Structural Differences among Procollagens Associated with Rough and Smooth Microsomes from Chick Embryo Cartilage*

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Epiphyseal cartilages of 12-day chick embryo were labeled in vitro with $[^{14}C]$proline. By subjecting the $[^{14}C]$proline-labeled tissues to subcellular fractionation, it was possible to obtain radioactive procollagens as they were associated with smooth and rough microsomes. Analyses of the procollagens thus obtained indicated that the components in the rough microsome fraction comprised both single- and triple-stranded forms in nearly equal proportions while those in the smooth microsome fraction were present predominantly as triple-stranded forms. When hydroxylation of procollagen polypeptides was inhibited by 2,2'-dipyridyl, unhydroxylated procollagens accumulated in the rough microsomal fraction as single- and triple-stranded forms.

Labeling experiments using $[^{14}C]$glucose revealed a marked difference in the extent of glycosylation among the single- and triple-stranded procollagens in different submicrosomal fractions. Digestion of the procollagen components with bacterial collagenase yielded, in each case, a large glycopeptide fraction characterized by the presence of glucose, galactose, mannose, and N-acetylglucosamine, plus a fraction of small peptides characterized by the presence of glucose and galactose. The characterization and comparison of these glycopeptide fragments further defined the nature of the additional peptide region in the various procollagen components, and, when combined with information derived from studies of $[^{14}C]$proline-labeled procollagens, supported the view that glycosylation of the additional region is completed in the rough endoplasmic reticulum prior to formation of the triple-stranded form, whereas glucose and galactose continue to be incorporated into the region after the formation of triple strands and the movement of the triple strands into the Golgi complex.

It is now well established that both type I collagens in dermis, bone, tendon, etc. and type II collagens in cartilage are initially synthesized in a precursor form, termed procollagen, in which the constituent a chains have terminal extensions (for a review see Ref. 1). By using matrix-free tendon cells and cranial bone it has been possible to study the synthesis and secretion of type II procollagen. This approach has enabled several investigators (4, 5) to demonstrate the occurrence of a disulfide-linked trimer of pro-a(II) chains in the medium of cultured cartilage cells. More recent studies (6, 7) have shown that the medium pro-a(II) contains both NH$_2$- and COOH-terminal extensions and the interchain disulfide bonds are located in the COOH-terminal region.

Surviving cartilage slices, as the system having all the complex series of steps essential for the formation of type II collagen, would appear to be another favorable object in which to study the synthesis and transport of the collagen molecules as well as the factors regulating these processes. However, studies with intact cartilages have been handicapped by the fact that extraction with neutral salt solutions as well as dilute acetic acid are totally ineffective as solvents for the collagen in cartilage unless the tissue is made lathyritic (8). In a previous work with surviving epiphyseal cartilages from 12-day-old chick embryo (9), we observed that extraction with 4 M guanidine hydrochloride of cartilages, previously incubated with $[^{14}C]$proline for 30 min, resulted in the solubilization of over 80% of the labeled proteins therein. Further evidence indicated that the radioactivity was largely in a single type of polypeptide chain which was sensitive to collagenase but somewhat larger than the authentic a1(II) chain because of a noncollagenous glycopeptide extension ($M_\text{r} = 13,200$) containing mannose, galactose, and glucose (10). In these initial studies, however, no special precautions were taken to prevent artificial cleavage of peptide and disulfide bonds during the extraction. Thus, the isolation of the single-stranded procollagen from intact cartilage remained a problem of whether the chain represents a physiological precursor in the conversion to a triple-stranded molecule or results from partial degradation of the triple strands during the manipulation.

By modifying the original technique for the tissue extraction, we now demonstrate that both single- and triple-stranded procollagen molecules can be isolated from cartilage slices in high yield. Whether the different forms of procollagen thus obtained represent different states of biosynthetic precursors is examined here. Special emphasis is laid on the studies to determine whether they exist in different subcellular compartments and whether they differ in the type and content of carbohydrate moieties linked to polypeptide chains.

**EXPERIMENTAL PROCEDURES**

**Materials**

Fertile eggs were obtained from a local supplier on Day 9 and were incubated in a moist atmosphere at 38°C until they were used on Day 12. A highly purified preparation of bacterial collagenase (from Clostridium histolyticum) was a gift from Dr. T. Ohya, Amaro Pharmaceutical Co., Nagoya. The specific activity determined by the method of Mandl et al. (11) was 2,200 units/mg. Pepstatin was given by...
Banyu Pharmaceutical Co., Tokyo. Acid-soluble collagen of rat skin was prepared by the method of Piez et al. (12).

The following commercial materials were used: L-[U-14C]proline, 205 Ci/mmol, and L-[3,4-14C]tryptophan, 58 Ci/mmol, from New England Nuclear, Boston; n-[U-14C]glucose, 180 Ci/mmol, from International Chemical and Nuclear Corp., Irvine; carrier-free Na235S04 from Radionuclide Association, Tokyo; chymotrypsinogen A and cytochrome c from Behringwerke Mannheim, Yamanouchi, Tokyo; fetal calf serum from Gibco Corp., Berkeley; Diallo PM-10 membrane from Amicon Corp., Lexington; DEAE-cellulose (Whatman DE32) from H. Reeve Angel & Co., London; and Bio-Gel A-15m, 200 to 400 mesh, from Bio-Rad Laboratories, Richmond.

