Formation of Collagen Fibrils in Vitro by Cleavage of Procollagen with Procollagen Proteinases*

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A new system was developed for studying the assembly of collagen fibrils in vitro. A partially purified enzyme preparation containing both procollagen N-proteinase and C-proteinase (EC 3.4.24.00) activities was used to initiate fibril formation by removal of the N- and C-propeptides from type I procollagen in a physiological buffer at 35–37 °C. The kinetics of fibril formation were similar to those observed for fibril formation with tissue-extracted collagen in the same buffer system, except that the lag phase was longer. The longer lag phase was in part accounted for by the time required to convert procollagen to collagen. Similar results were obtained when an intermediate containing the C-propeptide but not the N-propeptide was used as a substrate. Therefore, removal of the C-propeptide appeared to be the critical step for fibril formation under the conditions used here. The fibrils formed by enzymic cleavage of procollagen or pCCollagen appeared microscopically to be more tightly packed than fibrils formed directly from collagen under the same conditions. This impression was confirmed by the observation that the fibrils formed by cleavage of procollagen were stable to temperatures 1.5–2 °C higher than fibers formed from extracted collagen under the same conditions. When smaller amounts of procollagen proteinase were used, the rate of cleavage of procollagen to collagen was markedly reduced. The fibrils which formed under these conditions were up to 3 μm in diameter. Some appeared to contain branch points.

Collagen monomers and polymers extracted from connective tissues with cold acidic or neutral buffers readily form characteristic fibrils when solutions containing the protein are adjusted to neutrality and warmed to 25–37 °C (1–7). The fibrils formed have the typical cross-striated pattern of collagen fibrils seen in vitro. However, the kinetics of fibril formation, and in part the morphology of the structures, are dependent on the history of the protein solution in that they are altered by the loss of telopeptides from the ends of the molecule during extraction or purification (5, 8–14), the presence of cross-links (6–9), the presence of aldehydes which can form cross-links in the protein (14, 15), and other variables such as the composition of the buffer used for fibril formation (1, 3, 5, 16, 17).

We report here the development of a new system for the assembly of collagen fibrils in vitro in which a partially purified enzyme preparation containing both procollagen N-proteinase1 and C-proteinase activities was used to initiate fibril formation by enzymic removal of the N- and C-propeptides from type I procollagen in a physiological buffer at 35–37 °C.

MATERIALS AND METHODS

Preparation of Procollagen Substrates—Type I procollagen substrates were prepared using matrix-free cells from chick embryo tendons as described previously (18, 19), but conditions were altered so that pCCollagen was obtained in addition to procollagen. The cells were incubated at a higher concentration (1.5 × 10^7/ml) for 6 h instead of the 4–5 h used previously. Also, the ammonium sulfate precipitate of the medium was chromatographed on a DEAE-cellulose (Whatman DE32) column at pH 9.2 instead of 7.8 (20). Under these conditions, about 20% of the nondialyzable 14C-labeled protein was recovered in the void volume of the column as collagen. About 25% eluted in a peak just after the void volume and consisted of pCCollagen. The remainder eluted as a peak in the latter two-thirds of the gradient and consisted of procollagen (20). The fractions containing pCCollagen and procollagen were pooled separately and concentrated by precipitation with 180 mg/ml of ammonium sulfate for 4–5 h at 4 °C. After centrifugation, the samples were stored at −20 °C in 0.1 M Tris/HCl buffer, pH 7.5, at 25 °C, containing 0.4 M NaCl. The protein concentration was about 400 μg/ml.

Preparation of Type I Collagen—Collagen was extracted from lathyritic chick embryo tendon as described before (21). Collagen was precipitated from the extract by adding solid NaCl to a final concentration of 20% and collected by centrifugation at 10,000 × g for 30 min. The pellet was washed three times with 20% NaCl and dissolved in 0.5 M acetic acid. The collagen solution was further purified according to ChandrasakAn et al. (8).

Preparation of Procollagen N- and C-proteinases—Procollagen N- and C-proteinases were prepared from calvaria of 17-day-old chick embryos. The initial steps in the purification involved extraction of the tissue with a high salt buffer containing a non-ionic detergent and chromatography on DEAE-cellulose as described previously (22). Some experiments were performed with this enzyme preparation. It contained about 570 units of C-proteinase activity/mg of protein. For other experiments, the enzymes were purified further by chromatography on a column of heparin-Sepharose.1 The heparin-Sepharose column-purified preparation contained about 4000 units of C-proteinase activity/mg of protein.

