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Collagen Fibril Formation *in Vitro*

THE ROLE OF THE NONHELICAL TERMINAL REGIONS*

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We showed previously that fibril formation *in vitro* from rat tail tendon collagen requires a temperature-dependent initiation (Step 1) following which linear assembly to form thin filaments (Step 2) proceeds as rapidly at 4°C as at 26°C. Step 3, lateral assembly of filaments to form fibrils, is again temperature-dependent. We now find that Step 1 is complete in 6 min at 26°C and the time is independent of collagen concentration in the range 0.08 to 0.39 mg/ml. Collagen treated with pepsin, which removes the nonhelical ends but leaves the triple helix intact, forms fibrils by a similar mechanism. However, Step 1 is altered or absent and early temperature changes produce a complex response consistent with an alternate, counterproductive pathway. Assembly is also much slower, particularly Step 2, and the fibrils formed are abnormal in that native banding is often absent and short tactoidal forms are common. These results suggest that in the assembly of fibrils from normal collagen the nonhelical ends are involved in an early conformational change and critically regulate later steps.

We have previously selected a set of optimal conditions for *in vitro* assembly of native collagen fibrils from rat tail tendon collagen (1). Kinetic and morphologic studies have shown that assembly under these conditions is a multistep process (2). Step 1 involves a temperature-dependent change which leads to an unidentified intermediate. Step 2 is formation of long thin filaments by a process that is not reversed by cooling. The filaments have not been characterized but may be 5-fold helical microfibrils. Step 3, which is associated with the characteristic turbidity increase, is lateral association of thin filaments to form native banded fibrils. This last step is temperature-dependent and is reversed by cooling, if covalent cross-linking is prevented by prior reduction of the aldehydes that are normally present.

A variety of studies have shown that the nonhelical ends of the collagen molecule must play a critical role in fibril formation since their removal by limited proteolysis affects both the kinetics of assembly (3, 4) and the morphology of the product (5, 6). The experiments reported here were designed to examine the effect of the absence of the nonhelical ends on the individual steps of assembly. For this purpose, we have studied Step 1 in more detail and we have compared the behavior of pepsin-treated collagen to normal and reduced collagen.

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METHODS

Collagen—Soluble collagen was prepared from rat tail tendon and characterized as described by Chandrakasan *et al.* (7) and Williams *et al.* (1). Reduced collagen was prepared from normal collagen by sodium borohydride reduction (2). Pepsin-treated collagen was prepared as follows: Pepsin (Sigma) was added to a solution of collagen (2 mg/ml in 0.5 M acetic acid) to give a 1:10 pepsin:collagen weight ratio. All procedures were performed at 4°C. After stirring for 24 h, the same amount of pepsin was added as initially and stirring was continued an additional 24 h. The pepsin was inactivated and the collagen precipitated by dialysis against 0.02 M Na_2HPO_4. The precipitate was isolated by centrifugation at 8000 × *g* for 30 min and redissolved in 0.5 M acetic acid. After exhaustive dialysis against 0.01 M acetic acid, the solution was lyophilized and the collagen was stored at −20°C. The pepsin-treated collagen was recharacterized as before (1, 7). The tyrosine content was reduced from 4.0 to 0.3 residues/1000 and the content of cross-linked components was reduced to <5%. Tyrosine occurs only in the nonhelical ends and the expected value for intact collagen is 4 to 5 residues/1000 (7). These results indicate extensive removal of the nonhelical ends. The melting profile was indistinguishable from that obtained with the original collagen, showing that the triple helical portion of the molecule was unaffected.

Fibril Formation—As described previously (1, 2), collagen dissolved in 5 mM acetic acid at twice the desired final concentration was mixed with an equal volume of double strength NaCl/*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid/phosphate buffer at 4°C. The sample was placed in a jacketed cell in a recording spectrophotometer and the temperature was raised to the desired temperature to initiate fibril formation. Turbidity was monitored as the optical density at 313 nm. Temperature changes during the course of an experiment were achieved by switching between preset circulating water baths. Temperature was controlled to ±0.1°C and equilibrium was reached in less than 1 min.

