer (0.05 M NaP, and 0.1 M NaCl, pH 7.0) containing either 0.6 M (noninhibitor collagen) or 0.5 M (inhibitor collagen) glycerol and incubated at 30 °C for 24–72 h. The collagen fibrils formed were separated by centrifugation at 12,000 × g for 10 min. The pellets were dispersed in 0.1 M H2OAc and dialyzed exhaustively against 1 M HAc. This collagen will be referred to as "oligomeric collagen." The protein in the supernatant was then precipitated by adding solid NaCl to 3 molal and collected by the same centrifugation. These pellets, too, were dispersed in 0.1 M H2OAc, dialyzed overnight against 1 M HAc, and stored under liquid nitrogen. The latter collagen will be referred to as "monomeric collagen."

1 In the present context, the terms "monomer" and "oligomer" refer to the M, 285,000 triple-helical collagen molecule and the covalently cross-linked species with molecular weights greater than 285,000, respectively.

2 The abbreviations used are: GdnHCl, guanidine hydrochloride; Ac, acetyl; SDS, sodium dodecyl sulfate.

3 Reference of company or product names does not constitute endorsement by the United States Department of Agriculture over others of similar nature.

SDS-Polyacrylamide Gel Electrophoresis—The oligomer content of the collagen was determined by electrophoresis using 4% SDS-polyacrylamide disc gels prepared by following the method of Murray et al. (1982). A concentrated collagen solution at approximately 2 to 3 mg/ml in PS buffer was prepared by adding to PS buffer a small aliquot of the stock collagen that contained about 100 mol% of acid, and an equal volume of a double strength PS buffer. The mixture was dispersed through gentle shaking at 4 °C. A Microman pipette (Rainin Instrument Co. Inc., Woburn, MA) with a piston-type disposable tip was used to measure and deliver the viscous collagen stock solution. Unless otherwise specified, the concentrated collagen solution was passed through a Millex-GV 0.22-μm filter (Millipore) before its concentration was measured. The formation of collagen fibrils was monitored by measuring the solution turbidity at 350 nm using a Perkin-Elmer Lambda-7 UV-visible spectrophotometer. Both the cuvettes and the cell holder were jacketed and connected in series with the circulating water. A temperature controller automatically achieved simultaneous measurement of up to six samples. Two methods were used to initiate the fibril assembly. In the first method, 800 to 900 μl of PS buffer was added to each cuvette and was equilibrated at 30 °C. An aliquot of the concentrated collagen solution in PS buffer at 10 °C was then added to each cuvette to bring the total volume to 1 ml. The solution was mixed by turning the cuvette upside down three times and the turbidity measurement was then started. With this method, the solution warmed up to 30 °C within 10 s. It is thus suitable for samples such as the oligomeric collagen with rapid rates of fibril assembly. In the second method, the cuvettes were kept at room temperature (20–25 °C) whereas the collagen solutions were maintained at 10 °C. The solutions were degassed under vacuum for 2 min and added to the cuvettes. This was followed immediately by opening the valve of the water bath and starting the turbidity measurement. The temperature of the sample reached 30 °C within 2 min. Care was taken to ensure that no air bubble was created during the transfer of the solution to the cuvette. This method was used with the monomeric collagens that have slow rates of fibril assembly. With the monomeric collagen, the extra care of avoiding vigorous mixing and eliminating air bubbles is essential for obtaining consistent results. Since the SDS-PAGE fibril formation of these samples are slow, the 2-min warm-up time can be ignored.

The validity of the solution turbidity as a quantitative measure of the weight concentration of collagen fibrils was verified experimentally by comparing the turbidity results with those of direct measurement. An aliquot of the fibril concentration of the monomeric collagen determined turbidimetrically was used to prepare a standard curve for turbidity measurements. The standard curve is a straight line. The amount of fibrils formed was determined by centrifuging the fibril solution in a 1.5-ml microcentrifuge tube at 12,000 × g for 2 min using a Sorvall SH-MT rotor. The protein concentration of both the solution prior to the assembly and the supernatant after the centrifugation were measured and the difference between them was taken as the amount of fibrils formed. Both the kinetics and the equilibrium of the fibril assembly reaction were examined in this manner. Fig. 1 shows the kinetics of fibril assembly measured by both turbidimetry (solid curve) and direct concentration determinations (circles). It is evident from the data that there were two stages of the lag phase where the direct concentration measurements consistently showed the formation of small amounts of sedimentable aggregates before the appearance of the solution turbidity. The growth and plateau stages of the fibril assembly measured by the two methods were identical.

