Intracellular Collagen and Protocollagen from Embryonic Tendon Cells*

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SUMMARY

When matrix-free tendon cells from chick embryos were incubated with [14C]proline and then extracted with sodium dodecyl sulfate and mercaptoethanol, a major part of the newly synthesized [14C]-collagen was found to consist of polypeptides that were in part derived from the intracellular precursors. When these cells were incubated with [14C]proline and then extracted with sodium dodecyl sulfate, a large fraction of the intracellular collagen, and amino acid analyses indicated that 40 to 50% of the protein in the extracts was collagen. The protein extracted from cells incubated with [14C]proline and then extracted with sodium dodecyl sulfate and mercaptoethanol, a major part of the newly synthesized [14C]-collagen was found to consist of polypeptides that were in part derived from the intracellular precursors. When these cells were incubated with [14C]proline and then extracted with sodium dodecyl sulfate, a large fraction of the intracellular collagen, and amino acid analyses indicated that 40 to 50% of the protein in the extracts was collagen. The protein extracted from cells incubated with [14C]proline and then extracted with sodium dodecyl sulfate and mercaptoethanol, a major part of the newly synthesized [14C]-collagen was found to consist of polypeptides that were in part derived from the intracellular precursors. When these cells were incubated with [14C]proline and then extracted with sodium dodecyl sulfate, a large fraction of the intracellular collagen, and amino acid analyses indicated that 40 to 50% of the protein in the extracts was collagen.

Collagen is assembled by a series of sequential steps consisting of assembly of proline-rich and lysine-rich polypeptides, hydroxylation of some of the prolyl and lysyl residues either while the nascent chains are still being assembled or after the completed polypeptides are released from ribosomes, and glycosylation of some of the hydroxylysyl residues before the molecule is secreted into the extracellular matrix (for recent review, see Reference 1). Recent studies have demonstrated that collagen is first synthesized as a precursor form which is larger than the collagen found in extracellular fibers (1-13) because of an extension of about 130 A at the NH2-terminal end (9-11). This precursor form has been known either as "procollagen" (5, 9, 10, 12, 13) or "transport form" (3, 8, 11), and it apparently cannot self-assemble into fibers until the NH2-terminal extension is cleaved off (9, 11). In the case of embryonic tendon it has been shown that the extension is not removed until after the molecule is secreted into the extracellular matrix (8).

The two enzymes which synthesize the hydroxyproline and hydroxylysine in collagen require as co-factors or co-substrates O2, Fe3+, α-ketoglutarate, and perhaps ascorbate (for review, see Reference 1). When cartilage from chick embryos was incubated under conditions where the two hydroxylases were inhibited either by excluding O2 from the system (14, 15) or by adding an iron chelator such as α,α'-dipyridyl (14-16), the tissue was found to accumulate [14C]-collagen and [14C]-proline hydroxylase and protocollagen lysine hydroxylase, secretion of [14C]-protein was inhibited and [14C]protocollagen accumulated within the cells. [14C]Protocollagen extracted from the cells was comprised of polypeptides which were of the same size as polypeptides of the intracellular collagen or about 125,000 as estimated by gel filtration in sodium dodecyl sulfate. In contrast, the small amount of peptide-bound [14C] still secreted in the presence of α,α'-dipyridyl was shown to consist of small peptides which were in part derived from the intracellular degradation of [14C]protocollagen. The results demonstrated therefore that in freshly isolated tendon cells collagen itself is not secreted. Since the rate of protein synthesis in the presence of α,α'-dipyridyl was the same as under control conditions for about 90 min, the data suggested that the intracellular [14C]protocollagen accumulated in some postribosomal compartment.

Extraction of control cells with acetic acid solubilized a large fraction of the intracellular collagen, and amino acid analyses indicated that 40 to 50% of the protein in the extracts was collagen. The protein extracted from cells incubated with α,α'-dipyridyl was similar in amino acid composition, but it contained essentially no hydroxylysine and hydroxyproline and was correspondingly rich in lysine and proline. In addition, 40% of the proline in the protein extract was converted to hydroxyproline after incubation with pure proteocollagen proline hydroxylase.

Experiments involving limited pepsin digestion provided the first demonstration that intracellular collagen and propeptocollagen are largely in a native, triple-helical conformation. After acetic acid extracts from control cells and from cells incubated with α,α'-dipyridyl were dialyzed against ATP, segment long spacing aggregates were obtained. The aggregates were similar to those formed by extracellular, fibrillar collagen except that they had a 130-A NH2-terminal extension which was indistinguishable from that seen in aggregates of the procollagen form of collagen secreted by the same cells.
minced feet (18) and calvaria of chick embryos (19). However, attempts to characterize the protein more fully were handicapped by the limited amounts available, its contamination with matrix collagen, and the tendency of the procollagen to form large insoluble aggregates (17-19).

The studies with embryonic cartilage in vitro strongly suggested that procollagen was not secreted from cells and that it accumulated intracellularly (15). After the hydroxylases were inhibited for several hours and then the inhibition was reversed by exposing the tissue to O2 or iron, the procollagen which had accumulated during the period of inhibition was hydroxylated to the same extent as collagen in control tissues. Studies with a metal chelator which does not enter cells (20) and experiments with puromycin (21) demonstrated that procollagen proline hydroxylase is an intracellular enzyme. Accordingly, the fact that procollagen was hydroxylated in the tissue up to 2 hours after it was synthesized (15) indicated that it remained intracellular. Autoradiographs of the cartilage incubated with [3H]proline supported the conclusion that procollagen was not secreted from the cells (15, 22).

Subsequent studies with fibroblasts in monolayer cultures raised the possibility that under certain conditions some procollagen was secreted from cells (23-25). However, most of the reports were not clear as to how much of the total procollagen synthesized by the cells was secreted, or as to the size of the protein recovered in the culture medium. A recent paper (7) in fact suggested that most of the procollagen-like material recovered in the medium from fibroblast cultures consisted of small peptides which were probably degradative products.