Electron Microscopy—Samples at the temperature of the experiment were placed on carbon films laid over 400 mesh grids. After 30 s, they were drained and immediately stained at room temperature with 2% phosphotungstic acid, pH 7.4. Electron micrographs were taken as previously described (1, 2).

RESULTS

Turbidity Curves—Preliminary experiments showed that pepsin-treated collagen formed fibrils much more slowly than normal and reduced collagen. In addition, at concentrations below about 0.3 mg/ml, the fibrils formed from pepsin-treated collagen tended to settle out making turbidity measurements unreliable. For these reasons, higher concentrations and temperatures than used for normal and reduced collagen were necessary. For example, while 0.1 mg/ml and 26°C were suitable for earlier experiments (1, 2), 0.39 mg/ml and 35°C were commonly used in the present series.

Typical turbidity curves obtained with these conditions are shown in Fig. 1. The $t_{1/2}$ values, defined as the times to reach half-maximal turbidity, for normal, reduced, and pepsin-treated collagen were 12, 21, and 131 min, respectively. All three samples gave similar patterns with a turbidity lag followed by a sigmoidal increase. However, in addition to showing a slower rate of assembly, the turbidity curve for pepsin-

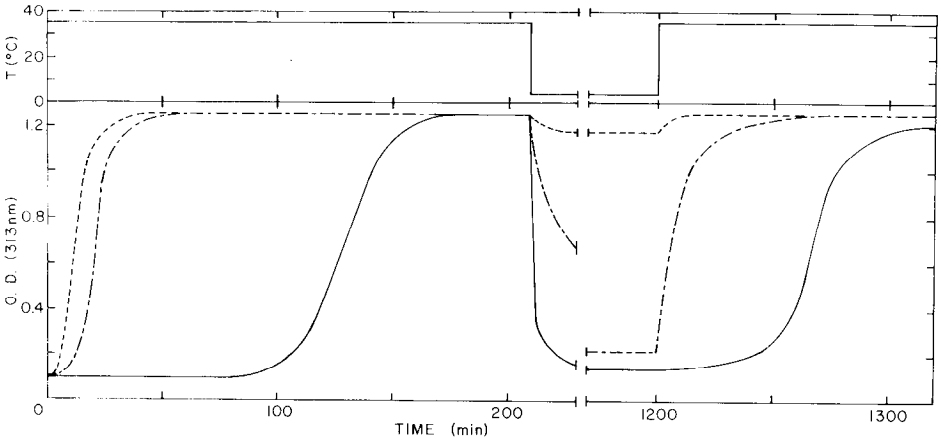

FIG. 1. **Turbidity change (bottom) measured as optical density during fibril formation from solutions of normal (-----), reduced (— - —), and pepsin-treated (——) collagen.** The collagen concentration was 0.39 mg/ml for all samples. The temperature at zero time was 4°C and was changed as shown at the top. The baseline was arbitrarily set at 0.1.

treated collagen had a different character. If the data were plotted as a function of log time, the slopes at $t_{1/2}$ were the same for normal and reduced collagen showing that they assembled by the same mechanism (1, 2), while the slope for pepsin-treated collagen was steeper (not shown). Another way to express this difference is by the ratio $t_{lag}/t_{1/2}$ where t_{lag} is a measure of the length of the turbidity lag and can be approximated as $t_{1/2}\ t_{1/4}/t_{3/4}$ (2), the latter two terms being the times to one-quarter and three-quarters of the turbidity change. This ratio was 0.74 for both normal and reduced collagen but was 0.84 for pepsin-treated collagen.

When the samples were cooled after fibril formation was complete, the turbidity decreased only slightly for normal collagen but returned nearly to baseline for reduced and pepsin-treated collagen (Fig. 1). This behavior of reduced collagen was previously observed and explained by the inhibition of cross-linking that occurs through lysine-derived aldehydes that are normally present in collagen (2). A similar behavior for pepsin-treated collagen was expected since the lysine-derived aldehydes are located in the nonhelical ends. The more rapid and complete reversal of turbidity for pepsin-treated collagen than reduced collagen (Fig. 1) presumably reflects a more complete loss of aldehydes by proteolysis than reduction.