Fig. 2 shows the equilibrium of the fibril assembly measured by these two techniques. The inset of this figure indicates that, at the plateau of the fibril assembly carried out in PS buffer, collagen assembled essentially entirely into fibrils, in agreement with what we reported earlier (Na et al., 1986). The main figure shows plots of the solution turbidity (absorbance), measured at 350 nm at the plateau of the fibril assembly, against collagen concentration. For the crude and monomeric collagens, the solution turbidity increased linearly with the total collagen concentration below 0.2 mg/ml. Above about 0.2 mg/ml, the data deviated from the straight line. In the linear
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FIG. 1. Measurement of the kinetics of collagen fibril assembly by two different methods. In the first, the formation of collagen fibrils was monitored by measuring the solution turbidity (absorbance) at 350 nm. The solid curve represents the average of three simultaneous measurements. In the second method, the weight concentration of the fibrils in each tube (O) was obtained by pelleting the fibrils by centrifugation and measuring the change in collagen concentration. For the latter data points, the ordinate indicates the starting time of the centrifugation which was at 12,000 x g and lasted for 2 min. For both measurements, the crude inhibitor collagen at 0.16 mg/ml and in 0.03 M NaPi, 0.15 M NaCl, pH 7.0, buffer was used. The temperature of the solutions in the cuvette and in the centrifuge tubes was critically adjusted with a microthermistor to agree within 0.01 °C. In calculating the percent assembly, the amount of fibrils formed at 210 min was taken as 100%.

FIG. 2. Measurement of the equilibrium of collagen fibril assembly by two different methods. The solution turbidity was measured at the plateau of fibril assembly. The symbols correspond to crude inhibitor collagen (O), crude noninhibitor collagen (●), monomeric inhibitor collagen (●), oligomeric inhibitor collagen (Δ), and oligomeric noninhibitor collagen (▲). The straight lines are the linear least squares fits of the data. For crude and monomeric collagens, only the data below a concentration of 0.2 mg/ml were fitted. The inset shows the weight concentration of the fibrils at the plateau of fibril assembly determined by pelleting the fibrils and measuring the change in collagen concentration. The symbols used in the inset are the same as in the main figure.

FIG. 3. SDS-polyacrylamide gel electrophoresis patterns of collagen samples that contained different amounts of oligomer. Four percent polyacrylamide tube gels were used. The samples were first run at 6 mA/tube for 30 min and then at 3 mA/tube for 10.5 h. They were deliberately overloaded (80–90 μg/tube) to give better visualization of the high molecular weight components. Lanes 1 and 3–6 correspond to the samples 1 and 3–6 in Table I. The sample of Lane 2 was similar to those of Lanes 3–6 except a collagen concentration of 0.5 mg/ml was used in purifying the monomer. The α and β bands were labeled according to their conventional names (Miller, 1982). Two distinct bands were found on the gels with mobilities similar to that of the γ band. Since they have not been given specific names in the literature, they were labeled as γ_1 and γ_2 here. Those bands with molecular weights greater than that of the γ bands were collectively labeled as HMW, standing for high molecular weight components.
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<table>
<thead>
<tr>
<th>Sample</th>
<th>Collagen Type</th>
<th>Distribution*</th>
<th>Oligomer Contents and Fibril Assembly Kinetics of Calf Skin Collagens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α</td>
<td>β</td>
<td>γ</td>
</tr>
<tr>
<td>Noninhibitor collagen</td>
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<td>31.5</td>
</tr>
<tr>
<td>1</td>
<td>O</td>
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<td>37.7</td>
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<tr>
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<tr>
<td>3</td>
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<td>5</td>
<td>M</td>
<td>1.30</td>
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<td>Inhibitor collagen</td>
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</tr>
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</table>

* These are the percent distribution of collagen in bands in SDS-gel electrophoreses as measured by a gel scanner.