We have recently obtained cells by enzymic digestion of chick embryo tendon which are free of extracellular matrix and which under the conditions we have employed remain in suspension and synthesize collagen at a rapid rate for several hours (6, 26). We here report the use of this biological system to demonstrate directly the intracellular accumulation of procollagen in the presence of α,α'-dipyridyl and to prepare sufficient quantities of both intracellular collagen1 and procollagen for the partial characterization of these proteins.

**EXPERIMENTAL PROCEDURE**

**Materials**—White Leghorn chick embryos were obtained from Shaw Hatcheries (West Chester, Pa.) and they were incubated in a moist atmosphere at 37°C until used. [14C]Proline, 218 μCi per μmole, was purchased from New England Nuclear Corporation. Trypsin solution, 2.5% in 0.15 M NaCl, Eagle's Minimum Essential Medium with glutamine for monolayer culture, and fetal calf serum were purchased from Grand Island Biological Co. Purified bacterial collagenase, SDS, and mercaptoethanol were purchased from Sigma Chemical Corporation. α,α'-Dipyridyl was purchased from Fisher Scientific Corp.

**Assay**—In experiments in which the incorporation of [14C]proline was followed, the medium and cell fractions were dialyzed against running tap water for 24 hours, and the retentates were hydrolyzed in sealed tubes in 6 N HCl at 120°C overnight. The hydrolysates were evaporated on a steam bath and they were dissolved in 4 ml of distilled water. The [14C]hydroxyproline content was assayed by a specific chemical procedure and a separate aliquot of each sample was taken for assay of total [14C] (27).

Amino acid analyses on protein fractions were carried out after hydrolysis in 6 N HCl at 120°C overnight. The samples were evaporated in a rotary evaporator and they were analyzed on a Spinco model 116 amino acid analyzer. The amino acid analyzer was sensitive enough to carry out either a long column or a short column analysis on about 15 μg of collagen.

Specific assays for hydroxyproline were carried out with a chemical procedure using half of the volumes previously recommended (28). With this modification the method was sensitive to about 0.5 μg of hydroxyproline.

**Isolation and Incubation of Matrix-free Cells from Tendon**—Cells were isolated from leg tendons of 17-day-old chick embryos by digestion with trypsin and purified bacterial collagenase as described previously (6, 20). After the digestion the cells were filtered through lens paper and isolated by centrifugation. The cells were washed three times with modified Krebs medium (6) containing 10% fetal calf serum and were then resuspended in Krebs medium for the incubation. In the experiments in which the synthesis of [14C]collagen and [14C]procollagen was followed, 5 × 10⁶ cells isolated from the tendons of four to six embryos were incubated in 20 ml of modified Krebs medium. The samples were preincubated with or without 0.3 mM α,α'-dipyridyl for 30 min and then 30 μCi of [14C]procollagen were added to both the control samples and the samples containing α,α'-dipyridyl. The incubation was carried out in a siliconized Erlenmeyer flask with shaking at 37°C. At each time point an aliquot of 2.0 ml was removed with a plastic pipette and immediately pipetted into 0.2 ml of medium containing 220 μg of cycloheximide and 2.2 μmoles of α,α'-dipyridyl in an ice bath in order to stop any further incorporation of [14C] or hydroxylation of [14C]procollagen. The samples were centrifuged at 1600 × g for 12 min at room temperature in order to separate the medium from the cells. The pellet containing the cells was washed with modified Krebs medium containing 1 mM α,α'-dipyridyl and 100 μg per ml of cycloheximide and renatured. The wash solution was discarded.

**Gel Filtration in SDS-Agarose**—Gel filtration was carried out on SDS-agarose as described by Jimenez et al. (8). To prevent degradation of the intracellular protein by hydrolytic enzymes, the previous procedure was modified slightly so that the cell fraction was suspended in 3.0 ml of water at the end of the incubation period and immediately heated in boiling water for 3 min. The medium from the cells was also immediately heated in boiling water for 3 min. The samples were then stored frozen until denatured and reduced with SDS-mercaptoethanol.

The latter procedure was carried out by adjusting both the cell fraction and the medium to 1% SDS and 1% mercaptoethanol in 0.02 M sodium phosphate, pH 7.4, in a final volume of 4 ml, and incubating them at 37°C for 3 hours. The samples were dialyzed at room temperature for 4 hours and then 15 hours against 100 volumes of 0.1% SDS and 0.1 M sodium phosphate, pH 7.4, and chromatographed at room temperature on a column (1.5 by 90 cm) of 6% agarose (Bio-Gel A-5m, 200 to 400 mesh, from Bio-Rad) equilibrated and eluted with 0.1% SDS and 0.1 M sodium phosphate, pH 7.4. Fractions of 2 ml were collected and aliquots of 0.2 ml were assayed in a liquid scintillation counter with an efficiency of 76 to 78%. The recovery of [14C]-protein from the SDS-agarose column was over 90%. All of the chromatograms were obtained with the same SDS-agarose column.
and the same values for $V_e/V_I$ were obtained with standard proteins over a period of 4 months. However, the sharpness of the elution peaks decreased somewhat with time. The $V_I$ varied from 114 to 120 ml and the chromatograms were adjusted to a $V_I$ of 116 ml to facilitate comparison of the elution patterns obtained in different experiments.

**Extraction of Collagen and Protocollagen from Cells**—To obtain maximal amounts of intracellular collagen or protocollagen from the matrix-free cells, from 0.4 to $1 \times 10^6$ cells from the leg tendons of about 60 embryos were used for a single experiment. The cells in a concentration of $5 \times 10^6$ per ml were incubated either under control conditions or with 0.3 mM $\alpha,\alpha'$-dipyridyl as described above except that 10% fetal calf serum was added to the medium of cells incubated with $\alpha,\alpha'$-dipyridyl to obtain slightly better yields of protocollagen. Although the rate of collagen synthesis was not consistently linear for more than 2 hours, the deviation from linearity was generally not great and the total amount of collagen in the system continued to increase for up to 8 hours (see below). For this reason incubation periods of 4 or 6 hours were used in many of the experiments for the preparation of quantitative amounts of collagen or protocollagen.

