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Partial Characterization of Protocollagen from Embryonic Cartilage

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1. Attempts were made to isolate and characterize the protocollagen that accumulates in connective tissue when the hydroxylation of proline and lysine is inhibited. The term protocollagen has been used to describe the proline-rich and lysine-rich polypeptide or polypeptides that serve as substrates for the formation of hydroxyproline and hydroxylysine during the synthesis of collagen. 2. Both protocollagen and newly synthesized collagen from embryonic cartilage were isolated as complex aggregates, which contained sulphated mucopolysaccharides and other proteins or polypeptides from the same tissue. The complexes containing protocollagen were similar to those containing newly synthesized collagen when examined with several different techniques. 3. After the complexes were denatured and disaggregated, zone centrifugation and gel filtration indicated that the denatured protocollagen was similar to the denatured newly synthesized collagen obtained from cartilage in which the hydroxylation was not inhibited, and it was also similar to purified α-collagen. The results suggest that, when the hydroxylation is inhibited, most of the protocollagen polypeptides that accumulate are as large as complete α-chains of collagen. 4. Significant purification of the protocollagen polypeptides was obtained with a new technique for DEAE-Sephadex chromatography in which urea was used to prevent aggregation of the samples and the column was eluted with guanidine thiocyanate. 5. Protocollagen polypeptides were completely hydrolysed to diffusible peptides by a specific collagenase. 6. It is not entirely clear whether the hydroxylation normally begins while relatively short protocollagen molecules are still attached to polysomes, or whether protocollagen molecules of the size of α-collagen are synthesized even when the hydroxylation is not inhibited. 7. Results obtained with puromycin suggest that some hydroxylation occurs with smaller polypeptides, but polypeptide chains approaching the size of α-collagen are required to obtain complete hydroxylation of the appropriate amino acid residues of protocollagen.

Isotopically labelled proline is readily incorporated into the peptide-bound hydroxyproline of collagen, but labelled hydroxyproline is not incorporated into collagen (Stetten & Schoenheimer, 1944; Stetten, 1949). Similar conditions hold for the incorporation of labelled lysine and hydroxylysine into collagen (Sinex, Van Slyke & Christman, 1959). These findings have suggested that either proline and lysine are hydroxylated after they are incorporated into 'activated' intermediates such as prolyl-t-RNA* and lysyl-t-RNA, or the hydroxylations occur after the synthesis of proline- and lysine-rich polypeptide precursors of collagen (see Lukens, 1965; Prockop & Kivirikko, 1966). Over the past few years a number of investigators have reported indirect evidence for the existence of polypeptide precursors of collagen (Peterkofsky & Udenfriend, 1963; Juva & Prockop, 1964, 1966*a*; Lukens, 1965; Hurych & Chvapil, 1965; Gottlieb, Peterkofsky & Udenfriend, 1965).

It was recently reported from this Laboratory (Prockop & Juva, 1965*a*,*b*; Juva & Prockop, 1965*a*,*b*) that a proline-rich polypeptide intermediate for the synthesis of hydroxyproline was prepared by incubating embryonic cartilage with [^{14}C]proline under anaerobic conditions. Because atmospheric oxygen is required for the hydroxylation of proline (Fujimoto & Tamiya, 1962; Prockop, Kaplan & Udenfriend, 1963), the synthesis of hydroxy[^{14}C]proline in the cartilage was inhibited to greater extent than the incorporation of [^{14}C]proline into protein. When protein fractions

* Abbreviations: t-RNA, transfer RNA; s-RNA, 'soluble' RNA.

from the anaerobically labelled cartilage were subsequently incubated aerobically with chick-embryo homogenates, up to 30% of the protein-bound [^{14}C]proline was converted into hydroxy-[^{14}C]proline. The enzymic system for the hydroxylation of the polypeptide intermediate has been purified about 200-fold (K. I. Kivirikko & D. J. Prockop, unpublished work), and it has been shown to require Fe^{2+}, K^+ and one or more additional unidentified cofactors (Prockop & Juva, 1965*b*). The proline-rich intermediate was shown to be a large polypeptide that readily forms insoluble aggregates, and lysine was also hydroxylated in the same polypeptide as proline (Prockop, Weinstein & Mulveny, 1966). The name 'protocollagen' has been suggested for the proline- and lysine-rich polypeptide or polypeptides that serve as the substrates for the synthesis of the hydroxyproline and hydroxylysine of collagen (Juva & Prockop, 1965*a*,*b*).

In the present study we have attempted to characterize protocollagen from embryonic cartilage. The results indicate that, when the hydroxylating reaction is inhibited, protocollagen molecules of about the same size as complete polypeptide chains of collagen are synthesized. It was found that the newly synthesized collagen and protocollagen from cartilage occur in complex aggregates with some unusual properties.

MATERIALS AND METHODS

Materials. Fertilized chicken eggs (5–9 days old) were obtained from Shaw Hatcheries, West Chester, Pa., U.S.A., and were incubated in a moist atmosphere at 38° until used. L-[U-^{14}C]Proline (186μc/μmole) and sodium [^{35}S]-sulphate (168μc/μmole) were obtained from the New England Nuclear Corp., Boston, Mass, U.S.A.; purified collagenase (collagenase B) and hyaluronidase (testicular) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. $\alpha\alpha'$-Bipyridyl was purchased from Fisher Scientific Co., Pittsburgh, Pa., U.S.A., and puromycin hydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Labelled α-[^{14}C]collagen (4000 counts/min./mg.) and non-radioactive α-collagen from rat skin were kindly supplied by Dr George R. Martin, National Institute of Dental Research, Bethesda, Md., U.S.A.