** The letters C, O, and M stand for crude, oligomeric, and monomeric collagen, respectively.

The high molecular weight (HMW) consists of all components with molecular weight greater than that of the γ band.

† Prior to the kinetic measurement of fibril assembly, the concentrated collagen solutions were filtered with Millipore Millex-GV (0.22 μm) filters except for samples 2 and 8, which were filtered with Millex-PF (0.80 μm) filters.

‡ These are the collagen concentrations used in the glycerol-PS buffer to separate the oligomers from the monomeric collagen.

§ These results were unattainable due to poor experimental accuracy (see "Discussion").

Fig. 4. Kinetics of fibril assembly from noninhibitor collagen. The reaction was monitored by measuring the solution turbidity at 350 nm. The length of the lag phase was obtained by linearly extrapolating the growth phase to the baseline. Also shown are the definitions of t₀ and t₀. The curves numbered 1 to 5 correspond to oligomeric collagen at 0.188 and 0.106 mg/ml, crude collagen at 0.183 and 0.107 mg/ml, and monomeric collagen at 0.204 mg/ml, respectively.

The gel patterns of the inhibitor collagens (not shown) were qualitatively identical to those of the corresponding noninhibitor collagens. The relative distributions of the individual bands of the inhibitor collagens are listed at the bottom half of Table I.

Kinetics of Fibril Assembly of the Noninhibitor Collagen—As shown in Fig. 4, the kinetics of the in vitro collagen fibril assembly can be divided into two phases. After the solution is heated to 30 °C, there is usually a lag phase with no change in solution turbidity. This is followed by a growth phase with a sigmoidal increase of the solution turbidity. The length of the lag phase was measured by extrapolating the linear region of the growth phase to the base line. As shown by curves 1–4 of Fig. 4, for the crude and oligomeric collagens, the lag time increased with decreasing total collagen concentration. This was also true for the monomeric collagen. Comparing at the same concentration the oligomeric, crude, and monomeric collagens, the lag phase lengthens in the above order, apparently reflecting the decreasing amount of oligomers in the sample. In fact, at concentrations above 0.3 mg/ml the solution turbidity of the oligomeric collagen seemed to emerge instantaneously after heating and did not display a measurement...
weight species. However, the kinetics of fibril assembly in PS
double logarithmic plot if used without being filtered and an
hibitor collagen. As depicted in Fig. 6 and listed in Table
collagen was comparable to that of the corresponding nonin-

netics of fibril assembly was generally faster for the inhibitor colla-
oligomeric collagen gave a less negative slope, whereas the monomeric
collagen showed a less negative slope, whereas the monomeric
collagen showed more negative slopes than that of the crude
collagen in the double logarithmic plot. Also, among the
monomeric collagens, the smaller the amount of oligomers
present in the solution, the more negative the slope became.

Collagen Aggregates Formed at the Lag Phase and the Effects
of Centrifugation on the Kinetics of Fibril Assembly—Since
the results of the kinetic studies shown in Fig. 1 revealed the
formation of small amounts of sedimentable aggregates in the
lag phase of fibril assembly, their morphologies were examined
by electron microscopy. Fig. 7a shows a representative electron
micrograph of samples taken at the lag phase of fibril
assembly. Microfibrils with diameters in the range of 3-15
nm were abundant. To ascertain that the microfibrils were
indeed formed in the lag phase and that they serve as an
intermediate of fibril assembly, the solution in the lag phase
was centrifuged, the supernatant was removed immediately
after the centrifugation and incubated continuously, and the
kinetics of fibril assembly were examined. As shown in Fig. 8,
the fibril assembly of the supernatant was similar to that in
the uncentrifuged solution except that the lag time was pro-
longed. As a result, by starting the centrifugation at different
times into the lag phase, the fibril assembly curves were
shifted in parallel to different degrees to the right. The inset
of Fig. 8 shows a plot of $t_w$ versus the incubation time of the
sample prior to the centrifugation. Linear least squares fitting
of the data gave a slope of 0.68.