Incubation of Cartilage Slices

Cartilage slices were prepared from the tibias and femurs of 12-day-old chick embryos as described previously (10). The slices, 300 mg, were preincubated at 37°C for 20 min with gentle shaking in (a) 1 ml of Krebs Medium II (13) enriched with 10% (v/v) fetal calf serum containing 50 µg of sodium ascorbate, (b) 1 ml of MgSO4-free Krebs Medium II with 10% fetal calf serum containing 30 µg of sodium ascorbate and 240 µg of MgCl2·6H2O, or (c) 2.5 ml of glucose-free Krebs Medium II with 1% fetal calf serum containing 125 µg of sodium ascorbate. Following this preincubation, 1 µCi of [14C]proline, 5 µCi of [14C]tryptophan, 10 µCi of Na235S04, or 25 µCi of [14C]glucose was added to the suspension in Medium a, b, or c, respectively, and incubation was allowed to continue for the periods indicated in individual experiments. At the end of the incubation periods, the cartilages were thoroughly rinsed with ice-cold cold 2% perchloric acid/iodoacetamide solution produced little radioactive materials.

In studies on the effect of 2,2'-dipyridyl on procollagen synthesis, the slices, 100 mg, were incubated with [14C]proline (see above) were washed twice with 5 ml of Krebs Medium II supplemented with 4 mM proline (chase medium), and then replaced in fresh chase medium at 37°C for various times of nonradioactive chase.

Extraction of Procollagen

Procollagen was extracted from labeled cartilage slices by three different methods. **Method A** This method, a modification of the procedure of Sajdera and Hascall (14) for proteoglycan extraction, has been described in detail (9, 10). Briefly, the procedure consisted of the following: sliced cartilages were treated successively with cold 2% perchloric acid, 50% ethanol, and 4 M guanidine hydrochloride containing 50 mM Tris-HCl, pH 7.5. The tissue residue was removed by centrifugation.

**Method B**—Labeled cartilage slices, 460 mg, were extracted by immediate homogenization in a Potter-Elvehjem glass homogenizer with 11 ml of ice-cold 4 M guanidine hydrochloride containing 50 mM Tris-HCl and 50 mM NaCl, pH 7.5. The tissue residue was removed by centrifugation (30,000 × g, 30 min). Further treatment of the precipitate as described above with fresh guanidine hydrochloride/Tris-HCl solution produced little radioactive materials.

**Method C**—The procedures were the same as in Method B except that 10 mM EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, and 0.36 mM pepstatin were added to 4 M guanidine hydrochloride containing 50 mM Tris-HCl, pH 7.5, before the tissue was homogenized.

**Subcellular Fractionation of Cartilages**

The homogenization procedures and fractionation schemes described by Kinata et al. (15) were used with a slight modification as follows. All the operations were performed at 0-4°C.

Sliced cartilages, 1 g, were placed in 5 ml of 0.24 M sucrose containing 0.1 M Tris-HCl, pH 7.5, 5 mM NaCl, 0.34 M NaCl, 0.3 M guanidine hydrochloride, 0.3 mM 2,2'-dipyridyl, 0.3 mM cycloheximide, and 0.3 mM pepstatin, and disrupted in a loose fitting Potter-Elvehjem glass homogenizer (consisting of a tube (1.8 × 18 cm) and a pestle (1.7 × 6 cm)) with 25 up and down strokes. The homogenate was centrifuged at 5,000 × g for 10 min. The precipitate was treated as described above with 4 ml of the sucrose solution, after which the suspension was centrifuged at 5,000 × g for 10 min. The first and second supernatant fluids were combined and a 10-ml aliquot of the fluid was layered over a discontinuous sucrose gradient consisting of a 7-ml layer of 0.64 M sucrose, an 8.5-ml layer of 1.24 M sucrose, and a 4.5-ml layer of 2 M sucrose, each containing the salts and inhibitors described above. Upon centrifugation at 60,000 × g, for 3 h, sharp white zones were observed at the bottom of the 1.24 M sucrose layer (P-1 fraction) and at the bottom of the 0.64 M sucrose layer (P-2 fraction). The supernatant solution, 10 ml, was pipetted from each tube (soluble fraction). The P-1 and P-2 layers were then collected by pipette and diluted with 3 volumes of 0.34 M sucrose containing the salts and inhibitors, see above. After centrifugation at 77,000 × g, for 60 min, the resulting pellets were collected.

**Electron Microscopy**

The submicromolar pellets, P-1 and P-2, were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated, and embedded in Epon 812 in the usual manner. Ultrathin sections were prepared and subjected to double staining with uranyl acetate and lead citrate. Electron microscopy was carried out with a JEOL-JEM-100B electron microscope.

**Other Methods**

N-Acetylhexosamine was measured by a modified Morgan-Elsden method (16). Reducing sugars were determined by the method of Park and Johnson (17). N-Acetylation of hexosamine was carried out as described by Roesman and Dafner (18). SDS* gel electrophoresis was carried out on 5% acrylamide gels (0.5 × 7 cm) as described by Weber and Osborn (19).