Fibril Formation—The substrates and the enzyme preparations were dialyzed separately against a buffer which had a composition similar to a standard cell culture salt solution (23): 110 mM NaCl, 4.4 mM KCl, 1.8 mM CaCl_2, 0.81 mM MgSO_4, and 0.91 mM Na_2HPO_4. The buffer also contained 15 μM N-(tris(hydroxymethyl)methyl)-2-aminoethanesulfonylic acid as a buffer. The final pH was about 7.2 at 35 °C. In order to prevent bacterial growth, 0.02% NaN_3, 100 units/ml of penicillin G, and 0.1 mg/ml of streptomycin were added to the buffer.

The abbreviations used are: procollagen N-proteinase, the enzyme that removes the NH₂-terminal propeptide from type I procollagen; procollagen C-proteinase, the enzyme that removes the COOH-terminal propeptide from type I procollagen; pCCollagen, intermediate in the conversion of procollagen to collagen containing the COOH-terminal propeptide but not the NH₂-terminal propeptide.

were added to the buffer. Before use, the substrate and enzyme solutions were clarified by centrifugation at 80,000 × g for 90 min. They were then mixed at 5 °C. Within 1 h after mixing, the temperature was raised to 35–37 °C. The increase in turbidity of the solution was monitored in a Gilford 240 spectrophotometer at 313 nm. For this purpose, about 200 µl of the solution were placed in a microcuvette after degassing on ice for 4 min. Evaporation of the solution was avoided by layering paraffin oil (0:119, Fisher) on the top of the solution.

In control experiments, collagen in 0.5 M acetic acid was dialyzed against 4.5 mM phosphate buffer (pH 3.1), clarified as above, and neutralized. The conditions for fibril formation were the same as above.

The extent of the cleavage of procollagen was estimated by electrophoresis. The enzymic activity was quenched by adding 15 µl of solution containing 250 mM sodium ethylenediaminetetraacetate, 0.1% Triton X-100, and 150 mM NaCl in 50 mM Tris/HCl buffer (pH 7.5 at 25 °C) to aliquots of 50 µl. The samples were then prepared for electrophoresis by adding 20 µl of 200 mM iodoacetamide and 15 µl of a solution containing 8 mM urea, 2% sodium dodecyl sulfate, and 0.01% bromphenol blue in 50 mM Tris/HC1, pH 6.8, at 25 °C, and heating to 100 °C for 3 min.

Collagen concentration was estimated by the microbiuret method adapted from that of Lawrence (23) at 310 nm (24). The concentration %C-labeled procollagen or pCcollagen was also determined by the microbiuret method. The %C content was assayed with a liquid scintillation counter, and the specific activity was then used to estimate substrate concentrations in each experiment.

**Microscopy**—Samples for electron microscopy were prepared on grids coated with carbon-coated collodion films. The collagen fibrils were suspended in water, a drop of the suspension was placed on a grid, and the drop allowed to dry at room temperature. The fibrils were then negatively stained with 1% sodium phosphotungstate at pH 7.5. Electron micrographs were taken in a JEM 100B electron microscope at an acceleration voltage of 80 kV.

**Heat Denaturation Experiments**—Collagen fibrils in microcuvettes were denatured by raising the temperature at a rate of about 12 °C/h. The melting of collagen fibrils was monitored by recording absorbance (25) at 313 nm. The temperature was estimated by measuring the temperature of water in another cuvette on the same cuvette holder.

The stability of collagen fibers was also assayed by heat denaturation and then trypsin/chymotrypsin digestion at 20 °C (25, 26). Polypropylene centrifuge tubes (0.5 ml) containing 50 µl of procollagen or collagen fibril solution were heated at a temperature of 37–60 °C for 5 min. After cooling for 1 min at 20 °C, protease digestion (26) was initiated by adding 15 µl of the buffer solution used for fibril formation containing trypsin (40 µg/ml) and chymotrypsin (400 µg/ml). The digestion was terminated after 2 min by adding 15 µl of 12 mM p-aminobenzamidine/HCl and 6 mM phenylmethylsulfonylfluoride in the same buffer. The solution was then cooled on an ice bath and left overnight at 4 °C. The samples were prepared for gel electrophoresis by adding 10 µl of a solution containing 8 mM urea, 20% sodium dodecyl sulfate, and 0.01% bromphenol blue in 50 mM Tris/HCl (pH 6.8 at 25 °C), and heating to 100 °C for 3 min. The samples were then electrophoresed on polyacrylamide gels. The amount of trypsin/chymotrypsin-resistant chains was estimated by densitometry at 550 nm in a Gilford 240 spectrophotometer of gels stained with Coomassie brilliant blue or of fluorograms prepared from the gels.