Preparation of [^{14}C]*protein fractions.* Tibiae which consisted almost entirely of unossified cartilage were removed from 10-day-old embryos by microscopic dissection with sterile precautions. From 20 to 60 tibiae were incubated with 50μc of [^{14}C]proline or 40μc of [^{35}S]sulphate for 2 or 3hr. at 37° in a simple medium containing inorganic salts, phosphate–bicarbonate buffer and glucose (Prockop & Juva, 1965*a*). The hydroxylation of [^{14}C]proline was inhibited in the test samples with 1mM-$\alpha\alpha'$-bipyridyl. After the incubation, the tibiae were homogenized at 4° in a Teflon–glass homogenizer in 4–6ml. of distilled water, and the homogenate was centrifuged at 1200*g* for 30min. at 4°. In experiments involving further fractionation of the homogenate, the 1200*g* supernatant was centrifuged at 100000*g* at 4° for 1hr. The NaCl or KCl extract of the 100000*g* sediment was prepared by extracting the pellet overnight at 4° with 1M-NaCl or 1M-KCl buffered with 50mM-tris, pH7·6, and the sample was then centrifuged at 100000*g* at 4° for 1hr. Some of the [^{14}C]protein fractions were dialysed against 1M-NaCl or 1M-KCl and stored frozen. Other [^{14}C]protein fractions were dialysed against 7M-urea and stored at 4°.

Hydroxylation with cell-free hydroxylating system. The conversion of [^{14}C]proline into hydroxy[^{14}C]proline in the protein fractions from inhibited cartilage was tested by incubating the samples in 8ml. of hydroxylating system that contained 6ml. of 100000*g* supernatant of homogenates obtained from 10–11-day-old chick embryos, 0·5mM-$FeSO_4$, 5μM-EDTA, 0·1M-KCl, 50mM-tris, pH7·6, and 1·5mM-ascorbic acid. If the labelled protein fractions contained urea, they were dialysed against 1M-KCl for 15hr. and 0·2M-KCl for 4hr. before addition to the hydroxylating system. After incubation with the hydroxylating system at 37° for 1hr., the samples were hydrolysed with an equal volume of conc. HCl at 120° for 15hr., and assayed for total radioactivity and hydroxy[^{14}C]proline.

Treatment with enzymes. Before treatment with the enzymes indicated, the labelled protein fractions containing urea were dialysed against 1M-NaCl for 15hr. and 0·2M-NaCl for 4hr. In the treatment with hyaluronidase, 40μg. of testicular hyaluronidase was used per 0·7ml. of 1200*g* supernatant in a final volume of 1ml. containing 0·15M-NaCl and 0·1M-NaH_2PO_4 buffer, pH5·3. In the treatment with collagenase, 500μg. of collagenase B was used per 0·5ml. of 1200*g* supernatant or 0·5 ml. of 1M-KCl extract of the 100000*g* pellet in a final volume of 1ml. containing 0·1M-NaCl and 50mM-tris buffer, pH7·2. After incubation with enzymes for 3hr. at 37°, urea was added to the samples to a final concn. of 4M or 7M, and they were heated briefly at 100°.

Zone centrifugation. A 0·2 or 0·3ml. portion of the sample was layered on the top of 1–10% (w/v) sucrose gradient, which, in most experiments, contained 1M-NaCl, 1M-KCl or 4M-urea uniformly throughout the gradient. The tubes were centrifuged at 125000*g* for 20hr. in a Spinco SW39 swinging bucket at 4°. Five-drop fractions were collected from the bottom of the tubes directly into the counting vials for liquid-scintillation counting.

Gel filtration. A 1·5cm. × 24cm. column of polyacrylamide gel (Bio-Gel P-300; Calbiochem, Los Angeles, Calif., U.S.A.) was equilibrated and eluted with 7M-urea. The sample volume was usually 0·7ml., and 0·8ml. fractions were collected.

Ion-exchange chromatography. A 1·65cm. × 14·0cm. column of DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) was equilibrated with a solution of 0·05M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0. The column was eluted with 160ml. of a gradient containing 0·05–0·4M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0. Before chromatography the sample was dialysed against 0·05M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0. The sample was placed on the column in a final volume of 3ml., and 2ml. fractions were collected. A new batch of DEAE-Sephadex was used for each experiment.

Assays. The total ^{14}C or ^{35}S radioactivity of the fractions from zone centrifugation, gel filtration and ion-exchange

chromatography was assayed with the solvent system for liquid scintillation described by Prockop & Ebert (1963). Assays for hydroxy[^{14}C]proline were performed by oxidation to pyrrole with the method of Juva & Prockop (1966*b*). Before assay of hydroxy[^{14}C]proline, the samples containing urea were dialysed against 0·2M-NaCl and hydrolysed with an equal volume of conc. HCl at 120° for 15hr. To determine the ratio of hydroxy[^{14}C]proline to total radioactivity, a portion of the hydrolysate was assayed for ^{14}C radioactivity. Observed counts/min. for the [^{14}C]pyrrole obtained in the hydroxy[^{14}C]proline assay were corrected for recovery in the procedure and other losses (Juva & Prockop, 1966*b*). Efficiency for hydroxy[^{14}C]proline and total ^{14}C radioactivity with the counting system employed was 58%, and the background was 11 counts/min. The non-radioactive α-collagen was assayed by determining the extinction at 218mμ in Cary model 15 spectrophotometer. Samples containing guanidine thiocyanate were dialysed against distilled water before assay of extinction.

RESULTS

Characterization of protocollagen and collagen in native forms

When embryonic cartilage was incubated with [^{14}C]proline and a homogenate of the tissue was fractionated by centrifugation, the distribution of protein-bound label in the fractions was similar in control samples and in samples in which the hydroxylation was inhibited with $\alpha\alpha'$-bipyridyl (Table 1). When the tissue was homogenized at 1500rev./min. (Juva & Prockop, 1966*a*), most of the non-diffusible label was recovered in the 1200*g* sediment, and smaller amounts in the other fractions. In general, the distribution of hydroxy-[^{14}C]proline in control samples paralleled the distribution of total radioactivity. Extraction of the 100000*g* sediment of control samples with cold 1M-potassium chloride yielded a fraction with the highest relative content of hydroxy[^{14}C]proline. When the fractions from inhibited samples were hydroxylated with the cell-free hydroxylating system, the results indicated that the distribution of [^{14}C]protocollagen roughly paralleled the distribution of hydroxy[^{14}C]proline and of [^{14}C]collagen in the control samples. The differences indicated are probably explained by the difficulty of obtaining complete hydroxylation of the particulate fractions (Prockop & Juva, 1965*b*).