**RESULTS**

**Extraction Experiments**—When cartilage slices, previously incubated with [14C]proline for 60 min, were extracted by Method A (the method used in our earlier papers, Refs. 9 and 10) and the extract was chromatographed on Bio-Gel A-15m (Fig. 1), the elution pattern was similar to that described (9), i.e., a large peak with one shoulder was obtained between Fraction 60 and Fraction 73, which have subsequently been identified as α1(II) (shoulder) and a precursor form of the α1(II) chain (main peak). In contrast, rapid homogenization

*The abbreviation used is: SDS, sodium dodecyl sulfate.
and extraction of similarly labeled tissues with 4 M guanidine hydrochloride in the presence of 50 mM iodoacetamide (Method B) revealed a different elution pattern (Fig. 1) with an additional large peak (Peak X) that was eluted in the area expected for triple-stranded procollagens. The small peak eluting in the void volume represents noncollagenous proteins (as assessed by its insusceptibility to digestion by bacterial collagenase). The total yield of radioactive proteins was about the same for the extracts obtained by Methods A and B (approximately 90% of the total radioactivity incorporated into tissue proteins). The results indicate that the extraction with 4 M guanidine hydrochloride alone (Method A) may have caused some nonspecific reduction of disulfide bonds but where appropriate precautions were taken (Method B) such nonspecific reduction has largely been prevented. When various protease inhibitors (ethylenediaminetetraacetate, N-ethylmaleimide, phenylmethylsulfonyl fluoride, and pepstatin) were included during the extraction (Method C) the elution pattern of the extracted proteins was essentially the same as that obtained without the inhibitors.

To test whether Peak X component contained interchain disulfide bonds, the peak fractions (Fig. 1, Tubes 48 to 54) were pooled and examined by polyacrylamide gel electrophoresis in SDS either without reduction or after the sample was reduced with 2 mercaptoethanol. Prior to reduction, the sample was recovered entirely in the position expected for triple-stranded components. After reduction, it was recovered as a band of the mobility expected for single-stranded pro-α chain. The molecular weight of the pro-α chain, estimated from the positions of rat skin α1(I) (Mr = 95,000) and β1I (Mr = 190,000), was 122,000 ± 5,000.

To assess metabolic relationships between the collagenous components of different size, a pulse-chase experiment was performed. A pulse period of 10 min with [14C]proline was followed by a 50-min chase with medium containing an excess of unlabeled proline. At the initiation of chase, a large portion of the radioactivity was found in the position expected for monomeric pro-α component. With increasing time of chase, the radioactivity of this component was gradually transferred first to a component corresponding in position to trimeric pro-α chains and then to a component corresponding to monomeric α chains. The data are exactly what one would expect if the triple-stranded, disulfide-linked pro-α component is assembled through utilization of the monomeric pro-α component and subsequently converted, by scission of a peptide portion involved in disulfide cross-linking, to a noncovalently bonded trimer of α chains (which could only be recovered as the single type of α chain under the conditions of the gel chromatography).

Analysis of Procollagen Variants in Submicrosomal Fractions—In preliminary experiments (20), slices of epiphyseal cartilage from 12-day chick embryos were labeled for 60 min with [3,4-3H]proline. Comparison by electron microscope radioautography of the concentration of silver grains did not indicate any marked difference of the distribution of grains between the rough endoplasmic reticulum and Golgi complex. If, however, cartilages were labeled in the presence of 1 mM 2,2'-dipyridyl, there was a marked increase in the grain concentration over the rough endoplasmic reticulum and a significant reduction over the Golgi complex. The results are consistent with studies (21, 22) suggesting that at least one pathway for procollagen transport in cartilage cell involves a passage of procollagen from the rough endoplasmic reticulum to the Golgi complex, and further suggest that various forms of collagen precursor might be obtained by cell fractionation as they exist in subcellular compartments.

To test this idea, cartilage slices were incubated at 37°C for 60 min in Krebs-ascorbate medium containing [14C]proline and submicrosomal fractions were obtained as described under "Experimental Procedures." The procedures were similar to those employed previously (15) except that iodoacetamide and the inhibitors of proteolysis and protein synthesis (see "Experimental Procedures") were added to the sucrose solution used for tissue homogenization. The result is given in Fig. 2, and shows that the particulate fractions from Fraction 2 to 4 (P-1) and Fraction 6 to 8 (P-2) contained collagenase-digestible radioactive materials in nearly equal proportions. The remainder of radioactivity was recovered in the soluble fraction from Fraction 11 to 15.

When cartilage slices, which had been 60-min labeled with [14C]-proline and 60-min chased with unlabeled proline, were submitted to the subcellular fractionation, over 95% of the collagenase-digestible radioactivity was recovered in the soluble fraction. It is suggested therefore that the source of the radioactivity in the soluble fraction is largely molecules transferred to the extracellular space. The available data, however, cannot rule out the possibility that some part of

![Fig. 2. Distribution of 14C-proline-labeled collagenous and non-collagenous proteins and 35S sulfate-labeled proteoglycans in sub-microsomal fractions obtained from labeled cartilages by sucrose density gradient centrifugation.](http://www.jbc.org/content/133/3/3578/F2.large.jpg)
When the above experiments were conducted with cartilage slices that had been labeled with [14C]proline in the presence of 1 mM 2,2′-dipyridyl, a different result was obtained (Fig. 2b), i.e. there was a large increase in collagenous materials in P-1 and a significant decrease in P-2. Indeed, these figures correlate well with the radioautographic profiles in regard to the distribution of radioactivity and provide evidence suggesting that P-1 and P-2 originate from the rough endoplasmic reticulum and the Golgi complex, respectively.