Reheating the cold samples again resulted in the formation of fibrils but with greatly shortened $t_{1/2}$ values (Fig. 1). The turbidity of the reduced collagen increased abruptly on reheating while the turbidity of the pepsin-treated sample increased more slowly in a sigmoidal manner with $t_{1/2} = 66$ min. This value was the same whether the sample was cooled 16 h (Fig. 1) or 64 h (not shown). This time was about one-half the time observed on the first heating and an initial period of constant turbidity was brief or absent. The different behavior of the reduced and pepsin-treated collagen may reflect incomplete reversal in the former case as a result of a small amount of cross-linking. The slightly lower plateau on reheating the pepsin-treated collagen compared to the first time (Fig. 1) is unexplained. It may be related to the impression of somewhat improved order of the fibrils formed, as noted below, since turbidity depends on the nature of the aggregate as well as its concentration.

Concentration Dependence—We have shown that the rate of assembly of normal and reduced collagen, expressed as the reciprocal of $t_{1/2}$ or t_{lag} is directly proportional to the concentration (1, 2). The same result was obtained for pepsin-treated collagen; the slope of the line through data plotted as $-\log t_{1/2}$ as a function of log concentration was 1.0 (Fig. 2).

Critical Concentration—For many assembly processes, it is found that there is a concentration below which no product is formed. For normal and reduced collagen, this concentration, termed the critical concentration, is very small and not significantly different from zero (1, 2). Similarly, when the change in turbidity, a measure of amount of product, for fibril formation from pepsin-treated collagen was plotted as a function of initial collagen concentration, the least squares line through the data extrapolated to a value close to zero (Fig. 3). This result may be subject to a large error because it was not possible to measure low concentrations, but the critical concentration is certainly very small.

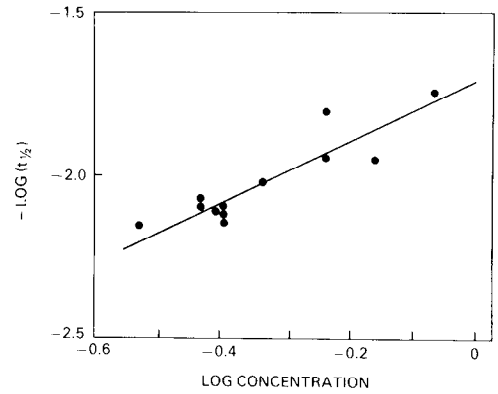

FIG. 2. **Dependence of $t_{1/2}$ of fibril formation on initial concentration of pepsin-treated collagen.** The least squares line through the points has a slope of 1.0.

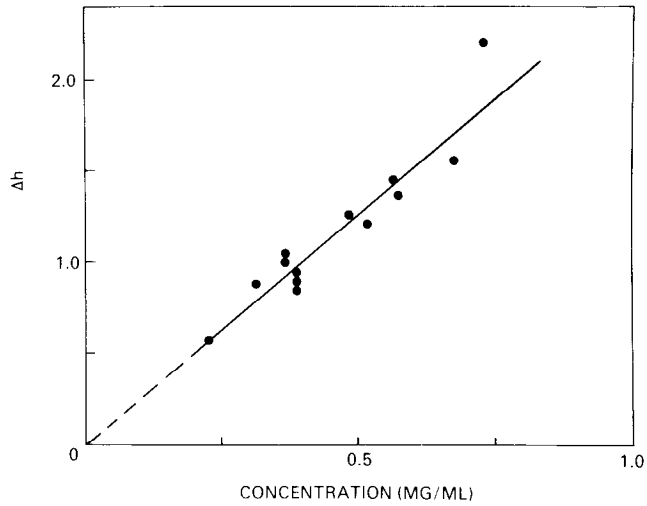

FIG. 3. **Dependence of the total turbidity change, Δ*h*, during fibril formation on initial concentration of pepsin-treated collagen.** The intercept of the least squares line is not significantly different from zero.