The cell pellet was homogenized in about 30 ml of 0.1 M acetic acid and dialyzed exhaustively at 4° against 0.1 M acetic acid for 48 to 72 hours. The suspension was centrifuged at 20,000 $\times$ g for 30 min and the supernate was dialyzed against 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.5. After dialysis for 24 to 48 hours, the protein in the sample was precipitated by adding 176 mg per ml of solid ammonium sulfate (Baker Chemical Co.). The precipitate was sedimented by centrifuging the sample at 20,000 $\times$ g for 30 min and the supernate was dialyzed against 0.1 M acetic acid overnight. After centrifugation at 20,000 $\times$ g for 30 min, the clear supernate was dialyzed against 0.1 M acetic acid and then taken directly either for amino acid analysis or for other studies. All these procedures were carried out at 4°.

**Hydroxylation of Isolated Protocollagen with Protocollagen Proline Hydroxylase**—To observe the synthesis of chemical amounts of hydroxyproline, a total of 90 $\mu$g of protein extracted from cells incubated with $\alpha,\alpha'$-dipyridyl was divided into two equal samples. The test sample was incubated with 100 units of pure protocollagen proline hydroxylase (29) with a specific activity of 1500 units per mg, 2 mM ascorbic acid (Fisher Scientific), 0.05 mM FeSO$_4$, 1 mg per ml of bovine serum albumin, 0.1 mM dithiothreitol (Eastman Organic Chemicals), 0.5 mM $\alpha$-keto- glutarate (Calbiochem), and 50 mM Tris-HCl buffer, adjusted to pH 7.8 at 25°, in a final volume of 1 ml (30). The control sample was incubated under the same conditions except that no enzyme was added. After incubation for 2 hours at 37°, the reaction was stopped by adding an equal volume of concentrated HCl. The samples were then hydrolyzed and assayed for hydroxyproline with the specific chemical assay (see above).

In experiments in which $[\text{C}]$protocollagen was used as a substrate and the synthesis of $[\text{C}]$hydroxyproline was measured, the hydroxylation was carried out under the same conditions except that the amount of enzyme used was 40 to 60 units, catalase (Sigma Chemical Co.) was added to a final concentration of 0.2 mg per ml, and the final volume was 4.0 ml. The amount of $[\text{C}]$protocollagen added was 5,000 to $60,000 \text{ dpm}$ which on the basis of amino acid analysis was equivalent to 0.5 to 6 $\mu$g of protein. When $[\text{C}]$protocollagen from the SDS-agarose column was used as substrate, the phosphorylase was removed by dialysis against distilled water and the SDS was removed by precipitating the protein with 10 volumes of cold acetone. The protein was isolated by centrifuging at 15,000 $\times$ g for 20 min and resuspended in 0.5 ml of 0.1 M Tris-HCl buffer, pH 7.8, at 25°. The protein was again precipitated with acetone and dissolved in the 0.1 M Tris-HCl buffer.

**Treatment of Collagen Precursors with Pepsin**—To determine whether the intracellular collagen and the protocollagen were resistant to pepsin, the cells were extracted with acetic acid and precipitated with ammonium sulfate as described above. The pellets were dissolved in 0.1 M acetic acid, dialyzed against 0.1 M acetic acid, and then stored at 4°. For the enzymic treatment (3, 11, 31), an aliquot of 0.2 ml was adjusted to 0.5 M acetic acid and incubated with 100 $\mu$g per ml of pepsin (Sigma Chemical Co.). The amounts of protein were 3 to 15 $\mu$g, and the final volume for the incubation was 2.0 ml. The enzymic digestion was carried out at 15° for 6 hours and then the reaction was stopped by adding 0.35 ml of 3 N NaOH. The samples were adjusted to a final volume of 3.0 ml containing 1% EDS, 1% mercaptoethanol, and 0.02 M sodium phosphate, pH 7.4, by adding concentrated stock solutions. The samples were incubated at 37° for 3 hours and then dialyzed at room temperature against 0.1% SDS and 0.1 M sodium phosphate buffer, pH 7.4, before chromatography on the SDS-agarose column.

**Preparation of Segment Long Spacing Aggregates**—To prepare SLS aggregates, 0.8 to $1 \times 10^6$ cells were incubated under control conditions or with 0.3 mM $\alpha,\alpha'$-dipyridyl for 4 hours, and the cells were separated from the medium by centrifugation. The cells were extracted with 0.1 M acetic acid, and the extracts were precipitated with ammonium sulfate as described above. The ammonium sulfate pellet was dissolved in 1.5 ml of 0.1 M acetic acid and dialyzed against 0.01 M acetic acid and then directly either for amino acid analysis or for other studies. All these procedures were carried out at 4°.

**Results**

**Synthesis of Intracellular $[\text{C}]$Collagen and $[\text{C}]$Protocollagen**—Matrix-free cells from tendon were incubated with $[\text{C}]$proline under control conditions or in the presence of 0.3 mM $\alpha,\alpha'$-dipyridyl for up to 3 hours. Under control conditions 17.5 to 20.9% of the $\text{C}$ in intracellular proteins was $[\text{C}]$hydroxyproline (Table I). From 37.5 to 41.4% of the $\text{C}$ in the protein secreted into the medium was $[\text{C}]$hydroxyproline. As discussed elsewhere (6), the ratio of $[\text{C}]$hydroxyproline to total $\text{C}$ should be about 43% for pure tendon collagen in which only the prolyl and hydroxyprolyl residues are radioactively labeled. In the $\alpha,\alpha'$-dipyridyl-treated samples hydroxylation of proline was markedly inhibited so that less than 0.3% of the protein-bound $\text{C}$ in either the cells or the medium was accounted for by $[\text{C}]$hydroxyproline.