Electron micrograph of P-1 (Fig. 3a) shows that this fraction was composed mainly of membrane-bounded vesicles with attached ribosomes. Smooth-surfaced microsomes were rare in this fraction. In contrast, the predominant feature of P-2 was the presence of smooth-surfaced, membrane-bounded vesicles typical of Golgi apparatus (Fig. 3b).

In studies on the biosynthesis of proteochondroitin sulfate in chick embryo epiphyses, a number of radioautographic and biochemical data have shown that the Golgi complex is the site for initial sulfation (15, 23). It would be possible therefore to demonstrate the initial appearance of radiosulfate in a Golgi-rich fraction if cartilages are subjected to subcellular fractionation after an appropriate pulse incubation with inorganic [35S]sulfate. The results presented in Fig. 2c indicate that after a 5-min pulse with [35S]sulfate, radioactivity was recovered almost exclusively in P-2.

Thus, the data obtained by the experiments of different principle indicate that P-1 and P-2 are composed largely of elements derived from the rough endoplasmic reticulum and the Golgi complex, respectively.

To determine the extent of disulfide bonding of the procollagens located in different subcellular sites, the subcellular fractions obtained as above were examined by Bio-Gel A-15m chromatography (Fig. 4). The results presented in Fig. 4a indicate that both single- and triple-stranded procollagens were present in P-1 in an approximate radioactivity ratio of 1:1, and that if hydroxylation was inhibited by 2,2′-dipyridyl a large amount of triple strands tended to accumulate. The results obtained with P-2 (Fig. 4b) are quite different and demonstrate that the procollagens in this fraction were present predominantly as triple strands.

Table 1 shows the results of analysis of the peak fractions obtained by gel chromatography. It is clear from these data that more than 90% of the radioactivity associated with each fraction behaved as collagenous material, according to its

![Fig. 3. Electron micrographs of submicrosomal fractions isolated from intact cartilage. The submicrosomal pellets, P-1 and P-2, obtained as described in the text were examined in an electron microscope. The P-1 fraction comprises ribosome-studded vesicles (RV). The P-2 fraction comprises smooth membranous vesicles (SV) which are devoid of attached ribosomes. For each submicrosomal fraction, five batches of preparation were examined and essentially identical results were obtained.](http://www.jbc.org/)

![Fig. 4. Bio-Gel A-15m chromatography of the [3H]proline-labeled proteins obtained from (a) P-1 submicrosomal pellets and (b) P-2 submicrosomal pellets. The P-1 and P-2 pellets obtained from 2 g of cartilages which had been labeled with [3H]proline for 60 min were separately suspended in 2 ml of 4 M guanidine hydrochloride, 50 mM iodoacetamide, 50 mM Tris-Cl (pH 7.5), treated in a 20 kHz sonic oscillator for 3 min during continuous cooling with ice water. The resulting solution (410,000 dpm from the P-1 pellets, 426,000 dpm from the P-2 pellets) was chromatographed on a column (1.5 × 50 cm) of Bio-Gel A-15m which was equilibrated with 20 mM Tris-Cl, pH 7.5, at 20°C (flow rate, 5 ml/h). Fractions of 2 ml were collected and assayed for radioactivity. Symbols: elution pattern of [3H]labeled proteins synthesized in the absence (O) and in the presence (●) of 2,2′-dipyridyl.](http://www.jbc.org/)
The Golgi complex, it can be postulated that the procollagen even after the conversion to triple strands and movement into microsomal fractions might be different from one another in regard to the extent of glycosylation.

Prompted by the previous observation (10) that the carbohydrate components obtained in association with the different submicrosomal fractions were undertaken. The studies were consistent with procollagen following the synthetic route from membrane-bound ribosomes. Furthermore, gel filtration of the peptide chains is not a critical requirement for the disulfide bonding between procollagen monomers.

**Variation in Sugar Moieties of Procollagens from Different Submicrosomal Fractions**—The results presented above are consistent with procollagen following the synthetic route from single strands to triple strands in the rough endoplasmic reticulum soon after release of procollagen chains from membrane-bound ribosomes. Furthermore, the accumulation of the unhydroxylated procollagen trimer supports the view (26, 27) that hydroxylation of the peptide chains is not a critical requirement for the disulfide bonding between procollagen monomers.