Initiation of Fibril Formation, Step 1—We have shown that with normal or reduced collagen, when the temperature is raised to 26°C to initiate fibril formation, after 10 min the sample can be cooled to 4°C for the remainder of the turbidity lag and then returned to 26°C without affecting $t_{1/2}$ or any other feature of assembly (2). Prior to doing similar experiments with pepsin-treated collagen, we examined this initiation step in more detail with normal collagen. We found that if the initial time of heating at 26°C was less than 6 min, $t_{1/2}$ increased. However, the minimal 6-min period was not dependent on collagen concentration in the range 0.08 to 0.39 mg/ml (Fig. 4).

When similar experiments were undertaken with pepsin-treated collagen, a very complex behavior was observed. Cooling to 4°C after an initial period at 35°C not only increased $t_{1/2}$ but increased it in excess of the time at 4°C; it seemed that cooling led to some change incompatible with normal assembly that had to be undone. These experiments were not pursued in detail. However, we also examined the effect of an initial period at 4°C after mixing with buffer to raise the pH of the collagen solution. With normal and reduced collagen we previously reported that $t_{1/2}$, measured from the time of heating, was independent of the earlier period at 4°C. Assembly does not start until the temperature is raised. Therefore, measured from the *time of mixing*, $t_{1/2}$ will have a slope of 1.0 when plotted as a function of the initial time at 4°C (Fig. 5, *dashed line*). However, with pepsin-treated collagen a change occurred after the initial mixing at 4°C such that $t_{1/2}$, measured from the time of mixing, was not increased for cooling times up to about 25 min (Fig. 5). Longer periods at 4°C increased $t_{1/2}$ more than the extra time at 4°C. Apparently, early changes at 4°C were compatible with assembly but later changes were not.

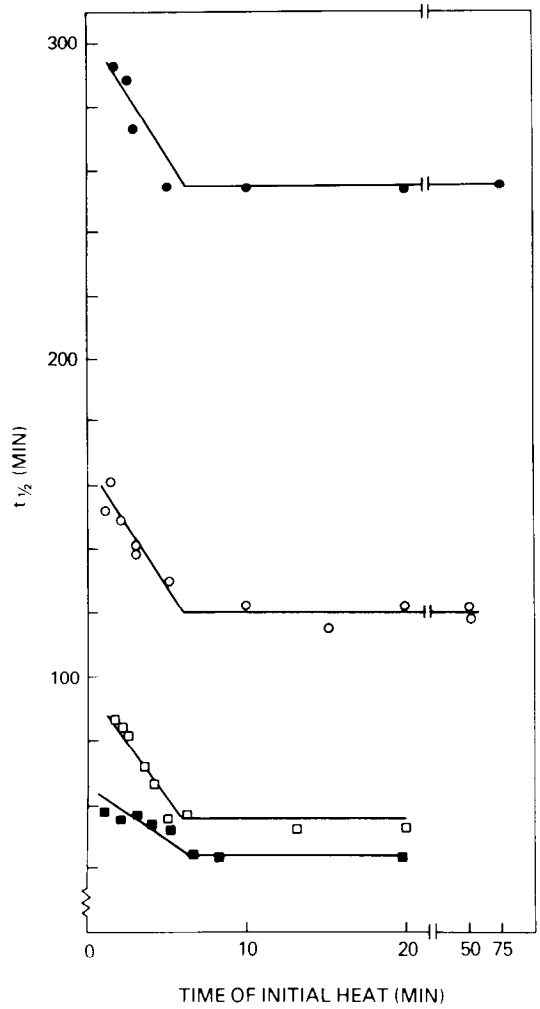

FIG. 4. **Dependence of $t_{1/2}$ of fibril formation on initial time at 26°C for normal collagen at 0.08 (●), 0.10 (○), 0.20 (□), and 0.39 (■) mg/ml.** The temperature of the samples was initially 26°C for the times shown, then reduced to 4°C for the remainder of the turbidity lag, and raised again to 26°C. The last point in each set was taken at approximately the end of the turbidity lag and therefore the time at 4°C was zero for that point. The lines were approximated to show the two steps, the first ending at 6 ± 1 min.