**Gel Electrophoresis**—Polyacrylamide gel electrophoresis was performed as described by King and Laemmli (27) with minor modifications (20). A slab of 1.5-mm thickness was used as a separating gel; the stacking gel was 4% polyacrylamide. The gels were either stained with Coomassie brilliant blue or used to prepare fluorograms (28).

**RESULTS**

**Fibril Formation from Collagen and Enzymic Cleavage of Procollagen**—Procollagen proteinases were prepared with a recently developed procedure for purification of type I procollagen C-proteinase (22). During the purification, considerable amounts of procollagen N-proteinase activity were discarded in fractions which did not contain C-proteinase activity. The amount of N-proteinase activity in the final preparation, however, was still sufficient to remove the N-propeptides under the conditions employed here (Fig. 1).

In preliminary experiments, efforts were made to develop a buffer system which was optimal for studying fibril formation. In selecting the buffer system, two criteria were established: (a) the buffer should approximate physiological conditions, and (b) the buffer should not inhibit either procollagen N- or C-proteinase activities. The buffer selected to satisfy these criteria was Earle's balanced salt solution (23). The buffer has an ionic composition similar to extracellular fluid; it contains 1.8 mM Ca++ and 0.9 mM phosphate, and it has an ionic strength of 0.19. Because of the instability of the bicarbonate ion, which was a major component of the buffer, the buffering capacity was enhanced by adding the synthetic buffer N-(tris(hydroxymethyl)methyl)-2-amino)ethanesulfonic acid to a final concentration of 15 mM.

As indicated in Fig. 2, collagen extracted from the tendons of lathyritic chick embryos readily formed fibrils when warmed to 35 °C in the buffer. The kinetic data for fibril formation were similar to the kinetics observed in buffer systems employed by other investigators (see Refs. 3 and 5) in that there was a well defined lag phase followed by a sigmoidal increase in turbidity. The time course and the kinetics of fibril formation were approximately the same as those recently obtained by Williams et al. (5) except that the lag time was longer, i.e. 1.3 h instead of 0.5 h. Under the same conditions, procollagen from chick embryo tendon cells did not form fibrils which were detectable by turbidimetric measurements in 10 h (Fig. 2) or even after 4 days (not shown). Fibrils readily formed, however, when a mixture of procollagen N- and C-proteinases was added to the procollagen. The kinetic data of fibril formation were similar to that seen with collagen solutions. As anticipated, the kinetic data were dependent on the amount of enzymic activity added to the solution. With the maximal amounts of enzymic activities employed here, the lag phase was approximately twice the length of the lag phase.
observed with an equimolar concentration of collagen under the same conditions (Fig. 2). The longer lag phase was in part accounted for by the time required to convert procollagen to collagen in the system. As indicated in Fig. 1, complete removal of the propeptides required 4 to 8 h (Fig. 1).

**Thermal Stability of the Fibrils Formed by Cleavage of Procollagen—Electron microscopy (not shown) of the fibrils formed by cleavage of procollagen with the proteinases (Fig. 2) suggested that the fibrils were more tightly packed than fibrils obtained directly from lathyritic collagen under the same experimental conditions. To evaluate the quality of the fibrils quantitatively, we examined them with two tests: their thermal stability by loss of turbidity on heating and their sensitivity to proteinases.

To examine the thermal stability of the fibrils, fibrils formed under conditions shown in Fig. 2 were heated at a rate of about 12 °C/h, and melting of the fibers was followed by turbidity. A similar approach was previously used by Snowden and Swan (25) to test the effects of glycosaminoglycans and proteoglycans on fibril formation. As indicated in Fig. 3, there was a relatively sharp decrease in turbidity as a function of temperature. The midpoint for the thermal transition of fibrils formed directly from collagen was about 48.2 °C. Under the same conditions, fibrils formed by the cleavage of procollagen had a thermal transition with a midpoint of about 50.5 °C. In repeated experiments, the midpoint of the thermal transition seen with fibrils formed by cleavage of procollagen was consistently 1.5-2.0 °C higher than the midpoint of the transition seen with fibrils formed directly from lathyritic collagen. In control experiments, it was found that addition of the enzyme preparation to lathyritic collagen did not increase the thermal stability of the fibrils formed. Therefore, the greater stability of the fibrils formed by cleavage of the procollagen was not explained by the presence of the enzymes per se or the presence of any other component of the enzyme preparation.