To test this possibility, studies on the relative proportions and type of carbohydrate residues of procollagens in different submicrosomal fractions were undertaken. The studies were prompted by the previous observation (10) that the carbohydrate components of procollagens were readily labeled by the following criteria. (a) Cartilage slices that had been incubated with [U-14C]proline or [3-14C]tryptophan were extracted with 4 mM guanidine hydrochloride and the extracted proteins were thoroughly washed by repeating the precipitation with 75% ethanol containing 1% potassium acetate (four cycles). Aliquots (10,000 dpm each) of the protein mixture thus prepared were treated with collagenase (100 μg each) for up to 2 h. When the reaction was followed by the increase of ethanol-unprecipitable radioactivity (which should represent small peptide fragments released from the labeled proteins), there was a marked difference between the [14C]proline-labeled sample and the [14C]tryptophan-labeled sample; i.e. from the former about 60% of the total 14C was released as small peptide fragments during the initial 1-h incubation, and there was no further release for the next 1 h, whereas from the latter not more than 0.7% of the total radioactivity was released as ethanol-unprecipitable material even after 2-h incubation. The results indicate that collagenous proteins in the sample were entirely degraded within 1 h while noncollagenous proteins (characterized by the presence of tryptophan) remained undegraded. (b) A similar experiment was performed on cartilage slices that had been labeled with [U-14C]glucose for 60 min. When ethanol-precipitable material was prepared (by the same method used for the [14C]proline-labeled sample) and treated with collagenase (100 μg/10,000 dpm of substrate), it was shown that ethanol-unprecipitable radioactivity increased rapidly and reached a limiting value (5% of the total 14C) in 1 h. On addition of 100 μg of fresh collagenase no further increase in ethanol-unprecipitable radioactivity occurred for about 1 h. The reaction bears a striking resemblance with respect to the time course to the reaction with the [14C]proline-labeled substrate. Furthermore, gel filtration of the 2-h digest on Bio-Gel A-15m (for the conditions, see the legend for Fig. 1) indicated that a significant reduction of radioactivity had occurred only in the fractions corresponding to procollagens and collagens, with a comparable increase of radioactivity in fractions displaying higher retention on the gel. Using this collagenase preparation as an analytical tool, studies were performed on the carbohydrate components of the procollagens in the submicrosomal fractions. Cartilage slices were labeled with [14C]glucose for 60 min and submicrosomal fractions, P-1 and P-2, were obtained as above. As was the case with the [14C]proline-labeled tissue, significant radioactivity was recovered in both P-1 and P-2 (Fig. 5). Of the total radioactivities found in P-1 and P-2, about 40% and 13%, respectively, were found to be susceptible to destruction with the collagenase.

The labeled materials were extracted and chromatographed on a column of Bio-Gel A-15m with 2 mM guanidine hydrochloride in 20 mM Tris-HCl, pH 7.5 as eluant (Fig. 6). The material from P-1 was separated into a large peak at the void volume and relatively small included peaks (Fig. 6a). The profiles of the included peaks suggest the presence of radioactive procollagen monomer and trimer. The material from P-2 showed a quite different profile (Fig. 6b), i.e. a much higher proportion of radioactivity was recovered in the region expected for procollagen trimer.

Collagenase treatment of the labeled materials recovered in the void volume fractions showed that they were not digested to ethanol-unprecipitable fragments and therefore are not related to collagenous components. Evidence that these fractions represent sulfated proteoglycans has already been provided by our previous studies (9, 28).

To remove contaminating proteoglycans, each of the included fractions corresponding to procollagen monomer and trimer was chromatographed on a small DEAE-cellulose column (1.6 x 3 cm) by elution with 50 mM Tris-HCl (pH 7.5) containing 2 mM urea, 0.2 mM NaCl, and 0.1% Triton X-100. It

### Table I

**Analysis of 14C-Proline-Labeled Samples from P-1 and P-2**

<table>
<thead>
<tr>
<th>Sample *</th>
<th>Total 14C</th>
<th>(A) Collagenase-Digestible 14C</th>
<th>(B) 14CHydroxyproline</th>
<th>R/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm x 10^3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From P-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-α</td>
<td>1168</td>
<td>1112 (1.0)*</td>
<td>0.509</td>
<td>0.46</td>
</tr>
<tr>
<td>[Pro-α]</td>
<td>1315</td>
<td>1170 (1.1)</td>
<td>0.518</td>
<td>0.44</td>
</tr>
<tr>
<td>From P-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-α</td>
<td>2141</td>
<td>2013 (1.8)</td>
<td>0.926</td>
<td>0.46</td>
</tr>
<tr>
<td>[Pro-α]</td>
<td>2306</td>
<td>2214 (2.0)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* See Fig. 4.

* Amount of radioactivity appearing in the supernatant after incubation with collagenase followed by ethanol precipitation.

* Analyzed by the method of Juva and Prockop (24).

* Values in parentheses indicate relative radioactivities.
and 0.1% Triton X-100, and was shown to be resistant to recovery almost quantitatively from the column by elution with 50 μM Tris-HCl (pH 7.5) containing 2 M NaCl, 1.0:1.1:1.8 (Table II), compared to the corresponding ratio of 1.0:0.9:1.4, is consistent with the population of small peptide fractions. The radioactivity ratio of the large peptide fractions, 1.0:0.9:1.4, is consistent with the population of the intact procollagen components in P-1 and P-2 (1:0:1:8, see Table I), whereas the counts of the small peptide fractions exhibit a marked increase in the order, P-1 monomer < P-2 trimer (radioactivity ratio = 1.0:2.0:5.6).

The data can be interpreted as indicating that although the three procollagen components were similar in the extent of glycosylation in the noncollagenous extension, they had variation in the extent of glycosylation in a region reflecting transition from P-2 procollagen chain isolated from chick embryo cartilage containing an additional glycopeptide region (M_r = 13,200) which differed from the α region not only in amino acid composition but also in the type of bound sugars. One may postulate that the carbohydrate chains in the additional peptide region and those in the α region are introduced at different stages as procollagen traverses the intracellular compartments. Procollagens at different stages of biosynthesis and transport may thus be expected to show structural heterogeneity in regard to the distribution of carbohydrates.