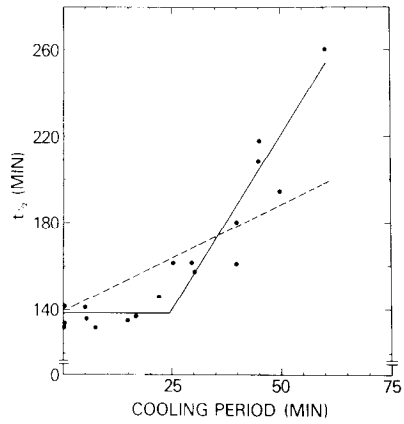

FIG. 5. **Dependence of $t_{1/2}$ of fibril formation for pepsin-treated collagen on the initial time at 4°C (*solid line*).** The pH was raised at zero time and the temperature was raised to 35°C at the times indicated. Therefore, $t_{1/2}$ includes the cooling period. The *solid lines* were drawn to emphasize the difference between early and late changes. The *dashed line* has a slope of 1.0 and illustrates the independence exhibited by normal collagen on initial cooling.

Morphology—Samples were taken for electron microscopy during the course of fibril formation from pepsin-treated collagen in the same manner as for normal and reduced collagen in our earlier experiments (1, 2). At the turbidity plateau (Fig. 1, 150 min), fibrils formed from pepsin-treated collagen were usually rope-like with a twisted filamentous substructure and occasional areas of native banding. Fully formed fibrils tended to form dense clumps on the grid. Although pictures were taken in less populated areas, the general morphological characteristics of the fibrils themselves did not vary in any observable manner. The banding sometimes was oblique as observed by others using protease-treated collagen (4, 8). Ends were visible in every field and short tactoidal forms were common. Samples taken during the turbidity increase or immediately after cooling contained a larger proportion of these tactoids; they varied in size, the smallest being about 1 μm long, and appeared to be intermediate forms. These features, shown in Fig. 6, *A* and *B*, are in sharp contrast to fibrils formed from normal and reduced collagen. Although normal fibrils are not as well ordered when made at 35°C as at lower temperatures (1), native banding was common and the fibrils were very long with ends rarely seen (Fig. 6*D*). Short tactoids were not present.

Samples taken, either just before the turbidity increase (Fig. 1, 80 min) or after cooling overnight after the turbidity plateau, showed thin filaments as illustrated in Fig. 6*C*. They were usually seen only in tangled clumps probably because isolated filaments would not hold sufficient stain to be easily recognized. It is unlikely that these filaments were artifacts since similar filaments were often present with fibrils and indeed seemed to form the substructure of fibrils. The filaments from pepsin-treated collagen were similar to those from normal and reduced collagen (2) in that minimal diameters were 2 to 4 nm and wider filaments were often seen to be composed of two or more thinner filaments. They differed in