In the assay of the thermal stability of the fibrils (Fig. 3), there was a residual turbidity when the solutions were heated to 55 or 60 °C, and in some experiments the turbidity changed erratically after the sharp transition (see Fig. 6 below), apparently because of unevenness in the distribution of denatured collagen and heat-induced convection currents. For these reasons, we also examined the stability of the fibers by proteinase digestion.

To assay the thermal stability of the fibrils by proteinase resistance, fibrils formed under the conditions used in Fig. 2 were heated for 5 min at various, predetermined temperatures. The samples were then rapidly cooled to 20 °C and digested with a mixture of chymotrypsin and trypsin. The conditions for digestion with chymotrypsin and trypsin were similar to those employed recently to study the folding of procollagen molecules into a triple helical conformation in vitro and in cellulo (26). After digestion at 20 °C with chymotrypsin and trypsin for 2 min, the proteinases were inhibited by adding the proteinase inhibitors p-aminobenzamidine and phenylmethylsulfonyl fluoride. The digestion products were then examined by polyacrylamide gel electrophoresis. Fluorograms of the gels were prepared and scanned with a densitometer. With this assay procedure, procollagen itself was resistant to proteinase digestion after heating to 46 °C (Fig. 4). It was 50% digested after exposure to a temperature of about 43.5 °C. This value for 50% digestibility is consistent with a midpoint

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**Fig. 2. Turbidity change due to fibril formation.** Procollagen (120 μg/ml) was incubated with and without a DEAE-column-purified enzyme preparation containing procollagen N- and C-proteinase activities (110 units of the C-proteinase/ml) in a physiological buffer at 36 °C. See Fig. 1 for the extent of cleavage of procollagen. The collagen concentration (80 μg/ml) was at about the same molar concentration as the procollagen. Lathyritic collagen, ○; procollagen incubated with enzyme, □; procollagen incubated without the enzyme preparation.

**Fig. 3. Melting curves for collagen fibrils formed by in vitro enzymic cleavage of procollagen (□) and by self-assembly of collagen (△).** Decrease of turbidity with melting was measured as optical density at 313 nm. The fibrils were formed as in Fig. 2 and melted by raising the temperature at a rate of about 12 °C/h after incubation at 35 °C for 28 h.

**Fig. 4. Thermal stability of procollagen (○) and collagen fibrils formed by in vitro enzymic cleavage of procollagen (△) and self-assembly of collagen (△).** The collagen fibrils were formed under conditions similar to those used in Figs. 1 and 2 by incubating a solution of collagen (80 μg/ml) with an enzyme preparation containing procollagen N- and C-proteinase activities (DEAE-column-purified; 40 or 110 units/ml of the C-proteinase) at 36 °C for about 30 h. Procollagen (○), 120 μg/ml, was also incubated at 36 °C for a similar period. The thermal stability was assessed by measuring the trypsin/chymotrypsin-resistant α1 chains at 20 °C after heating the samples at the temperature shown for 5 min. The data are means from two experiments and are expressed as the percentage of protease-resistant α1 chains relative to the value obtained with procollagen which was incubated at 36 °C for 26 to 30 h.
of 42 °C for the helix to coil transition of chick embryo type I procollagen as assayed by its circular dichroism spectrum (21). With the same assay system, fibrils formed from lathyritic collagen were more resistant to proteinase digestion than procollagen, and about 50% of the protein was proteinase-resistant after exposure to 48 °C. Fibrils formed by enzymic cleavage of procollagen were more resistant than those formed from collagen; about 50% of the protein was not digested after exposure to 51 °C. The proteinase digestion data provided an assay of all the 14C-labeled protein. Under the conditions employed here, triple helical collagen resists proteinase digestion (26). Since essentially all the 14C-protein cleaved by the procollagen proteinase was more resistant to digestion than the initial procollagen, the data indicated that essentially all the initial procollagen was incorporated into fibrils after cleavage with the enzymes.