An attempt was made to isolate purer samples of protocollagen and newly synthesized collagen from the tissues by using a common procedure for the isolation of neutral-salt-soluble collagen (Jackson & Bentley, 1960). However, after the cartilage was

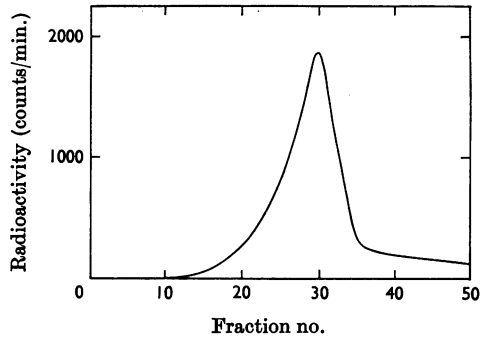

Fig. 1. Zone centrifugation of the native 1M-KCl extract from the 100000*g* sediment of cartilage that was incubated under conditions in which the hydroxylation of proline was inhibited. The gradient contained 1–10% sucrose and 1M-KCl. Fractions were collected from the bottom of the tube. A similar distribution of radioactivity was obtained with a preparation from cartilage in which the hydroxylation was not inhibited.

Table 1. *Distribution of* [^{14}C]*protein in fractions of homogenates from cartilage incubated under control conditions and under conditions in which the hydroxylation of proline was inhibited*

Homogenates were fractionated by centrifugation as indicated. All samples were dialysed and hydrolysed before assay of total radioactivity and hydroxy[^{14}C]proline. Fractions from inhibited cartilage were assayed before and after incubation with the cell-free hydroxylating system.

		Hydroxy[^{14}C]proline/total ^{14}C ratio (%)		
			Inhibited sample	
	$10^{-5}\times$ Total ^{14}C* (counts/min.)	Control sample	Before hydroxylation	After hydroxylation
1200*g* sediment	12·8	23	<1	6–8
100000*g* supernatant	4·7	20	<1	10–20
100000*g* sediment	3·6	23	<1	8–16
KCl extract of 100000*g* sediment	1·5	33	<1	25–30

* Values were similar in control samples and in samples in which the hydroxylation was inhibited.

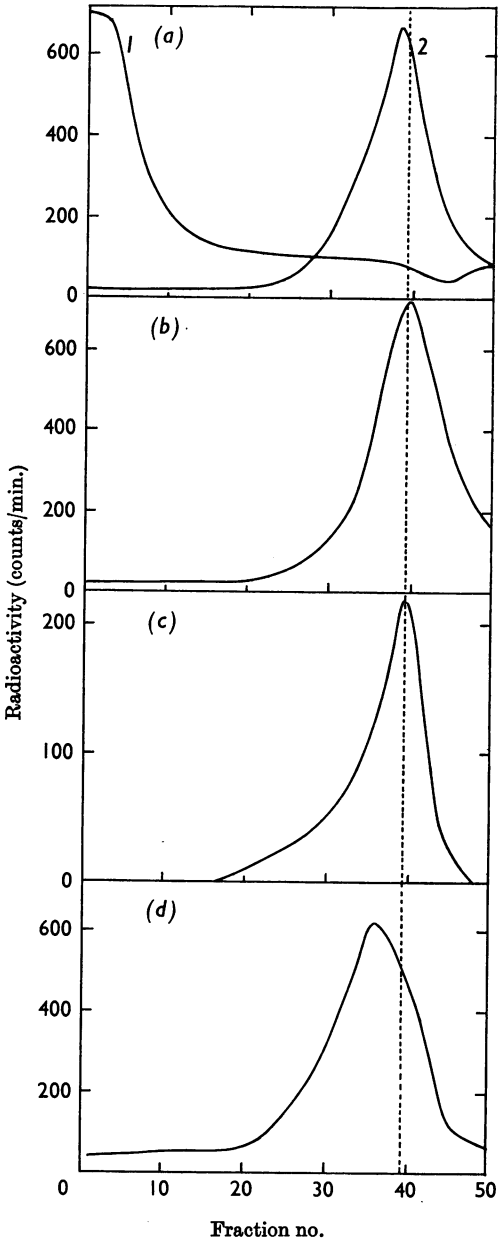

Fig. 2. Zone centrifugation of denatured preparations on gradients containing 1–10% sucrose and 4M-urea. All samples were diluted to 4M-urea before being layered on the top of the gradient. Similar results were obtained with preparations from cartilage that were incubated under control conditions and under conditions in which the hydroxylation of proline was inhibited. The broken vertical line indicates the maximum of the peak of purified α-collagen. (*a*) Curve *1*: a 1200*g* supernatant preparation that was heated at 60° in 7M-urea for 15min. Curve *2*: a 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 1hr. (*b*) A 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 3hr. (*c*) Purified α-[^{14}C]collagen from rat skin. (*d*) A 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 1hr. and then stored at 4° for 3 weeks.

homogenized in 0·2M-sodium chloride and centrifuged at 12000*g*, less than one-quarter of the non-diffusible label in the supernatant fraction was precipitated with 20% (w/v) sodium chloride, and the recoveries in subsequent steps were too low to allow purification of the labelled protein with this procedure. Results were similar with both inhibited and control samples.