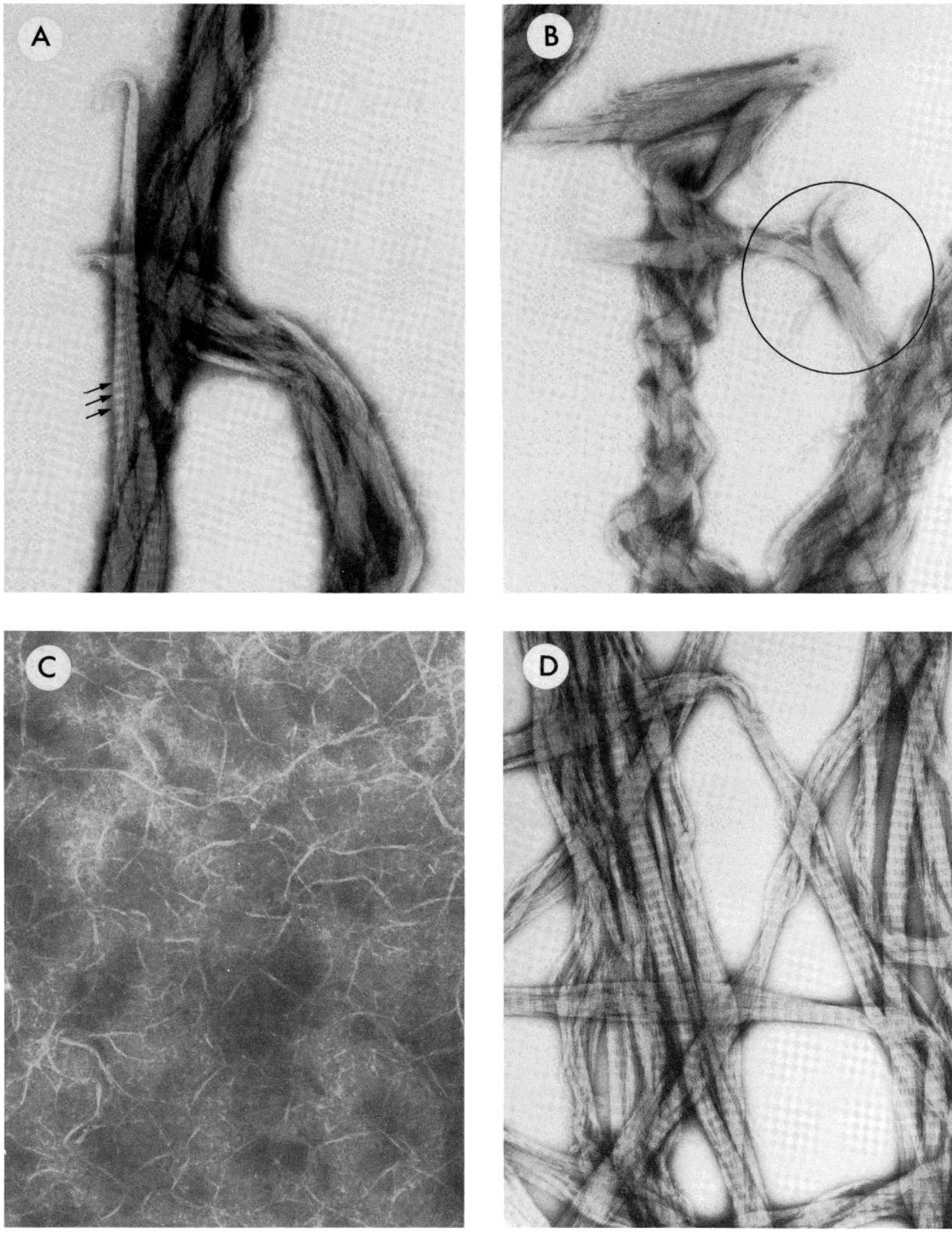

FIG. 6. **Electron micrographs of collagen preparations negatively stained with 2% phosphotungstic acid, pH 7.4.** *A* and *B*, fibrils formed from pepsin-treated collagen at 35°C. Normal banding, which is sometimes oblique (*arrows*), is occasionally present. The fibrils are filamentous and twisted and short tactoidal forms are common. A typical tactoid is *circled*. Magnification, × 33,000. *C*, filaments formed from pepsin-treated collagen fibrils by cooling to 4°C. Similar filaments are present at the end of the turbidity lag period and reversibly form fibrils as in *A* and *B*. Magnification, × 46,000. *D*, fibrils formed from normal collagen at 35°C. Normal banding is common and fibrils are very long. Magnification, × 33,000.

that the distance over which a smooth contour could be followed was much less, suggesting that the filaments from pepsin-treated collagen were much shorter. However, ends were difficult to identify and lengths could not be measured. No order could be seen in the filaments. When filaments were reheated to form fibrils a second time, the impression was gained that the fibrils were less distorted and that native banding was more frequent than the first time.