In the control samples the incorporation of $[\text{C}]$proline was linear for about 180 min from the time the isotope was added (Fig. 1A). In the samples incubated with $\alpha,\alpha'$-dipyridyl the incorporation of $\text{C}$ was the same as in the control for 60 min after the isotope was added. Thereafter the apparent rate of incorporation decreased. When the $\text{C}$-protein in the cells and medium was assayed separately, it was apparent that a major effect of the $\alpha,\alpha'$-dipyridyl was a decrease in the secretion of
The amount of intracellular W-protein increased progressively in the treated sample so that, at the end of the 3-hour incubation time with W-proline, the amount of intracellular W-protein was about twice that in the control (Fig. 1B). In contrast, the amount of W-protein secreted into the medium was only about 15% of the control (Fig. 1C).

Size of W-Peptides Secreted in Presence of α,α'-Dipyridyl—To determine the size of the W-peptides secreted under control conditions and in the presence of α,α'-dipyridyl, the medium from the samples was removed, treated with SDS-mercaptoethanol, and chromatographed on an SDS-agarose column. As reported previously (S), about 85% of the nondialyzable W recovered from the control medium appeared in a sharp symmetrical peak (Fig. 2A) with an apparent molecular weight of about 125,000. Assays for W-hydroxyproline indicated that

### Table I

| Sample  | Treatment | Incubation time | Total W incorporated | W-hydroxyproline synthesized | W-hydroxyproline/total W |
|---------|-----------|-----------------|----------------------|-----------------------------|--------------------------|
| Cells... | None      | 60 min          | 3.00 × 10^4 dpm      | 0.86 X 10^3 dpm             | 25.9                     |
| Medium  | None      | 60 min          | 2.20 × 10^4 dpm      | 0.88 X 10^3 dpm             | 40.0                     |
| Cells... | None      | 120 min         | 4.96 X 10^4 dpm      | 1.20 X 10^3 dpm             | 24.3                     |
| Medium  | None      | 120 min         | 8.21 X 10^4 dpm      | 3.40 X 10^3 dpm             | 41.4                     |
| Cells... | None      | 180 min         | 4.73 X 10^4 dpm      | 0.83 X 10^3 dpm             | 17.5                     |
| Medium  | None      | 180 min         | 14.25 X 10^4 dpm     | 5.35 X 10^3 dpm             | 37.5                     |
| Cells... | 0.3 mM α,α'-dipyridyl | 60 min         | 5.03 X 10^4 dpm      | <0.01 α                     | <0.2                     |
| Medium | 0.3 mM α,α'-dipyridyl | 60 min          | 3.00 X 10^4 dpm      | <0.001 α                    | <0.3                     |
| Cells... | 0.3 mM α,α'-dipyridyl | 120 min         | 9.04 X 10^4 dpm      | <0.02 α                     | <0.2                     |
| Medium | 0.3 mM α,α'-dipyridyl | 120 min          | 1.12 X 10^4 dpm      | <0.002 α                    | <0.2                     |
| Cells... | 0.3 mM α,α'-dipyridyl | 180 min         | 9.49 X 10^4 dpm      | <0.02 α                     | <0.2                     |
| Medium | 0.3 mM α,α'-dipyridyl | 180 min          | 2.56 X 10^4 dpm      | <0.004 α                    | <0.2                     |

*Observed values for counts per min in the chemical assay for W-hydroxyproline were less than three times the background.

**Fig. 2.** A, gel filtration on SDS-agarose of W-protein secreted into the medium by cells incubated with W-proline under control conditions. Matrix-free cells, 1.5 × 10^7, were incubated for 3 hours with 5 μCi of W-proline in 6.0 ml of medium. The medium was removed by centrifugation, boiled for 3 min, and treated with SDS-mercaptoethanol. After dialysis, an aliquot corresponding to 1.5 ml of original medium was chromatographed on an SDS-agarose column as described under "Experimental Procedure." The void volume was in fraction 18 and the total volume was in fraction 58. ○-○, elution of W; ●-●, elution of W-hydroxyproline. B, gel filtration on SDS-agarose of W-protein secreted into the medium by cells incubated with W-proline and 0.3 mM α,α'-dipyridyl. The number of cells, the incubation volume, and the microcuries of W-proline were the same as in A. Also the aliquot of original medium and the chromatographic conditions were as described in A. ●-●, elution of W; ○-○, W-hydroxyproline.

**Fig. 1.** A, incorporation of W-proline by matrix-free cells from embryonic tendon under control conditions and in the presence of 0.3 mM α,α'-dipyridyl. Two samples of 5 × 10^7 cells each were incubated in 20 ml of medium, and aliquots of 2.0 ml were removed at the times indicated. One sample was preincubated with and the other without α,α'-dipyridyl for 30 min and then 20 μCi of W-proline was added to each at "zero time." Other conditions were as described under "Experimental Procedure." Values indicates total nondialyzable W in total system (cell and medium). B, incorporation of W-proline into cell fraction under control conditions and in the presence of 0.3 mM α,α'-dipyridyl. Values are from experiment shown in A. C, incorporation of W-proline into proteins secreted into the medium under control conditions and in the presence of 0.3 mM α,α'-dipyridyl. Values are from the same experiment shown in A and B.
43.7% of the $^{14}$C in the peak fraction was in $[^{14}C]$hydroxyproline, a value that suggested that essentially all of the $^{14}$C was in collagen $[^{14}C]$proline and $[^{14}C]$hydroxyproline. In contrast, the smaller amount of nondialyzable $^{14}$C recovered from the medium of cells incubated with $\alpha,\alpha'$-dipyridyl eluted much later from the SDS-agarose column (Fig. 2B). The peak eluted at about the same position as cytochrome $c$ with a molecular weight of 13,500, and therefore it consisted of $^{14}$C-peptides just large enough to be retained during the dialysis steps. The amount of peptide-bound $^{14}$C which was recovered as small peptides (fractions of 39 to 52 in Fig. 2, A and B) was significantly greater in the medium of cells incubated with $\alpha,\alpha'$-dipyridyl than in the control sample, even though the number of cells, the amount of $[^{14}C]$proline, and other conditions were the same.