Because the 1M-sodium chloride or 1M-potassium chloride extracts of the 100000*g* sediments appeared to be the purest preparations, attempts were made to characterize these fractions by zone centrifugation with sucrose gradients. Preparations from both control and inhibited samples sedimented as a single peak in a 1–10% sucrose gradient containing 1M-potassium chloride or 1M-sodium chloride (Fig. 1), even though protocollagen or collagen could not have accounted for more than about one-half of the total radioactivity (see below). Both preparations sedimented rapidly and formed a firm pellet when salt was omitted from the gradient solutions. Further attempts to characterize these native preparations were abandoned, because the solutions were unstable and the labelled proteins formed insoluble aggregates on storage at 4° or −20°.

Characterization of denatured protocollagen and of newly synthesized collagen

For further studies, denatured 1200*g* supernatant fractions were a convenient source of stable solutions of labelled protein. In all experiments labelled 1200*g* supernatant fractions from cartilage where hydroxylation was not inhibited were used as a control to compare the characteristics of protocollagen with newly synthesized collagen from the same tissue.

Zone centrifugation. Initial experiments indicated that, after the 1200*g* supernatant fraction was heated at 60° in 7M-urea, pH8, for 15min., the resulting solution could be stored in 7M-urea at 4° without precipitation. Zone centrifugation of these preparations indicated that they sedimented rapidly in 1–10% sucrose gradients containing 4M-urea (Fig. 2). After the samples were heated at 100° for 1hr., almost all the radioactivity in both control and protocollagen samples appeared as a single peak near the top of the tube (Fig. 2*a*). Heating the preparations for 3hr. at 100° shifted the position of the peak slightly, and there was a small increase in the amount of radioactivity at the top of the gradient (Fig. 2*b*). The sedimentation

patterns obtained after boiling for 1 or 3hr. were similar to those obtained with purified α-[^{14}C]-collagen in the same gradient (Fig. 2*c*).

Although preparations heated at 100° for 1hr. remained soluble, they apparently reaggregated on standing for several weeks, since the labelled proteins began to sediment more rapidly in the sucrose gradient (Fig. 2*d*). Treatment of the preparation shown in Fig. 2(*d*) with hyaluronidase again converted it into a form that sedimented in a peak similar to those shown in Figs. 2(*a*), 2(*b*) and 2(*c*). Also, the peak sedimenting in the same position as α-collagen was obtained after hyaluronidase treatment of 1200*g* supernatant preparations that were only heated at 60° for 15min.

Further evidence for the formation of aggregates was obtained with one atypical preparation (K-5) of a 1200*g* supernatant fraction from cartilage. With the K-5 preparation, the initial incorporation of [^{14}C]proline was one-tenth that observed with any other preparation, and the labelled proteins sedimented rapidly even after the preparation was heated at 100° for 1hr. (Fig. 3*a*, curve *1*). Treatment of the K-5 preparation with hyaluronidase converted the labelled proteins into forms that sedimented in the same pattern as more typical denatured preparations and α-collagen (Fig. 3*a*, curve *2*). Mixing the K-5 preparation with other preparations of denatured 1200*g* supernatant (Fig. 3*b*, curve *2*) made essentially all the radioactivity in both preparations sediment rapidly (curve *3*). Similar results were obtained when the K-5 preparation was mixed with less rapidly sedimenting preparations from either control or inhibited cartilage. On the basis of the hydroxy-[^{14}C]proline values in Table 1 and the approx. equal amounts of proline and hydroxyproline in collagen (see below), only about 50% of the label in the 1200*g* supernatant fraction (Fig. 3*b*, curve *2*) could be accounted for by collagen or protocollagen, but essentially all the label was displaced by mixing with K-5.

Gel filtration. When the 1200*g* supernatant fraction was heated at 100° for 10min. in 7M-urea and then passed through a large-pore polyacrylamide column, all the radioactivity appeared as a slightly asymmetrical peak with a maximum in the exclusion volume of the column (Fig. 4*a*, curve *1*). As noted, this peak appeared before the peak obtained with purified α-collagen in the same column (curve *2*). A slightly broader peak was obtained after the α-collagen was boiled in urea for 1hr. (not shown). When the 1200*g* supernatant fraction was boiled for 1hr., the peak became more asymmetrical (Fig. 4*b*). Boiling for 3hr. shifted the maximum of the peak, and significant amounts of the radioactivity appeared in later fractions (Fig. 4*c*). When a preparation was heated at 100° for only 20min. and then treated with hyaluronidase, the elution pattern was similar to that obtained by heating at 100° for 3hr. (Fig. 4*d*). In all instances, similar elution patterns of total radioactivity were obtained with preparations of cartilage that were incubated under control conditions or under conditions in which the hydroxylation of proline was inhibited.

The distribution of collagen in the elution pattern of the control samples was examined by hydrolysing fractions from the column and assaying them for hydroxy[^{14}C]proline. When the control preparation was heated at 100° for 1hr., most of the hydroxy[^{14}C]proline appeared near the exclusion volume of the column (Fig. 4*b*). When the preparation was heated at 100° for 3hr., or when treated with hyaluronidase, the elution peak of hydroxy-[^{14}C]proline appeared to shift towards the position at which α-collagen was eluted, and small amounts