When the proteoglycan-free, [14C]glucose-labeled samples of procollagen (obtained as above from the DEAE-cellulose columns) were treated with 2-mercaptoethanol and then submitted to gel filtration on 0.1% SDS-Sephadex G-150, all of the labeled materials eluted in the void volume (Fig. 7). That these materials represented pro-α chains was indicated, in each case, by chromatography of a second aliquot following collagenase digestion (Fig. 7) when the majority of radioactivity now eluted in two included peaks. The chromatographic behavior, before and after collagenase treatment, is consistent with the previously described observations for [14C]proline-labeled pro-α1(II) chain, the identity and purity of which have been well established (9, 10). When combined with information derived from these studies on [14C]proline-labeled chain, it is suggested that the peak eluted near the end of the column represents a mixture of small peptides derived from the α region of pro-α1(II) and that eluted near cytochrome c (M_r = 13,500) represents a large peptide (or peptides) derived from an extension (or extensions) attached to the α chain.

As shown in Fig. 7 and Table II, there were some differences between the counts of the large peptide fractions derived from P-1 monomer, P-1 trimer, and P-2 trimer, but these were much smaller than the differences between the counts of the small peptide fractions. The radioactivity ratio of the large peptide fractions, 1.0:0.9:1.4, is consistent with the population ratio of the intact procollagen components in P-1 and P-2 (1:0:1:8, see Table I), whereas the counts of the small peptide fractions exhibit a marked increase in the order, P-1 monomer < P-2 trimer < P-2 trimer (radioactivity ratio = 1:0:2:0:5:6).

The data can be interpreted as indicating that although the three procollagen components were similar in the extent of glycosylation in noncollagenous extension, they had variation in the extent of glycosylation in a region reflecting transition.

![Figure 5](http://www.jbc.org/)

**Figure 5.** Distribution of 14C-labeled materials in submicrosomal fractions obtained from [14C]glucose-labeled cartilage by sucrose density gradient centrifugation. The labeling of cartilage was carried out with [14C]glucose for 60 min, and submicrosomal fractions were obtained as described in the text. Each fraction was assayed for total radioactivity (○--○) and for collagenase-sensitive radioactive materials (●—●). The conditions for the collagenase assay were as in the legend for Fig. 2.

![Figure 6](http://www.jbc.org/)

**Figure 6.** Bio-Gel A-15m chromatography of the [14C]glucose-labeled materials obtained from (a) P-1 submicrosomal pellets and (b) P-2 submicrosomal pellets. The experimental procedures were the same as described in the legend for Fig. 4, except that cartilage (2 g) had been labeled with [14C]glucose for 60 min.

TABLE II

<table>
<thead>
<tr>
<th>Sample†</th>
<th>Total 14C</th>
<th>14C eluted from DEAE with 0.2 M NaCl</th>
<th>14C released by collagenase digestion as†a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Large peptide</td>
<td>Small peptide</td>
</tr>
<tr>
<td>From P-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-α</td>
<td>1.963</td>
<td>1.523 (1.0)</td>
<td>0.358 (1.0)</td>
</tr>
<tr>
<td>[Pro-α]</td>
<td>3.147</td>
<td>2.360 (1.5)</td>
<td>0.337 (0.9)</td>
</tr>
<tr>
<td>From P-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Pro-α]</td>
<td>8.495</td>
<td>4.924 (3.3)</td>
<td>0.513 (1.4)</td>
</tr>
</tbody>
</table>

† See Fig. 6.  
‡ Amount of radioactivity appearing in the 0.2 M NaCl eluate from DEAE-cellulose column (see the text).  
§ See Fig. 7.  
†† Values in parentheses indicate relative radioactivities.

has been shown previously (2, 29) that procollagen and collagen eluted unretarded from the column under these conditions whereas elution of proteoglycan required a higher ionic strength buffer. With this procedure approximately 50 to 80% of the counts of each sample could be recovered in the eluates (Table II). In each case, the remainder of the counts was recovered almost quantitatively from the column by elution with 50 mM Tris HCl (pH 7.5) containing 2 M urea, 1 M NaCl, and 0.1% Triton X-100, and was shown to be resistant to collagenase.

It is of interest to note that the approximate radioactivity ratio of "P-1 monomer" to "P-1 trimer" to "P-2 trimer" was 1.0:1.5:3.2 (Table II), compared to the corresponding ratio of 1.0:1.1:1.8 for the [14C]proline-labeled samples (cf. Table I). The difference in radioactivity ratio between [14C]glucose-labeled samples and [14C]proline-labeled samples can be interpreted as reflecting variations in the extents to which procollagen polypeptides are glycosylated: P-1 monomer < P-1 trimer < P-2 trimer. As has already been reported (10), a procollagen chain isolated from chick embryo cartilage contained an additional glycopeptide region (M_r = 13,200) which differed from the α region not only in amino acid composition but also in the type of bound sugars. One may postulate that the carbohydrate chains in the additional peptide region and those in the α region are introduced at different stages as procollagen traverses the intracellular compartments. Procollagens at different stages of biosynthesis and transport may thus be expected to show structural heterogeneity in regard to the distribution of carbohydrates.