As indicated above (Table I), the $^{14}$C-peptides in the medium contained essentially no $[^{14}C]$hydroxyproline. When the $^{14}$C-peptides recovered from the column (Fig. 2B) were incubated with protocollagen proline hydroxylase in two separate experiments, 7.1 and 8.3% of the $^{14}$C was converted to peptide-bound $[^{14}C]$hydroxyproline, a result which indicated that these peptides were at least in part derived from collagen precursors.

**Size of Intracellular $[^{14}C]$Collagen and $[^{14}C]$Protocollagen—** The size and amount of $[^{14}C]$-protein in the cell fractions were also examined on the SDS-agarose column (Fig. 3). Preliminary experiments demonstrated that when cells which had been incubated with $[^{14}C]$proline were extracted with 1% SDS and 1% mercaptoethanol at $37^\circ$ for 3 hours, over 95% of the nondialyzable $^{14}$C was solubilized. The elution pattern obtained from the SDS-agarose column was not significantly different if the extraction at $37^\circ$ in 1% SDS-mercaptoethanol was carried out for 12 hours instead of 3 hours. In the control sample a large fraction of the nondialyzable $^{14}$C-protein was recovered in a peak with an elution position about the same as the major peak observed with $[^{14}C]$-protein from the medium (compare Fig. 3 with Fig. 2A). The ratio of $[^{14}C]$hydroxyproline to total $^{14}$C in the major peak was 36%. As indicated in Fig. 3, the total amount of nondialyzable $^{14}$C was increased in cells incubated with $\alpha,\alpha'$-dipyridyl and most of the additional $[^{14}C]$-protein was recovered in a peak in an apparent molecular weight of about 125,000.

![Fig. 3. Gel filtration on SDS-agarose of an acetic acid extract of cells incubated under control conditions. Cells, $6 \times 10^8$, were incubated in 56 ml of medium for 6 hours with 8 pCi of $[^{14}C]$proline. The total cell fraction was treated with SDS-mercaptoethanol as described under "Experimental Procedure" and the chromatographic conditions were the same as in Fig. 2A. The aliquot placed on the column corresponded to one-eighth of the total cell fraction.](http://www.jbc.org/)

The $[^{14}C]$collagen from control cells and the $[^{14}C]$protocollagen from the cells incubated with $\alpha,\alpha'$-dipyridyl was partially purified and fractionated by extraction with acetic acid and precipitation with ammonium sulfate (see "Experimental Procedure"). From 50 to 70% of the nondialyzable $^{14}$C was recovered with these procedures. When the partially purified $^{14}$C-protein was denatured and reduced with SDS-mercaptoethanol and then chromatographed on the SDS-agarose column, most of the $^{14}$C eluted in a sharp peak with an apparent molecular weight of about 125,000 (Fig. 4, A and B). With the control sample the ratio of $[^{14}C]$hydroxyproline to total $^{14}$C in the peak was 46.6%, indicating that the proline in the intracellular collagen was hydroxylated as fully as the collagen in the medium (6, 26). The $^{14}$C-protein extracted from cells incubated with $\alpha,\alpha'$-dipyridyl contained no $[^{14}C]$hydroxyproline, but, when the SDS in the fractions was removed and the $^{14}$C-protein was incubated with protocollagen proline hydroxylase, there was a synthesis of $[^{14}C]$hydroxyproline. The final value for the ratio of $[^{14}C]$hydroxyproline to total $^{14}$C after incubation with the enzyme was 29.6%.

![Fig. 4. A, gel filtration on SDS-agarose of an acetic acid extract of cells incubated under control conditions. Cells, $6 \times 10^8$, were incubated in 56 ml of medium for 6 hours with 8 pCi of $[^{14}C]$proline. The cells were treated as described under "Experimental Procedure" and an aliquot corresponding to one-fifteenth of the total extract was placed on the column. The chromatographic conditions were the same as in Fig. 2A. •—•, elution of $^{14}$C; O—O, elution of $[^{14}C]$hydroxyproline. B, gel filtration on SDS-agarose of an acetic acid extract of cells incubated with $0.3 \text{ mM } \alpha,\alpha'$-dipyridyl. Conditions were similar to those in the experiment shown in A but the total amount of $^{14}$C-protein is not directly comparable because the number of cells was $2.1 \times 10^8$, the cells were incubated in 40 ml of modified Krebs medium containing 10% fetal calf serum, 10 pCi of $[^{14}C]$proline were used, and the incubation time was 4 hours. •—•, elution of $^{14}$C; O—O, $[^{14}C]$hydroxyproline content of fractions before incubation with protocollagen proline hydroxylase; \(\Delta\) — \(\Delta\), $[^{14}C]$hydroxyproline content after incubation with protocollagen proline hydroxylase.](http://www.jbc.org/)
Control cells, but there were several major differences (Table III). The protein from the cells incubated with cr,ru'-dipyridyl was extracted with acetic acid and the extract was precipitated with a precipitating agent. On this basis it was concluded that the amount of hydroxyproline in the medium of 3.77 pg. When the tenon cells were incubated in the cell extracts. There was no significant difference in the net synthesis of collagen hydroxyproline when the cells were incubated in Eagle’s Minimum Essential medium instead of the Krebs medium, indicating that during the incubation period the amount of collagen synthesized was not affected by the presence of exogenous essential amino acids and vitamins.

From 0.4 to 1.1 × 10⁶ cells which had been incubated under control conditions or with α,α'-dipyridyl for 4 hours were extracted with acetic acid and the extract was precipitated with ammonium sulfate. The samples were then hydrolyzed and examined in an amino acid analyzer. Since collagen is the only protein in tissues such as tendon which contains any significant amount of hydroxylysine or hydroxyproline, and since collagen has an unusually high content of glycine, the contents of these three amino acids were used to estimate the collagen content of the cell extracts.