Toward the end of the lag phase, fibrils with faint band
patterns were observed together with the unbanded micro-

4 The oligomeric collagen was filtered with the 0.8-μm filter because it tended to block the 0.22-μm filter.
FIG. 7. Electron micrographs of collagen aggregates formed at various stages of fibril assembly. The sample was inhibitor collagen at 0.12 mg/ml. It was applied to the grid after 10 min (a) and 20 min (b-d) of incubation at 30 °C. In a, microfibrils of different diameters ranging from 3 to 15 nm were observed. b-d show the coexistence of unbanded microfibrils with banded fibrils toward the end of the lag phase. In b, loosely packed fibrils with faint band patterns were evident, whereas in c and d splitting of a single fibril into microfibrils was observed.

DISCUSSION

As stated in the Introduction, the general features of the kinetics of in vitro fibril assembly of type I collagen have been known for decades. The assembly of collagen fibrils usually starts with a lag period showing no solution turbidity, the length being inversely related to the collagen concentration. This is followed by a growth phase with a sigmoidal increase of the solution turbidity and the appearance of banded fibrils (Wood, 1960; Wood and Keech, 1960; Comper and Veis, 1977a, 1977b; Williams et al., 1978; Gelman et al., 1979a). Such assembly kinetics are characteristic of a nucleation-growth mechanism which has been shown to be true in the in vitro assemblies of actin filaments and microtubules and it has led to the initial proposal of the nucleation-growth mechanism for collagen fibril assembly (Wood, 1960). However, more recent analyses of fibril assembly kinetics by a double logarithmic plot defined above have shown a straight line with a slope of −1 (Williams et al., 1978; Gelman et al., 1979a, 1979b). This finding apparently refutes the cooperative nucleation-growth mechanism and lends support to an accretion mechanism for collagen fibril assembly.

The present study addresses mainly the effect on the kinetics of fibril assembly of the covalently cross-linked oligomers found in most collagen preparations and its implication on the mechanism of fibril assembly derived from the kinetic studies. In addition to the sigmoidal shape of the kinetics of fibril assembly, the observation that the more the oligomer in the solution the faster the rate of fibril assembly suggests a cooperative nucleation-growth mechanism for the reaction. For this mechanism, the monomeric collagen is expected to self-associate and form nucleation centers more slowly than the oligomeric collagen. The latter either already contains the nucleation centers or can form the nucleation centers much faster and, consequently, their lag phase should be much shorter. Since the kinetics of the in vitro collagen fibril assembly displayed all the signs of a cooperative nucleation-growth polymerization, the data were analyzed accordingly. A cooperative self-association involving spontaneous nucleation and growth from the monomer can be expressed as (Oosawa and Kasai, 1971; Timasheff, 1981; Na et al., 1986):
The energy change. Its reaction equilibria and kinetics can be expressed as:

$$nA \equiv \frac{k_+}{k_-} A_n$$  

where $k_+$ and $k_-$ are the apparent association and dissociation rate constants. It has been shown that the kinetics of a cooperative polymerization proceeding through the above nucleation-growth mechanism can be described by (Oosawa and Kasai, 1962; Oosawa and Asakura, 1975):

$$\ln \left( \frac{1 + t \cdot \left( c/c_0 \right)^{nA}}{1 - \left( c/c_0 \right)^{nA}} \right) = 2nA \cdot k_+ \cdot c_0 \cdot n^2 t$$  

where $t$ is time, $c_0$ is the total weight concentration of the protein, $c_i$ is the weight concentration of the monomer, and $n$ is the stoichiometry of the nucleation center. By adopting a constant ratio of $c_1/c_0$. Equation 5 can be reduced to:

$$\ln t = -\frac{n}{2} \ln c_0 + \text{constant}$$  

which indicates that for such a system if one plots $\ln t$, $t$ being the time it takes for a given percentage of the monomer to polymerize, versus $\ln c_0$, the data should be linear with a slope of $-n/2$, $n$ being the stoichiometry of the nucleation center. It should be noted, however, that this rather simple kinetic equation was derived under three important assumptions. First, it was assumed that the rate of dissociation of the monomer from the polymer is very slow and can be ignored. Second, it was assumed that during polymer growth only the monomer becomes associated with the polymer. Polymers do not associate with one another and they do not dissociate into smaller polymers. In other words, the nucleation center can be formed only from linear polymerization of the monomers and not from breaking down of the existing polymers. Third, it was assumed that the monomer is the only species in the solution at the beginning of the polymerization. We shall consider these three assumptions in turn.