When the proteoglycan-free, [14C]glucose-labeled samples of procollagen (obtained as above from the DEAE-cellulose columns) were treated with 2-mercaptoethanol and then submitted to gel filtration on 0.1% SDS-Sephadex G-150, all of the labeled materials eluted in the void volume (Fig. 7). That these materials represented pro-α chains was indicated, in each case, by chromatography of a second aliquot following collagenase digestion (Fig. 7) when the majority of radioactivity now eluted in two included peaks. The chromatographic behavior, before and after collagenase treatment, is consistent with the previously described observations for [14C]proline-labeled pro-α1(II) chain, the identity and purity of which have been well established (9, 10). When combined with information derived from these studies on [14C]proline-labeled chain, it is suggested that the peak eluted near the end of the column represents a mixture of small peptides derived from the α region of pro-α1(II) and that eluted near cytochrome c (M_r = 13,500) represents a large peptide (or peptides) derived from an extension (or extensions) attached to the α chain.

As shown in Fig. 7 and Table II, there were some differences between the counts of the large peptide fractions derived from P-1 monomer, P-1 trimer, and P-2 trimer, but these were much smaller than the differences between the counts of the small peptide fractions. The radioactivity ratio of the large peptide fractions, 1.0:0.9:1.4, is consistent with the population ratio of the intact procollagen components in P-1 and P-2 (1:0:1:8, see Table I), whereas the counts of the small peptide fractions exhibit a marked increase in the order, P-1 monomer < P-2 trimer < P-2 trimer (radioactivity ratio = 1:0:2:0:5:6).
were subjected to hydrolysis with 2 M HCl at 100°C for 4 h. The hydrolysates were evaporated to dryness under reduced pressure, dissolved in water, and passed successively through small columns (1.0 × 2.5 cm) of Dowex 50 (H⁺) and Dowex 1 (HCO₃⁻).

When the small peptide fraction obtained from P-1 monomer was examined, about 49% of the ¹⁴C eluted from the columns in the water effluents. Reducing value analysis indicated that the total recovery of reducing sugars in the water effluents was about 53% of the total amount of neutral sugars (carriers) added before hydrolysis. Upon paper chromatography in 1-butanol/pyridine/water (6:4:3, by volume), greater than 90% of the recovered ¹⁴C co-chromatographed with carrier glucose (Rₑ = 0.31) and galactose (Rₑ = 0.27) in an approximate radioactivity ratio of 3:1. No significant radioactivity was detected in the area corresponding to mannose (Rₑ = 0.36). An additional amount (28%) of ¹⁴C was recovered by subsequent elution of the Dowex 50 column with 1.0 M HCl. When the eluate was successively acid-treated (4 M HCl, 100°C, 16 h), N-acetylated, dissolved in water, and passed through the Dowex 50 (H⁺) and Dowex 1 (HCO₃⁻) columns, about 80% of the unlabeled hexosamine carriers were recovered in the water effluents as N-acetylhexosamines (as judged by the modified Morgan-Elson assay). However, total recovery of ¹⁴C from the Dowex columns was less than 1%, indicating that the labeled material in the 1.0 M HCl eluate from the initial Dowex 50 column is not hexosamine. The behavior toward Dowex columns before and after N-acetylation suggests that the ¹⁴C may represent amino acids or peptides.

Essentially identical results were obtained with the small peptide fractions from P-1 trimer and P-2 trimer, except that the amounts of ¹⁴C recovered in neutral sugar fraction were 42% and 54%, respectively, of the total ¹⁴C in starting procollagen sample.

When the large peptide fractions were analyzed in a similar way, it was remarkable that a large portion of the ¹⁴C was found in chromatographic fractions corresponding to mannose and N-acetylglucosamine. Thus, the hydrolysis products eluted from the Dowex columns with water (i.e. neutral sugars) accounted for about 24%, 38%, 15% of the total ¹⁴C in the large peptide fractions from P-1 monomer, P-1 trimer, and P-2 trimer, respectively. Paper chromatography of the neutral sugar fractions thus obtained indicated that in each case the ¹⁴C was found in three fractions corresponding to mobility to glucose, galactose, and mannose (approximate radioactivity ratio = 4:3:3). An additional amount of ¹⁴C appeared in the N-acetylated hexosamine fraction (after elution from the Dowex 50 column, acid hydrolysis, N-acetylation, and ion exchange chromatography; see above); radioactivity in this fraction accounted for about 12%, 19%, and 7% of the total ¹⁴C in the large peptide fractions from P-1 monomer, P-1 trimer, and P-2 trimer, respectively. For further characterization, the N-acetylated hexosamine fractions were again acid-treated (4 M HCl, 100°C, 1 h, for deacetylation), evaporated to dryness under reduced pressure, dissolved in water, and subjected to paper chromatography in ethyl acetate/1-butanol/pyridine/butyric acid/water (10:5:5:1.5, by volume), a procedure which separates glucosamine, galactosamine, mannosamine, and talosamine (30). In each case, over 75% of the ¹⁴C co-chromatographed with carrier glucosamine; less than 10% of ¹⁴C was found in the area corresponding to galactosamine.

**DISCUSSION**

Our molecular weight estimate, based on collagen standards, is 122,000 (± 5,000) for the individual chains obtained after reduction of the disulfide-bonded procollagen trimer.