Collagen from chicken tendons has been reported to contain 9.6 residues of hydroxylysine, 99 residues of hydroxyproline, and 331 residues of glycine/1000 amino acid residues (35). An acetic acid extract of the tendons from which the matrix-free cells were prepared was found to contain 10 residues/1000 of hydroxylysine, 80 of hydroxyproline, and 283 of glycine (Table III), suggesting that 80 to 90% of the protein in the extract was collagen. The amino acid analysis of the extract from the control tendon cells indicated that the protein fraction contained 8.3 residues of hydroxylysine, 37 residues of hydroxyproline, and 168 residues of glycine/1000. On this basis it was concluded that 40 to 50% of the protein extracted from the control cells was collagen.

Amino acid analyses of the protein extracted from the tendon cells which had been incubated with α,α'-dipyridyl for 4 hours were similar to the amino acid analyses of the protein from control cells, but there were several major differences (Table III). The protein from the cells incubated with α,α'-dipyridyl contained less than 0.4 residue of hydroxylysine and less than 2 residues of hydroxyproline. The protein contained 43 more residues of proline so that its total content of imino acid was 6.24% of the value for the protein extracted from the control tendon cells. Therefore the content of hydroxylysine plus lysine was within 6% of the value for the protein extracted from the control tendon cells.

The extracts from control tendon cells and from cells incubated with α,α'-dipyridyl were prepared as described under “Experimental Procedure.”

Amino acid analyses of acetic acid extracts of whole tendon, tendon cells, and tendon cells incubated with α,α'-dipyridyl

The amino acid from whole tendon were prepared by placing 20 mg of β-aminopropionitrile (General Biochemicals) in 0.1 ml of 0.15 M NaCl in the air sack of 14-day-old chick embryos, and the tendons were removed 3 days later. The tendons were washed with Krebs medium and then extracted in 0.1 M acetic acid at 4° for 24 hours. The supernatant was removed by centrifuging at 40,000 × g for 1 hour, lyophilized, and taken for hydrolysis.

The extracts from control tendon cells and from cells incubated with α,α'-dipyridyl were prepared as described under “Experimental Procedure.”
These fractions accounted for 79% of the initial fractions as chains of collagen (fraction 27 to 37 in Fig. 5B). These fractions contained 90% of the peptide-bound [14C]hydrosyproline in the chromatograph. After the digestion with pepsin, 55% of the total [14C]hydrosyproline in the SDS-agarose column was sedimented by about 5%, whereas the polypeptide chains appear to be about 20 to 30% larger when their size is examined by gel filtration. Under the conditions employed the length of the SLS aggregates increased to 42 residues/1000, a value comparable to the value of 37 residues/1000 observed in the peptide-bound [14C] in the acetic acid extract from a nondialyzable [14C]hydroxyproline.

| Source of acetic acid extract | Treatment | Amino acid content | Ratio of hydroxyproline to proline |
|------------------------------|-----------|--------------------|----------------------------------|
|                              |           | Glycine | Proline | Hydrosyproline |
| Control cells . . . . . . . . | None     | 30      | 9.7     | 6.9           | 0.71          |
| Cells incubated with α,α′-dipyridyl . | None     | 122     | 71      | <4.0          | <0.06         |
| Cells incubated with α,α′-dipyridyl . . . . . . | Hydroxy- | 122     | 47      | 28            | 0.55          |

* 4 × 10⁸ cells were incubated with 0.3 mM α,α′-dipyridyl for 4 hours and then homogenized in and extracted with 30 ml of 0.1 M acetic acid. The extract was precipitated with ammonium sulfate (see "Experimental Procedure"), resuspended in 1.5 ml of 0.1 M acetic acid, and dialyzed against 0.1 M acetic acid. The sample was centrifuged at 10,000 × g for 30 min and 0.3 ml of the supernatant was taken for incubation with or without procollagen proline hydroxylase (see "Experimental Procedure"). After incubation with enzyme, the hydroxyproline content was assayed with a specific chemical procedure (28). The glycine contents were measured by amino acid analysis of the extract which was used for the enzymatic hydroxylation. The proline content before hydroxylation was taken from the amino acid analysis, and the proline content after enzymatic hydroxylation was calculated as the proline content before the hydroxylation less the hydroxyproline found with the specific chemical assay after the hydroxylation.

* Sample incubated with the required co-factors for procollagen proline hydroxylase but without addition of the enzyme.

* Value indicates limit of sensitivity of the specific chemical assay for hydroxyproline.

* Sample incubated with the required co-factors and pure procollagen proline hydroxylase.

were incubated with procollagen proline hydroxylase and the required co-factors and co-substrates for the hydroxylation of proline. The results (Table IV) demonstrated the synthesis of quantitative amounts of hydroxyproline. After hydroxylation by the enzyme, the hydroxyproline content of the protein fraction increased to 42 residues/1000, a value comparable to the value of 37 residues/1000 observed in the acetic acid extracts from control cells (Table III). As indicated (Table IV), the ratio of hydroxyproline to proline in the protein increased over 10-fold.

**Resistance of Intracellular Collagen and Procollagen to Limited Digestion by Pepsin—**Cells were incubated with [14C]proline, and then acetic acid extracts were prepared as described above. The extracts were then incubated with 100 µg per ml of pepsin at 15° for 6 hours (Table V).

With extracts of control cells which were not treated with pepsin, 60% of the nondialyzable [14C]protein was recovered from the SDS-agarose column in about the same fractions as the precursor polypeptides secreted in the medium (fractions 22 to 35 in Fig. 5A). These fractions contained 90% of the peptide-bound [14C]hydroxyproline in the chromatograph. After the digestion with pepsin, 55% of the total [14C]eluted in the same fractions as α chains of collagen (fraction 27 to 37 in Fig. 5B). These fractions accounted for 79% of the initial [14C]hydroxyproline.

Similar results were obtained with the [14C]protein in acetic acid extracts from cells which had been incubated with α,α′-dipyridyl for 4 hours. Before treatment with pepsin, 67% of the [14C]eluted in the same fractions as the precursor polypeptides from the medium (Fig. 5C). After treatment with pepsin, 51% of the initial [14C]eluted in the same fractions as α chains (Fig. 5D). However, if the [14C]protein was denatured by heating at 100° for 10 min before treatment with pepsin, essentially all of the [14C]protein was digested to smaller peptides (Fig. 5D and Table IV).