The first condition can be met by taking a ratio of $c_1/c_0$ near 1, i.e., examining the initial rate of fibril assembly where the concentration of the polymer is low and its dissociation can be ignored. In the data analysis shown above, $t_i$ was used instead of $t_0$ as in earlier studies to better satisfy this condition. In fact, one can extrapolate the linear portion of fibril growth to the base line to obtain the length of the lag phase which represents the amount of time it takes for the formation of an infinitesimal amount of fibril. As shown in the last column of Table 1, double logarithmic plots of $t_{0.03}$ versus the collagen concentration gave slopes very similar to those of $t_i$. Such an analysis should alleviate the problems of polymer dissociation and the nonlinearity of the turbidity at high fibril concentrations shown in Fig. 2.

The second assumption mentioned above can also be assumed to be reasonably satisfied in the case of collagen fibril formation. If the fibril assembly proceeds through microfibrils as an intermediate as suggested by the results of Fig. 7a, it is unlikely that a significant amount of polymer can break down into smaller polymers. Such a polymer fragmentation would require a rather high activation energy. It is equally unlikely that there is a significant amount of end-to-end longitudinal association among the microfibrils or fibrils because these large asymmetrical structures should have slow translational and rotational movements and could not provide frequent enough mutual collisions with proper orientation of the molecules to result in polymer association.

With respect to the third assumption, the absence of oligomers from the collagen solution is uncertain. Depending on

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![FIG. 8. Effects of centrifugation at lag phase on the kinetics of fibril assembly.](image)
the age of the animal and the method of extraction, type I collagen obtained from skin or tendon usually contains a few percent of oligomers that are covalently cross-linked. The presence of covalent intercollagen cross-links is usually reflected in the appearance of component(s) with molecular weights greater than 285,000 under denaturing conditions such as in SDS-polyacrylamide gel electrophoresis. If polymer formation requires the initial formation of nucleation centers, it is reasonable to expect the oligomers in the solution to accelerate the rate of polymer formation. This effect could be particularly pronounced at low protein concentrations where spontaneous nucleation is slow but to a lesser degree at high protein concentrations where spontaneous nucleation is fast. As a result, the presence of the oligomers could significantly change the slope of the double logarithmic plot. Indeed, in Figs. 5 and 6, the slopes of the double logarithmic plots of the kinetics of fibril formation from crude collagens became more negative if the protein was passed through a 0.22-μm filter before being assembled. This observation suggested that there were aggregates in the solution affecting the kinetics of the assembly reaction which were retained by the 0.22-μm filter. The 0.8-μm filter seemed to have no measurable effect on the kinetics of fibril assembly, suggesting that the aggregates are relatively small in size. While these are qualitative observations, subsequent studies using collagen preparations that contained different amounts of oligomer confirmed that the slope is indeed inversely related to the oligomer content of the solution.

According to Equation 6, a slope more negative than −1 in the double logarithmic plot indicates that the fibril assembly reaction proceeds through a cooperative mechanism. In fact, a slope of −2.3 was obtained with both a noninhibitor and an inhibitor collagen sample that contained, respectively, 0.2 and 1.6% high molecular weight components as determined by SDS-polyacrylamide gel electrophoresis, suggesting that the nucleation center for the cooperative self-association could be a pentamer. It is interesting to note that this stoichiometry agrees with the Smith five-stranded microfibril model in which parallel collagen monomers are staggered by 1 D and packed in a helical manner (Smith, 1968). It should be emphasized, however, that, given the uncertainty of the slopes shown in Table I and the fact that a definitive slope could not be obtained for a monomeric collagen that contains absolutely no high molecular weight components, our results by no means rule out other association models that employ nucleation centers of slightly higher stoichiometries.