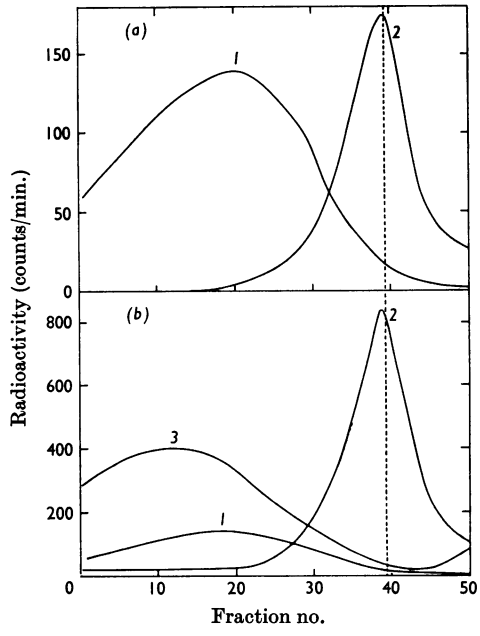

Fig. 3. Zone centrifugation of denatured preparations on gradients containing 1–10% sucrose and 4M-urea. The broken vertical line indicates the maximum of the peak of purified α-collagen. (*a*) Curve *1*: 1200*g* supernatant preparation K-5 that was heated at 100° in 7M-urea for 1hr. Curve *2*: the same preparation after treatment with hyaluronidase. (*b*) Curve *1*: 1200*g* supernatant preparation K-5 that was heated at 100° in 7M-urea for 1hr. Curve *2*: typical 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 1hr. Curve *3*: mixture of preparations indicated in curves *1* and *2*. Similar results were obtained when the K-5 preparation was mixed with typical preparations from cartilage that were incubated under control conditions and under conditions in which the hydroxylation of proline was inhibited.

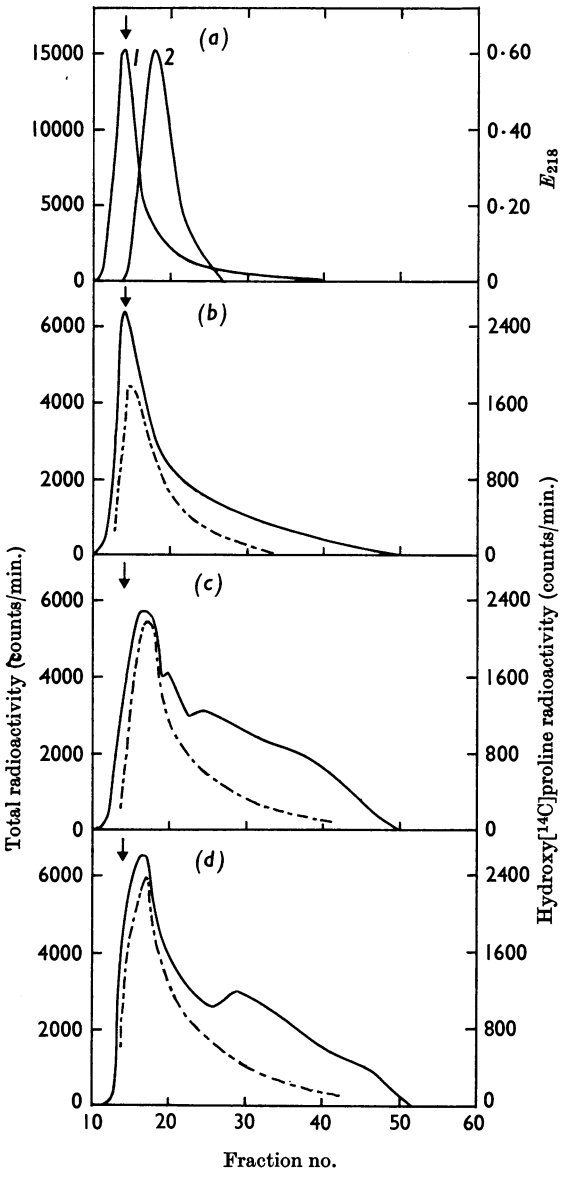

Fig. 4. Gel filtration of denatured preparations on a polyacrylamide gel (Bio-Gel P-300) in 7M-urea. Similar elution patterns of total radioactivity were obtained with preparations from cartilage that were incubated under control conditions or under conditions in which the hydroxylation of proline was inhibited. The broken lines in (*b*), (*c*) and (*d*) indicate the distribution of hydroxy[^{14}C]proline in the control samples. The peak of the exclusion volume of the column is indicated by the arrow. (*a*) Curve *1*: a 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 10min. Curve *2*: purified α-collagen from rat skin assayed by extinction at 218mμ (scale on right). (*b*) A 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 1hr. (*c*) A 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 3hr. (*d*) A 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 20min. and then treated with hyaluronidase.

of hydroxy[^{14}C]proline were eluted in later fractions (Figs. 4*c* and 4*d*). After boiling for 3hr. or after treatment with hyaluronidase, the hydroxy-[^{14}C]proline/total radioactivity ratio in the major elution peak increased significantly, and the highest ratios were observed in fractions that were slightly beyond the fractions with the highest ^{14}C content. When hyaluronidase-treated preparations from inhibited cartilage were passed through the column and the eluted fractions were tested in the cell-free hydroxylating system, the maximum conversion of [^{14}C]prolinei nto hydroxy[^{14}C]proline was observed with fractions 15–20, indicating that protocollagen appeared in about the same position as the newly synthesized collagen.

When cartilage was incubated with [^{35}S]sulphate and the non-diffusible 1200*g* supernatant was passed through the Bio-Gel P-300 column, all the [^{35}S]sulphate appeared in the exclusion volume of the column after the preparation was boiled in urea for 20min. Only a small amount of [^{35}S]sulphate appeared in later fractions even after the sample was boiled for 3hr. (Fig. 5).

Ion-exchange chromatography. Attempts to purify the protocollagen by chromatography on CM-cellulose (Piez, Eigner & Lewis, 1963) were unsuccessful, because even the preparations boiled in urea or treated with hyaluronidase formed insoluble aggregates under the conditions usually employed. Significant fractionation was obtained by chromatography on DEAE-Sephadex by equilibrating the system with 4M-urea and by using guanidine thiocyanate as an electrolytic eluent that did not produce precipitation of the samples in the column (Fig. 6).