**Limited pepsin digestion of the [14C]-protein extracted from control tendon cells and cells incubated with 0.3 mM α,α′-dipyridyl for 4 hours**

| Source of acetic acid extract | Amino size | Acetic acid extract | After dialysis in SDS-mercaptoethanol⁴ | [14C] in precursor polypeptides before pepsin digestion | [14C] in chains after pepsin digestion | % total |
|------------------------------|------------|--------------------|--------------------------------------|---------------------------------|-------------------------------------|--------|
| Control cells . . . . . . . . | None       | 122                | 71                                   | 4.0                             | 0.06                                | 100    |
| Cells incubated with α,α′-dipyridyl . | None     | 122                | 71                                   | 4.0                             | 0.06                                | 100    |
| Cells incubated with α,α′-dipyridyl . . . . . . | Hydroxy- | 122                | 47                                   | 28                             | 0.55                                | 100    |

* Extract was heated to 100° for 10 min before digestion with pepsin.

⁴ As noted under "Experimental Procedure," the dialysis in the presence of SDS-mercaptoethanol was carried out after the digestion with pepsin and prior to gel filtration on the SDS-agarose column. Control experiments indicated that the treatment with SDS-mercaptoethanol in itself converted 15 to 20% of the peptide-bound [14C] in the acetic acid extract from a nondialyzable [14C]hydroxyproline.

⁵ [%] Protein eluted in fractions 22 to 35 in the SDS-agarose column (see Fig. 5, A and C).

⁶ [%] Protein eluted in fraction 27 to 37 in the SDS-agarose column (see Fig. 5, B and D).

⁷ Calculated as 100 × [14C] in the α chains after pepsin treatment/total [14C] in initial acetic acid extract.

**Segment Long Spacing Aggregates from Intracellular Collagen—**Control cells and cells which had been incubated with α,α′-dipyridyl for 4 hours were extracted with 0.1 M acetic acid, and the [14C]-protein in the extracts was precipitated with ammonium sulfate. The samples were redissolved in acetic acid and dialyzed against diisumic acid ATP in acetic acid. A flocculent precipitate appeared in the bag and 70 to 90% of the [14C]-protein in the samples was sedimented when the sample was allowed to stand at 4° overnight. Electron microscopy indicated the presence of regular aggregates which were indistinguishable from SLS aggregates of collagen except that they contained an extension at the NH₂-terminal or C-end (Fig. 6). The NH₂-terminal extension was defined most clearly when the aggregates were stained with ammonium molybdate, and measurement of the extension in 40 aggregates indicated a length of 134.0 A ± 1.5 (S.E.M.). Under the conditions employed the length of the SLS aggregates was 2890 A ± 13 (S.E.M.), and therefore the NH₂-terminal extension accounted for 4.7% of the total length. As discussed elsewhere (11), there is no clear explanation as to why the NH₂-terminal extension increases the length of the SLS aggregates by about 5%, whereas the polypeptide chains appear to be about 20 to 30% longer when their size is examined by gel filtration (8, 12) or polyacrylamide gel electrophoresis (13).
FIG. 5. Gel filtration on SDS-agarose after limited pepsin digestion of the 14C-protein from cells incubated under control conditions and in the presence of 0.3 mM α,α'-dipyridyl. The control sample was prepared by incubating $9 \times 10^8$ cells for 6 hours in 120 ml of medium with 12 μCi of [14C]proline. The α,α'-dipyridyl sample was prepared by incubating $6 \times 10^8$ cells for 4 hours in 80 ml of medium with 6 μCi of [14C]proline. The cells were extracted with acetic acid, treated with pepsin at 15°, and then treated with SDS-mercaptoethanol as described under "Experimental Procedure." With the control samples an aliquot corresponding to one-eighth of the total extract was placed on the column. With the α,α'-dipyridyl sample an aliquot corresponding to one-sixteenth of the total sample was placed on the column. The chromatographic conditions were the same as in Fig. 2A. A, extract of control cells; sample not digested with pepsin. Note that pattern is similar to Fig. 4A. Also shown is elution of a mixture of α and β chains of collagen obtained by acetic acid extract of skin from lathyritic 21-day-old chicks (8). B, extract of control cells; sample digested with pepsin. C, extract of cells incubated with α,α'-dipyridyl; sample not digested with pepsin. D, extract of cells incubated with α,α'-dipyridyl; sample digested with pepsin under native conditions and after denaturation by heating to 100° for 10 min. ●, elution of 14C; ▲, elution of α and β chains; ○, elution of 14C-hydroxyproline; ◆, elution of 14C in sample which was heat-denatured before digestion with pepsin. Dotted vertical line indicates elution position of α chains.

There was no difference in the appearance of the SLS aggregates prepared from control cells and those prepared from cells incubated with α,α'-dipyridyl for 4 hours. Also the apparent yield of SLS aggregates was about the same as judged by the number of aggregates seen on the grids and by the relative amount of 14C-protein which sedimented when the samples were allowed to stand at 4° overnight.

Although it was previously reported (11) that the NH2-termina

nal extension of the collagen secreted by tendon cells did not stain positively with either phosphotungstic acid or uranyl acetate, further studies demonstrated that the NH2-terminal extension did stain positively (Fig. 7) when the SLS aggregates were exposed to uranyl acetate for 5 min instead of 1 to 2 min. After staining with uranyl acetate for 5 min a broad, positively stained band was seen at the NH2-terminal extension. The same pattern was seen in SLS aggregates from control cells, from cells incubated with α,α'-dipyridyl, and from the medium of control cells (11).