DISCUSSION

We have shown that *in vitro* assembly of fibrils from pepsin-treated collagen has many characteristics in common with assembly from normal or reduced collagen. (*a*) There is a period that is dominated by linear assembly without a change in turbidity followed by lateral assembly associated with the turbidity increase. (*b*) Lateral assembly but not linear assembly is reversed by cooling. (*c*) Fibril formation is most simply described as an accretion process since the reciprocal of $t_{1/2}$ is proportional to collagen concentration and the critical concentration is essentially zero. (*d*) The product is filamentous fibrils with, in some places, characteristic native collagen banding.

There are, however, some major differences between normal and pepsin-treated collagen which provide information about the process. These differences and possible interpretations based on our multistep model (2) are as follows.

1. The response to temperature changes early in assembly is very different. Since the initial temperature-dependent, concentration-independent response of normal collagen, Step 1, is abolished or altered by removal of the nonhelical ends of the collagen molecule, we conclude that these ends are critically involved in this step. How they are involved is not known, but the chemistry of the nonhelical ends and of regions in the helical portion of the molecule with which they probably associate in the native fibril (9, 10), suggest highly specific interactions. These interactions would require molecular association which is not reflected in the apparent absence of a concentration dependence, suggesting that there may be a rate-limiting conformational change as part of Step 1.

2. Electron microscopy of material taken before the turbidity begins to increase shows that although linear growth of thin filaments occurs with pepsin-treated collagen, they appear to be considerably shorter than those formed from normal collagen during Step 2 (2). Furthermore, the increased $t_{1/2}$ observed after an initial cooling of pepsin-treated collagen suggests that there is an alternate, counterproductive pathway. The difficulties in linear assembly are also reflected in the greatly increased t_{lag} and the greater proportion of t_{lag} to $t_{1/2}$ compared to normal collagen. Once formed, filaments appear to be stable since $t_{1/2}$ of reassembly is independent of the time at 4°C.

3. Lateral assembly, Step 3, occurs with pepsin-treated collagen but the fibrils are much shorter, distorted, and less commonly banded than fibrils formed from normal collagen. A characteristic feature of pepsin-treated collagen is the formation of short tactoids, particularly early in Step 3. They are similar to those reported by Trelstad *et al.* (11), but are larger and appear later in the assembly process. We do not see them with normal collagen.

Since fibrils form from pepsin-treated collagen, even though abnormal, the properties necessary for fibril formation must be inherent to a certain extent in interactions between the helical bodies of collagen molecules. However, it is evident that the nonhelical ends critically regulate the normal progression of events.

REFERENCES

1. Williams, B. R., Gelman, R. A., Poppke, D. C., and Piez, K. A. (1978) *J. Biol. Chem.* **253,** 6578–6585
2. Gelman, R. A., Williams, B. R., and Piez, K. A. (1979) *J. Biol. Chem.* **254,** 180–186
3. Bannister, D. W., and Burns, A. B. (1972) *Biochem. J.* **129,** 677–681
4. Comper, W. D., and Veis, A. (1977) *Biopolymers* **16,** 2133–2142
5. Comper, W. D., and Veis, A. (1977) *Biopolymers* **16,** 2113–2131
6. Leibovich, S. J., and Weiss, J. B. (1970) *Biochim. Biophys. Acta* **214,** 445–454
7. Chandrakasan, G., Torchia, D. A., and Piez, K. A. (1976) *J. Biol. Chem.* **251,** 6062–6067
8. Ghosh, S. K., and Mitra, H. P. (1975) *Biochim. Biophys. Acta* **405,** 340–346
9. Piez, K. A. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., and Reddi, A. H., eds) pp. 1–44, Plenum, New York
10. Fietzek, P. P., and Kühn, K. (1976) *Int. Rev. Connect. Tissue Res.* **7,** 1–60
11. Trelstad, R. L., Hayashi, K., and Gross, J. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **11,** 4027–4031

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