When a supernatant fraction of a control sample was boiled in urea for 3hr. and chromatographed, two peaks of radioactivity were obtained, and essentially all the hydroxy[^{14}C]proline was recovered in the first peak (Fig. 6*a*). A similar distribution of total counts was obtained with a sample from cartilage in which the hydroxylation was inhibited. When a supernatant fraction was treated with hyaluronidase (Fig. 6*b*), the ^{14}C elution pattern was similar to that obtained with samples boiled for 3hr., but large amounts of the radioactivity in the second peak (Fig. 6*a*) were adsorbed on the resin, and they were not eluted by the gradient. However, almost all the hydroxy[^{14}C]-proline was recovered. When supernatant fractions from inhibited cartilage were treated with hyaluronidase and the fractions eluted from the column were tested for hydroxylation in the cell-free system, the highest hydroxylation values were

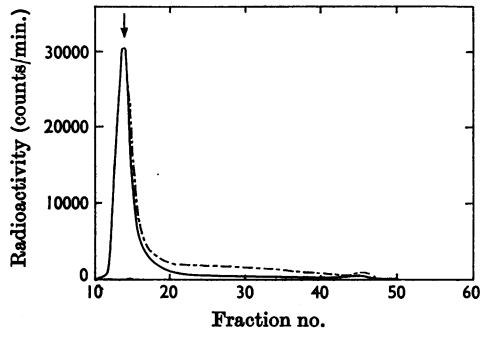

Fig. 5. Gel filtration of denatured preparations labelled with [^{35}S]sulphate on a polyacrylamide gel (Bio-Gel P-300). Solid line: a 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 20min. Broken line: a 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 3hr.

obtained with fractions corresponding to the first peak (Fig. 6*c*). When preparations of the 1200*g* supernatant fraction were denatured by boiling in urea before chromatography, the hydroxylation observed with the cell-free hydroxylating system was reduced from 15–20% in native preparations to 7–9% in denatured preparations. However, values as high as 18% were observed when the labelled protein eluted in the first peak from the column was hydroxylated.

In samples which were boiled for 3hr. before chromatography, the non-diffusible [^{35}S]sulphate of the system was separated from the two peaks of labelled proteins (compare Fig. 6*a* and Fig. 7). Pure α-collagen did not bind to the resin, and it appeared in the exclusion volume of the column (Fig. 7).

Collagenase treatment. When the denatured 1200*g* supernatant fraction from control cartilage was treated with a purified collagenase specific for the amino acid sequences found in collagen (Mandl, Keller & Manahan, 1964), all the hydroxy[^{14}C]-proline and essentially all the radioactivity that was calculated to be present in [^{14}C]collagen were lost on dialysis (Table 2). All the radioactivity in proteins other than collagen was still recovered in non-diffusible forms. Similar results were obtained with the 1M-potassium chloride extract of the 100000*g* pellet of control cartilage. When comparable experiments were performed with the same fractions from cartilage in which the hydroxylation was inhibited, similar amounts of labelled protein became diffusible, suggesting that all the radioactive protocollagen was hydrolysed. Control experiments with albumin and globulin indicated that the collagenase did not produce any significant hydrolysis of these substrates (Juva & Prockop, 1966*a*).

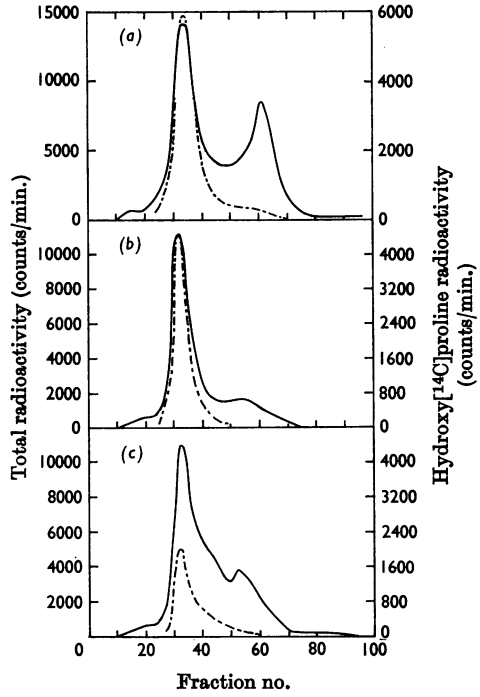

Fig. 6. Anion-exchange chromatography of denatured preparations on a DEAE-Sephadex column. The column was equilibrated with 0·05M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0, and it was eluted with a linear gradient of 0·05–0·4M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0, beginning in about fraction 14 and ending in fraction 95. The broken lines indicate the distribution of hydroxy[^{14}C]proline. (*a*) A 1200*g* supernatant preparation that was heated at 100° in 7M-urea for 3hr. Similar elution patterns of total radioactivity were obtained with preparations from cartilage that were incubated under control conditions and under conditions in which the hydroxylation of proline was inhibited. The broken line indicates the distribution of hydroxy[^{14}C]proline in the control sample. (*b*) A 1200*g* supernatant preparation from control cartilage that was heated at 100° in 7M-urea for 20min. and then treated with hyaluronidase. (*c*) A 1200*g* supernatant preparation from cartilage incubated under conditions in which the hydroxylation of proline was inhibited. The sample was heated at 100° in 7M-urea for 20min. and then treated with hyaluronidase. The eluted fractions were incubated with a cell-free hydroxylating system and then assayed for hydroxy[^{14}C]-proline. The fractions did not contain significant amounts of hydroxy[^{14}C]proline before incubation with the hydroxylating system.

Purity of isolated protocollagen. All the protein-bound radioactivity recovered from cartilage incubated for 1–4hr. with [^{14}C]proline was present as [^{14}C]proline and hydroxy[^{14}C]proline (Juva & Prockop, 1966*a*). If it is assumed that the specific

activities of the [^{14}C]proline and hydroxy[^{14}C]-proline in the collagen of the control preparations were the same, then the hydroxy[^{14}C]proline/total radioactivity ratio can be used to indicate the purity of labelled protein in the isolated collagen fraction.