DISCUSSION

Pulse-label and chase experiments (6) and quantitative assays of hydroxyproline (Table II) demonstrated that most of the collagen secreted by the matrix-free cells from tendon is recovered in the incubation medium. The system therefore makes it possible to isolate intracellular collagen which is not contaminated by extracellular fibrillar collagen. Extraction of the cells with SDS-mercaptoethanol under the conditions used here solubilized all of the 14C-protein, and gel filtration on SDS-agarose indicated that a major part of the newly synthesized intracellular 14C-protein consisted of polypeptides of about 125,000 daltons, or of about the same size as the polypeptides of the precursor form of collagen secreted into the medium by the matrix-free cells (8, 11). The 14C-protein extracted from cells incubated with [14C]proline contained large amounts of [14C]hydroxyproline, and, after partial purification by acetic acid ex-
Collagen rat tendon can be increased by incubating it with related collagen polypeptides. The 14C-protein which was accumulated with O, a'-dipyridyl prevented the conversion of peptide-bound 14C-proline to [14C]hydroxyproline. The 14C-protein contained protocollagen and little, if any, hydroxylated collagen. The conclusion was supported by the fact that 40% of the proline in the protein extracts was converted to hydroxyproline by pure protocollagen proline hydroxylase. The data also demonstrated that inhibition of the hydroxylation of protocollagen does not immediately affect protein synthesis, since the incorporation of [14C]proline was the same as in the control for up to 90 min from the time at which a', a'-dipyridyl was added. The synthesis time for a polypeptide chain of collagen has been estimated to be about 6 min (12).

The results suggested that after [14C]protocollagen had accumulated in the cells for 180 min, the amount of small 14C-peptides in the medium was markedly decreased, but after incubation of the cells with O, a'-dipyridyl for 90 min, the amount of small 14C-peptides in the medium was greater than in the control. Since the small 14C-peptide fractions served as a substrate for the synthesis of [14C]hydroxyproline by protocollagen proline hydroxylase, the results suggested that after [14C]protocollagen had accumulated in the cells for some time, some of the intracellular [14C]protocollagen was degraded to small 14C-peptides which were then secreted. A similar situation apparently occurs in fibroblast cultures incubated with a', a'-dipyridyl (7).

The precursor form of collagen known as “procollagen” or “transport form” had different solubility properties from the collagen which can be extracted from collagen fibers (2, 22, 23, 25-31). Because of this, and because of the small amount of peptide which were available, the intracellular collagen and proteoglycan could not be purified extensively. However, half or more of the intracellular collagen and proteoglycan was solubilized by extracting the cells with acetone acid under conditions known to solubilize many collagens in native form. After the extracts from control cells were fractionated with ammonium sulfate, 40 to 50% of the protein was collagen on the basis of its content of hydroxyproline, hydroxylysine, and glycine. Comparable extracts from cells incubated with a', a'-dipyridyl had about the same amino acid composition except that they contained essentially no hydroxyproline or hydroxylysine and they were correspondingly rich in proline and lysine. These results are compatible with the conclusion that the cells incubated with a', a'-dipyridyl contained protein and little, if any, hydroxylated collagen. The conclusion was supported by the fact that 40% of the proline in the protein extracts was converted to hydroxyproline by pure protocollagen proline hydroxylase.

Both the intracellular collagen and protocollagen extracted from the cells were largely in a native conformation. These observations provide the first experimental evidence to indicate that collagen becomes triple helical before it is secreted.

It was of interest that under the conditions used here, or when the cells were pulse labeled for only 2 to 15 min, no significant amounts of 14C-polypeptides were found with molecular weights of more than 125,000 as estimated by chromatography on SDS-agarose. Accordingly, there was no evidence that the matrix-free cells from tendon synthesized a collagen precursor with a molecular weight of 500,000 to 600,000 as has been recently described in cultures of fibroblasts (33).
into the extracellular matrix. It was previously demonstrated in cartilage (13, 22, 36) that [14C]protoplagen could be hydroxylated to a normal extent several hours after it accumulated in cells, and similar experiments have recently been carried out with tendon cells. It is apparent therefore that a triple-helical conformation does not prevent the intracellular hydroxylation of peptidyl proline by protocollagen proline hydroxylase. A similar conclusion has recently been reached on the basis of enzymic studies with a synthetic substrate which forms triple-helical structures in solution (38).

The helical conformation of the intracellular collagen was further demonstrated by the preparation of SLS aggregates, since previous work (39, 40) had demonstrated that only triple-helical collagen will form such regular structures. The major distinguishing feature of the aggregates from the intracellular collagen was the presence of a 130-A NH2-terminal extension which was indistinguishable from that seen in SLS aggregates of the precursor form of collagen secreted into the extracellular milieu (9, 10). With the extracts from tendon cells no SLS aggregates were seen which did not contain the NH2-terminal extension, an observation which was consistent with the fact that essentially all of the [14C]collagen polypeptides in the acetic acid extracts had an apparent molecular weight of 125,000 by gel filtration in SDS-gelose. The observation was also consistent with the earlier demonstration (8) that in tendon the NH2-terminal extension is not cleaved until after the collagen is secreted into the extracellular milieu.

The SLS aggregates from cells incubated with α,α'-dipyridyl were essentially the same as those from control cells and they contained the same NH2-terminal extension. The apparent yield of SLS aggregates from cells incubated with α,α'-dipyridyl was about the same as from controls, and, on the basis of the amino acid analyses, no more than 2% of the protein in the samples was fully hydroxylated collagen. Because it is not possible to quantitate the yields of SLS aggregates, it may be that some of the aggregates which were seen consisted of intracellular collagen which contaminated the preparation of protocollagen. However, the results suggested that protocollagen formed SLS aggregates similar to both intracellular collagen and the collagen secreted in the medium by the matrix-free cells.

Positive staining of the SLS aggregates for a longer time than was used previously (11) demonstrated that the NH2-terminal extension stained more readily with uranyl acetate than with phosphotungstic acid, a result which suggested that the extension contained an excess of negatively charged groups. This observation is consistent with reports indicating that the precursor forms of collagen from the skin of cows with dermatosparaxis (4, 9, 10), from membranous bone of chick embryos (19), and from the medium of matrix-free tendon cells (41) contain an excess of acidic amino acids when compared to fibrillar collagen.

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