Formation of Collagen Fibrils by Enzymic Cleavage of pCcollagen—Since the enzymic preparation employed contained both N- and C-proteinases, it was not possible to examine the effects of selective cleavage of the N- or C-propeptides from procollagen. It was possible, however, to use pCcollagen as a substrate and thereby examine fibril formation by cleavage of the C-propeptide from this protein.

As indicated in Fig. 5, pCcollagen did not form fibrils as assayed by turbidity when incubated at 35 °C in the standard buffer system. When N- and C-proteinases were added to pCcollagen, fibrils were formed. The kinetics of fibril formation was similar to that seen when procollagen was used as the substrate. In the same experiments, the cleavage of the C-propeptide from the pCcollagen was assayed by gel electrophoresis of the samples and densitometry of fluorograms of the gel. The results (Fig. 5) demonstrated that fibril formation did not begin until after most of the C-propeptide was cleaved from the substrate. Part of the lag phase, therefore, could...

**Fig. 5.** Turbidity change (○) with the formation of collagen fibrils and the release of C-propeptide (●) from pCcollagen. pCcollagen (130 μg/ml) was incubated with 60 units/ml of procollagen C-proteinase (DEAE-column-purified) in a physiological buffer at 35-36 °C. Control samples without the C-proteinase showed little change in turbidity (□) or release of C-propeptide (■).

**Fig. 6.** Melting curve, by turbidity, for collagen fibrils formed by enzymic cleavage in vitro of pCcollagen. The fibrils were formed as in Fig. 5, and melted by raising the temperature at a rate of about 12 °C/h after 50 h of incubation at 35 °C. As indicated, the absorbance at 313 nm did not return to the initial base-line value (see text).

**Fig. 7.** Distribution of C-propeptide and collagen after cleavage of procollagen and fibril formation. Procollagen (150 μg/ml) was incubated in a final volume of 100 μl with a heparin-Sepharose column-purified enzyme preparation (80 units/ml) for 48 h at 36 °C. The sample was centrifuged at 13,000 × g at room temperature for 15 min. The pellet was washed twice with buffer. The samples were then examined without reduction in 6% polyacrylamide gels in sodium dodecyl sulfate. Lane a, procollagen incubated without enzyme; Lane b, supernatant of procollagen incubated with enzyme preparation (10% of total sample applied to gel); Lane c, pellet from same experiment (20% of total sample applied to gel).

**Fig. 8.** Dependence of collagen fibril formation from procollagen on the amount of procollagen proteinases. Procollagen (120 μg/ml) was incubated with a heparin-Sepharose column-purified enzyme preparation containing procollagen N- and C-proteinase activities at 35 °C. Fibril formation was monitored by measuring the change in absorbance at 313 nm. The amount of the enzyme preparation in terms of the C-proteinase activity: X, 5 units/ml; △, 10 units/ml; ○, 20 units/ml.
FIG. 9. Optical micrographs of collagen fibrils. Procollagen (120 µg/ml) was incubated with heparin-Sepharose column-purified enzyme preparation for 3 days at 36 °C. The enzyme concentration was 10 units/ml of C-proteinase activity. A, dark field micrograph of fibrils formed by enzymic cleavage of procollagen. Magnification: × 100. B, dark field micrograph of fibrils formed from extracted lathyritic collagen under the same conditions. Magnification: × 100. Inset b, phase contrast micrograph of B. Magnification: × 1000. C, light microscopy of sample similar to that shown in A. Magnification: × 250. D, same as C. Magnification: × 1000. E, phase contrast micrograph of sample similar to those shown in A, C, and D. A branch point is seen. Magnification: × 2000.
again be accounted for by the time required to convert the pCcollagen to collagen. As indicated in Fig. 6, the fibrils formed by cleavage of pCcollagen had about the same thermal stability as fibrils formed by cleavage of procollagen in that the midpoint of the melting curve was about 50.6 °C.

**Distribution of C-propeptide and Collagen**—In further experiments, procollagen was cleaved with the enzymic preparation to generate collagen fibers, and the fibrils were isolated by centrifuging the sample. The supernatant and washed pellet of fibrils were then examined separately by gel electrophoresis in sodium dodecyl sulfate. As indicated in Fig. 7, the C-propeptide was recovered in the supernatant; no detectable amounts of C-propeptide were recovered in the fibril fraction. As also indicated in Fig. 7, the supernatant fraction contained α chains, and densitometric scanning of the fluorograms indicated that 29% of the total α chains were in the supernatant. In a control experiment with extracted collagen, 15% of the total α chains were recovered in the supernatant (not shown). The results indicated, therefore, that at least 71% of the collagen generated by cleavage of procollagen was incorporated into fibers. Since centrifugation under the conditions employed here is not likely to recover all the fibrils, this value is probably an underestimate. The data obtained by proteinase digestion (Fig. 4), indicating incorporation of essentially all the collagen into fibers, is probably a more reliable estimate.