In juxtaposition with the kinetic analyses we examined by electron microscopy the morphologies of the collagen aggregates formed at various phases of the assembly reaction. The electron micrographs shown in Fig. 7a confirmed the results reported earlier by Gelman et al. (1979a) indicating that collagen forms microfibrils during the lag phase of fibril assembly. It appears, as originally proposed by Gelman et al. (1979a), that the small diameters and the low concentration of these microfibrils render them undetectable by turbidity measurement. Unlike the fibrils, the microfibrils shown in Fig. 7a are unbanded. One explanation for the lack of banding is as follows. According to the model of D-staggered packing of collagen molecules within fibrils, the band pattern of a negatively stained collagen fibril arises from a heavier deposit of stain molecules at the 0.6 D gap between two coaxial collagen molecules (Hodge and Petruska, 1963; Petruska and Hodge, 1964; Hodge, 1967). One expects, as is true in large diameter fibrils, that in order for the extra stain to remain

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5 The unit D is equal to 67 nm, the combined length of the dark band and light band of the collagen fibril.
first tyrosine residue is located at the fourth position from the amino end and the third position from the carboxyl end (Miller, 1984). Such a deletion could weaken and slow fibril assembly (Helseth and Veis, 1981; Capaldi and Chapman, 1982, 1984).

With decreasing amounts of oligomer in solution, a gradual deterioration of the reproducibility of the fibril assembly kinetics was noted. In Table I, sample 6 showed no measurable amount of high molecular weight component. However, the rate of fibril assembly of this collagen in PS buffer was very slow and unreproducible. In particular, the duration of the lag phase varied substantially from experiment to experiment. This phenomenon is akin to many crystallization reactions in that environmental conditions such as vibration (from the automatic cell changer), the condition of the surface of the vessel, and a minute amount of impurity can greatly influence the rate of the reaction. It seems to reflect an extreme difficulty in the formation of the nucleation center but a much more favorable growth of the polymer once the nucleation center has taken its shape. Again, this is consistent with and supportive of a nucleation-growth mechanism for fibril assembly.

The preferential polymerization of collagen oligomers into fibrils in glycerol buffer could be explained in terms of either an equilibrium or kinetic effect. In an earlier study, it was noted that glycerol is preferentially attracted to the protein and that surface contact between collagen and glycerol solution is energetically favorable (Na, 1986). The oligomers have smaller surface areas per unit weight than the monomer and, therefore, should be less stabilized by the presence of glycerol in the solution than the monomer. Consequently, from the chemical equilibrium point of view, in glycerol solution the oligomers should have stronger propensities to form fibrils than the monomer. The presence of glycerol can also greatly reduce the rate of fibril assembly and perhaps the rate of the reaction. It seems to reflect an extreme difficulty in the formation of the nucleation center but a much more favorable growth of the polymer once the nucleation center has taken its shape. Again, this is consistent with and supportive of a nucleation-growth mechanism for fibril assembly.

It is interesting to note that fibrils formed from the oligomeric collagen displayed a substantially lower turbidity than those formed from crude and monomeric collagens. McPherson et al. (1985) reported that the solution turbidity of fibrils assembled from pepsin-solubilized bovine collagen was inversely related to the rate of fibril formation. Their morphological study indicated that the lower solution turbidity can be attributed to reduced fibril diameter. In the present study, the oligomeric collagen showed a faster rate of fibril initiation than the crude and monomeric collagen. It is thus likely that a greater number of nucleation centers were formed from oligomeric collagen during the initiation period. Consequently, oligomeric collagen could have formed shorter fibrils that scatter less light. This possibility needs to be investigated further.

In summary, the kinetics of the in vitro assembly of monomeric calf skin collagen into fibrils can be well described by a classic cooperative nucleation-growth mechanism. Electron microscopic studies showed the formation of microfibrils at an early stage, i.e. during the turbidity lag of fibril assembly. Double logarithmic plots of the kinetic data suggested that the nucleation center for the fibril assembly could be pentamer, although oligomers of similar stoichiometries were not ruled out. It appeared that the banded fibrils were formed through lateral parallel packing of the microfibrils. This mechanism of fibril assembly, derived from the kinetic data, is fully consistent with the conclusions we reached earlier from equilibrium observations (Na et al., 1986).

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