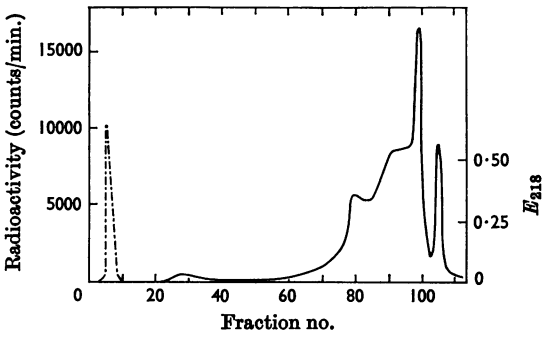

Fig. 7. Anion-exchange chromatography of purified α-collagen and of preparations labelled with [^{35}S]sulphate on a DEAE-Sephadex column. Conditions were as described in Fig. 6 except that, after completion of the gradient, the column was eluted further with 15ml. of 0·4M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0, and then with 20ml. of 1·0M-guanidine thiocyanate, 4M-urea and 5mM-tris buffer, pH8·0. Broken line: purified α-collagen from rat skin that was assayed by extinction at 218mμ. Solid line: a 1200g supernatant preparation from cartilage that was incubated with [^{35}S]sulphate. The sample was heated at 100° in 7M-urea for 3hr. Fraction numbers are comparable with those in Fig. 6.

The ratio of hydroxyproline residues to total hydroxyproline and proline residues is in the range 0·41–0·46 for pure collagen from mammals and birds (see Harrington & Hippel, 1961). When the control preparations were examined by gel filtration after digestion with hyaluronidase or after boiling for 3hr. (Fig. 4), the fractions corresponding to α-collagen had a hydroxy[^{14}C]proline/total ^{14}C radioactivity ratio 0·38. When similar control samples were chromatographed on DEAE-Sephadex (Fig. 6), the maximum of the first peak gave a ratio 0·41–0·43 with three different preparations. Identical incorporation of radioactivity into protein was obtained with a similar preparation from cartilage that was incubated with $\alpha\alpha'$-bipyridyl, and a similar elution pattern of radioactivity was observed after chromatography on the DEAE-Sephadex. Therefore it seems probable that the purity of [^{14}C]protocollagen was similar to that of the [^{14}C]collagen from the control sample, and that most of the radioactivity in the first peak from the column (Fig. 6) was in [^{14}C]protocollagen.

Even though the above assays of radioactivity suggest that the best fractions of [^{14}C]protocollagen and [^{14}C]collagen were relatively pure, preliminary attempts at amino acid analysis indicated that these samples were still contaminated by an approximately equal amount of non-radioactive proteins. The initial 1200g supernatant fraction of the control cartilage contained only about 130μg. of collagen or protocollagen and about 10mg. of other proteins per 100 tibiae, and attempts to purify the fractions further have not been successful as yet.

Table 2. *Effect of collagenase on the non-diffusible labelled proteins from cartilage incubated under control conditions and under conditions in which the hydroxylation was inhibited*

The 1200g supernatant and 1M-KCl extract of the 100000g pellet were prepared from control and inhibited cartilage and they were incubated with purified collagenase as described in the text. After incubation with collagenase, portions of the samples were assayed before and after dialysis.

	Before dialysis			After dialysis		
	Total ^{14}C* (counts/min.)	Hydroxy[^{14}C]-proline (counts/min.)	[^{14}C]Collagen† (counts/min.)	Total ^{14}C (counts/min.)	Hydroxy[^{14}C]-proline (counts/min.)	^{14}C loss (counts/min.)
1200g supernatant						
Control	10000	2200	5100	4700	50	5300
Inhibited	10000	<50		4800	<20	5200
1M-KCl extract						
Control	10000	3300	7700	2200	<20	7800
Inhibited	10000	<50		2000	<20	8000

* To facilitate comparison of the results, all values for observed counts/min. were expressed on the basis of initial samples containing 10000 counts/min.

† Calculated values assuming that the specific activities of [^{14}C]proline and hydroxy[^{14}C]proline in the collagen were the same and that the ratio of hydroxyproline residues to total proline and hydroxyproline residues in the collagen was 0·43.

Estimate of minimum size of the polypeptide substrate for hydroxylation

Tibiae were incubated with [^{14}C]proline and puromycin (8 or 20μg./ml.), and the 100000g supernatant fractions were examined by gel filtration after boiling for 1hr. in 7M-urea. The labelled polypeptides from samples incubated with 8μg. of puromycin/ml. were retarded to a greater extent than the polypeptides from the control sample (Figs. 8a and 8b), but most of the hydroxy[^{14}C]-proline appeared in the leading edge of the elution peak. The labelled polypeptides from samples incubated with 20μg. of puromycin/ml. were retarded further by the column (Fig. 8c). On the basis of complete exclusion equal to R_F 1·0, the maximum of the broad asymmetrical peak had R_F 0·49. The maximum of the hydroxy[^{14}C]proline peak appeared near the maximum of the total radioactivity elution pattern, but the hydroxy[^{14}C]-proline/total radioactivity ratio was considerably higher in the leading edge of the ^{14}C peak.