![Figure 7. SDS-Sephadex G-150 chromatography of the [¹⁴C]glucose-labeled procollagens before (---) and after (-----) treatment with bacterial collagenase. Elution patterns in experiments with the monomer fraction from P-1 (●), trimer fraction from P-1 (○), and trimer fraction from P-2 (▲) are shown. To each of the pooled fractions (about 12 ml) from Bio-Gel A-15m column (Fig. 6, indicated by the bars), 2 mg of acid-soluble collagen from rat skin and 12 ml of Triton X-100 were added. The mixture was dialyzed at 4°C against four changes (25 volumes each) of 50 mM Tris-HCl (pH 7.5) containing 2 M urea, 0.2 M NaCl, and 0.1% Triton X-100. The retentate was chromatographed on a column (1.6 × 3 cm) of DEAE-cellulose using the same buffer for equilibration and elution. The ¹⁴C-containing fractions eluted unretarded from the column were pooled (about 16 ml), concentrated to about 2 ml on a Diaflo PM-10 membrane. To this solution, 0.1 mg of bovine serum albumin and 2 ml of water were added, and proteins were precipitated with 12 ml of ethanol (containing 1.3% potassium acetate). The precipitation with ethanol was carried out four additional times. The final precipitate was suspended in 1.5 ml of the buffer solution for collagenase digestion (see the legend for Fig. 2). A 0.5-ml portion of the suspension was mixed with an equal volume of 4% SDS. The remainder (1.0 ml) of the suspension was treated with 100 µg of bacterial collagenase as described in the legend for Fig. 2. The collagenase digestion was terminated by addition of solid SDS to give a final concentration of 2%. For gel filtration, each of the mixtures was adjusted to 2% mercaptoethanol and, after incubation at 37°C overnight, loaded on a Sephadex G-150 column (1.2 × 50 cm) equilibrated with 0.1% SDS, 20 mM Tris-HCl, pH 7.5. The column was eluted with the SDS/Tris buffer at 20°C (flow rate, 6 ml/h). Fractions of 1 ml were collected and assayed for radioactivity.

The positions of elution of chymotrypsinogen A (Mᵦ = 25,000) and cytochrome c (Mᵦ = 13,500), used as standards, are indicated by bars 1 and 2, respectively. L, large peptide fraction; S, small peptide fraction.
Apparently, the value is lower than that reported for a procollagen chain with NH₂- and COOH-terminal extensions which has recently been isolated from the medium or cultured chondrocyte (7). It seems likely therefore that procollagen in intact cartilage is unusually susceptible to protease action and the lower value may be a consequence of partial proteolysis during isolation procedures. It should be noted in this regard that we have not fully characterized the peptides released by collagenase cleavage and therefore cannot ascertain whether the procollagen chains obtained here contain both NH₂- and COOH-terminal extensions. As shown in Fig. 7, the collagenase digest of the [¹⁴C]glucose-labeled procollagen yielded in the gel chromatography a somewhat broad band with a mobility similar to cytochrome c (Mr = 13,500) but we do not know whether the band represents a single peptide or a mixture of two (or more) kinds of peptide. If the latter is the case, an estimate of the molecular weight of the procollagen chain is approximately 122,000, resulting from the addition of two extensions (approximately 13,500 each) to an α1(II) chain (approximately 95,000). This matter requires additional study. A molecular weight of 150,000 has recently been reported by Monson et al. (31) and Davidson et al. (32) for a pro-α1(I) chain obtained from chick cranial bone, with values of 20,000 and 35,000 for an NH₂-terminal extension and COOH-terminal extension, respectively.

Although the carbohydrate constituents of interstitial collagen have been relatively well characterized (33), information on the carbohydrates of intracellular procollagen is still fragmentary. Recently, several groups presented evidence suggesting that procollagens contain, in their noncollagenous regions, some carbohydrate residues: e.g. hexose, hexosamine, and sialic acid in a pro-α1(I) from skin of calves with tarsparaxis (34); mannose and glucosamine in the cysteine-containing region of basement membrane collagen (35); mannose and glucosamine in pro-α1(I) and pro-α2 from chick tendon cells (36, 37); and mannose, glucose, galactose, and glucosamine in the extension of chick embryo epiphyseal proc-α1(II) (10). The studies described here demonstrate that the single- and triple-stranded procollagenes present in P-1 (rough endoplasmic reticulum-rich fraction) and the triple-stranded procollagen in P-2 (Golgi-rich fraction) are different from another in the extent of glycosylation in the collagenous α region relative to that in the noncollagenous region. The results are consistent with the possibility that glycosylation of both collagenous and noncollagenous regions is initiated early in the processing of polypeptides in the rough endoplasmic reticulum and, after the formation of interchisulfate bonds, the glycosylation of the noncollagenous region is not continued whereas glycosylation of the collagenous region is continued in the same place as well as in the Golgi apparatus where the appropriate transferases have been shown to occur (38). We should mention, however, that the above interpretations could be significantly affected if contamination of P-1 (or P-2) by the other submicrosomal fraction is significant. Another complication may arise if we consider a possibility that some carbohydrate residues in newly synthesized procollagenes are hydrolyzed by glycosidases during their subcellular transport, or, as discussed above, some glycosylated peptides are removed by proteolysis during tissue extraction. At the moment we cannot assess whether the observed differences between submicrosomal procollagenes have been affected by these factors.

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