**Formation of Large Collagen Fibrils**—One of the inherent properties of the system developed here was that the rate of fibril formation can be controlled by varying the amount of enzymic activity added to the solution of procollagen. When the amount of enzymic activity was reduced from 60 or 110 units/ml (Figs. 2 and 5) to 20 units/ml, complete conversion of the procollagen to collagen required over 10 h (not shown), and the maximal change in turbidity did not occur for 40 h or longer (Fig. 8). With 5 or 10 units/ml, complete conversion of procollagen to collagen required over 15 h, and the change in turbidity was again slower (Fig. 8). With 5 to 20 units/ml of enzyme, the final change in turbidity was less than in Fig. 2, apparently because the fibers which formed were large and settled to the bottom of the cuvette. A network of spicule-like structures was readily seen by inspection of the cuvettes (not shown). When examined by light microscopy (Fig. 9A), the fibrils were considerably larger than those formed from extracted collagen at the same temperature and in the same buffer (Fig. 9B). The diameter of the largest fibrils obtained by the slow cleavage of procollagen was 3 μm. Some of the fibrillar structures appear to have branch points in which new fibrils were attached at an angle of about 40° to the main shaft (Fig. 9, C, D, and E).

Two tests were used to establish that the fibrils shown in Fig. 9, A, C, D, and E were collagen. One was the observation that the structures melted when the sample was heated to about 68 °C. The other consisted of examining the fibrils by electron microscopy. As indicated in Fig. 10A, the fibrils had the typical cross-striated pattern of collagen fibrils. As expected from light microscopy (Fig. 9B) and from the tests of thermal stability (Fig. 3) and proteinase resistance (Fig. 4), the fibrils formed from extracted collagen under the same conditions were thinner and not as tightly packed (Fig. 10B).

**DISCUSSION**

The self-assembly of extracted collagen into collagen fibrils has been extensively investigated over the past several decades. The data have demonstrated that most of the essential information for directing fibril assembly is contained within the collagen monomer itself, and they have provided considerable information about the process of fibril formation (1-17, 29).

The system developed here is the first system in which collagen fibrils have been formed by enzymic cleavage of procollagen, the precursor of collagen. Therefore, the conditions approximate those seen with fibril formation in vivo. The procollagen substrate employed for the experiments was largely homogenous as tested by gel electrophoresis (20). The preparation of procollagen proteinases was not homogenous, but it was of sufficiently high specific activity so as to cleave about 25 μg of procollagen to collagen within 4 to 8 h. Also, the rate of cleavage of procollagen was sufficient to allow fibril formation to occur at a rate which was comparable to the rate of fibril formation seen with lathyritic collagen under the same conditions. Since the process of fibril formation appeared to be similar when either procollagen or pCCollagen was used as a substrate, the results suggested that removal of the C-propeptide was the critical step for initiating fibril formation in the system.

Microscopy of the fibrils suggested that the fibrils prepared by enzymic cleavage of procollagen were more tightly packed than the fibrils obtained directly from lathyritic collagen. This impression was supported by quantitative evaluation of the fibrils by their thermal stability. The observation that the fibrils formed by enzymic cleavage of procollagen were more stable does not necessarily mean that these structures are also more stable than fibrils obtained by warming extracted collagen in other buffer systems. In particular, the presence of
high phosphate appears to improve the morphology of collagen fibrils, and it is probable that the regular fibrils seen in high phosphate buffers (see Refs. 5 and 6) are comparable to the fibrils obtained here by cleaving procollagen in a physiological buffer.

When the procollagen was cleaved to collagen slowly and with the use of small amounts of enzymic activity, large fibrils were formed, and many contained branch points. The largest fibrils had diameters of 3 μm, or about five times the diameter of the largest type I collagen fibrils seen in animal tissues (30). The results, therefore, demonstrated that the diameter of collagen fibrils seen in vitro is not directly limited by the information present in the collagen monomer. Because of the large diameter of the fibrils, they may be particularly useful for studying the fibrillar structure of collagen by x-ray diffraction and other physical techniques.

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