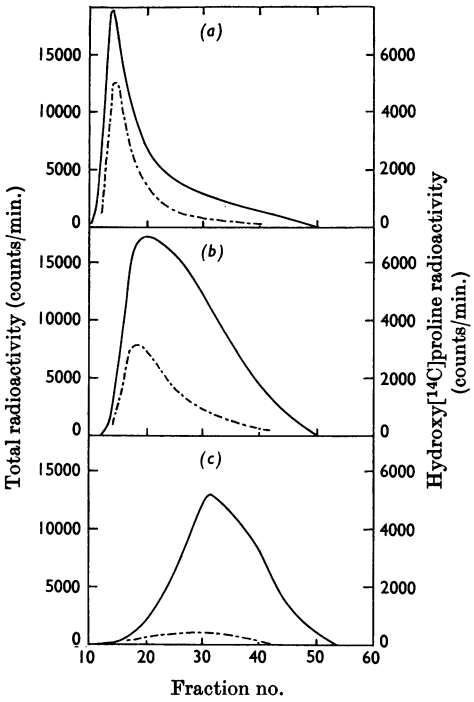

Fig. 8. Gel filtration of denatured preparations from control and puromycin-treated cartilage on a polyacrylamide gel (Bio-Gel P-300) in 7M-urea. The 100000g supernatant fractions of cartilage homogenates were heated at 100° in 7M-urea for 1hr. Portions of samples for chromatography were selected so as to contain 250000–400000 total counts/min. Broken lines indicate the distribution of hydroxy-[^{14}C]proline. (a) Control preparation. (b) Preparation from cartilage incubated with 8μg. of puromycin/ml. (c) Preparation from cartilage incubated with 20μg. of puromycin/ml.

DISCUSSION

Several Laboratories have reported evidence which suggests that small amounts of prolyl-s-RNA may be hydroxylated to hydroxyprolyl-s-RNA (Manner & Gould, 1963; Coronado, Mardones & Allende, 1963; Jackson, Watkins & Winkler, 1964; Urivetzky, Frei & Meilman, 1965), but Urivetzky, Frei & Meilman (1966) have shown that no significant amounts of hydroxy[^{14}C]prolyl-s-RNA are incorporated into collagen. Most recent studies (see the introduction) support the conclusion that the synthesis of both the hydroxyproline and hydroxylysine in collagen requires the synthesis of proline-rich and lysine-rich polypeptides. The polypeptide precursor or percursors, however, have not been previously isolated and characterized.

The disaggregated and denatured forms of protocollagen isolated here were shown to be similar in size to denatured newly synthesized collagen from the same tissue and to purified α-collagen from rat skin. The results therefore indicate that, when the hydroxylation is inhibited, most of the protocollagen polypeptides which accumulate are as large as the complete α-chains of collagen. The experiments with purified collagenase further suggest that the amino acid composition and amino acid sequences of protocollagen are similar to those of collagen. Chromatography on DEAE-Sephadex indicated that the protocollagen contained additional anionic groups not found in purified α-collagen from rat skin, but newly synthesized collagen from the same tissue was indistinguishable from the protocollagen. The hydroxy[^{14}C]proline/total radioactivity ratios suggest that the [^{14}C]protocollagen and [^{14}C]collagen were isolated in relatively pure form.

Although cartilagenous tibiae from 10-day-old chick embryos have a number of advantages as a system for studying collagen biosynthesis (Bhatnagar, 1964; Juva, Prockop, Cooper & Lash, 1966), several difficulties were encountered in isolating the collagen fractions. The protocollagen and newly synthesized collagen could not be purified by the procedures usually used for soluble collagen (Jackson & Bentley, 1960) or for denatured collagens (Piez *et al.* 1963). The impure, native preparations behaved as single components during zone centrifugation, and they had to be boiled in urea to obtain stable solutions. Other investigators (Levene & Gross, 1959; K. A. Piez, personal communication) also encountered difficulties in isolating newly synthesized collagen from embryonic tissues.

The unusual characteristics of both protocollagen and newly synthesized collagen from embryonic cartilage are apparently explained by the existence of complexes which contain these proteins, mucopolysaccharides, and other polypeptides or proteins from the same tissue. Cartilage is particularly rich in chondroitin sulphates, and several studies (see Muir, 1964; Mathews, 1965) have shown that acid mucopolysaccharides can form insoluble complexes with collagen and other proteins. The preparations of protocollagen and newly synthesized collagen were shown to contain at least two proteins and a sulphate-containing component, but it was necessary to disrupt the complexes under relatively vigorous conditions before they could be examined by gel filtration or chromatography. The prolonged boiling in urea probably produced some hydrolysis of collagen (see Veis, 1964) and of other components, but evolution of cyanate from the urea and other reactions may also have occurred. The treatment with hyaluronidase probably disrupted chondroitin sulphates in the complexes. Even after these treatments, a special elution system containing urea and guanidine thiocyanate had to be developed in order to chromatograph the samples on DEAE-Sephadex. This new chromatographic technique may be generally useful in examining insoluble proteins. In spite of the technical difficulties encountered studying the preparations, it was demonstrated by several different techniques that the complexes containing protocollagen were similar to the complexes containing newly synthesized collagen from the same system.

Although the results indicate that protocollagen molecules as large as α-collagen are synthesized when the hydroxylation is inhibited, it is not clear whether the hydroxylation normally begins when relatively short protocollagen molecules are still attached to polysomes (Manning & Meister, 1966; Bekhor, Mohseni & Bavetta, 1966), or whether protocollagen molecules the size of α-collagen must be synthesized and released before they can be hydroxylated. Experiments with the cell-free hydroxylating system and with synthetic polytripeptides of the structure $(prolylglycylpropyl)_n$ suggested that a molecular weight of greater than 15000 is necessary for complete hydroxylation of the appropriate proline residues (Juva & Prockop, 1965*a*), and that the polypeptide substrate may have to form a three-stranded structure similar to the triple helix of collagen (A. Berger & J. Engel, personal communication) before it can be hydroxylated.

It was previously reported (Juva & Prockop, 1964, 1966*a*) that, when large concentrations of puromycin were added to embryonic cartilage, the peptides which continued to be synthesized were of insufficient size to serve as substrates for the hydroxylating system. An attempt was made here to use moderate concentrations of puromycin in order to estimate the minimum size of the polypeptide required for the synthesis of hydroxyproline under conditions in which the hydroxylation was not inhibited. Labelled polypeptides of varying sizes appeared in the soluble protein fraction, but hydroxy[^{14}C]proline was primarily found in the largest polypeptides recovered by gel filtration. The results suggest therefore that only a limited amount of hydroxylation can occur with polypeptides smaller than α-collagen.

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